University of Alberta

Studies on the control of tRNA gene transcription by the replication stress checkpoint

by

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Abstract

RNA polymerase III (RNAPIII) pre-initiation complexes at tRNA genes naturally cause replication fork pausing in the yeast *Saccharomyces cerevisiae*, and interference with replication is known to have deleterious effects on genome stability. It follows that repression of tRNA gene transcription could be advantageous to minimize replication perturbation. Consistent with this idea, our lab has previously reported that the replication stress checkpoint inhibits tRNA gene transcription. Here, I describe how repression by checkpoint signalling, induced by treatment with the replication inhibitor hydroxyurea (HU), is associated with RNAPIII pre-initiation complex disassembly at tRNA genes. In addition, I show that active checkpoint signals likely impinge on Maf1, a key negative regulator of RNAPIII transcription, to signal to tRNA genes during HU exposure. Next, I report that checkpoint signalling affects the protein complex assemblage at tRNA genes during normal proliferation. Inactivation of the replication stress checkpoint, which is associated with an induction of tRNA gene transcription, results in greater RNAPIII occupancy at tRNA genes and a decrease in condensin association, condensin being an important tDNA localized complex that is vital for maintenance of genome integrity. Next, I extended these results by monitoring replication in cells with elevated tRNA gene transcription using cross-linking of replication proteins as proxy for replication fork movement. Despite the fact that tRNA gene transcription interferes with replication, by this method I detected no greater fork pausing at tRNA genes in strains with elevated transcription. These data are discussed in the context of current controversy in

the literature about this type of replication perturbation. One possibility is that in cells unable to repress transcription, replication interference promotes greater genome instability in a way that does not include amplified fork pausing. Altogether, the results presented here are in harmony with the idea that the replication stress checkpoint functions to disassemble RNAPIII transcriptional machinery, likely to maintain genome stability. Lastly, I present preliminary data that identifies potential cell division cycle links to tRNA transcription. We propose a possible new pathway that restrains tRNA gene transcription involving Cdc28, the main cyclin-dependent kinase in yeast.

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List of Abbreviations

Abbreviation	Definition
2D electrophoresis	neutral-neutral 2D agarose gel electrophoresis followed by detection by southern blotting
ARS	autonomously replicating sequence
CBP	calmodulin-binding protein
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
ChIP-on-chip or ChIP-chip	chromatin immunoprecipitation combined with microarray technology
Cln	G1 cyclin
DNA	deoxyribonucleic acid
DNAP	deoxyribonucleic acid polymerase
dNTP	deoxyribonucleotide triphosphate
GINS	**G**o, **I**ich, **N**ii, **S**an
H3K56ac	histone H3 lysine 56 acetylation
HU	hydroxyurea
IP	immunoprecipitation
MCM	minichromosome maintenance
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PIC	pre-initiation complex
RNA	ribonucleic acid
RNAPIII	ribonucleic acid polymerase III
RPA	replication protein A
RSC	remodel the structure of chromosome
RT-PCR	real-time polymerase chain reaction
SGA	synthetic genetic array
SMC	structural maintenance of chromosomes
snRNA	small nuclear ribonucleic acid
ssDNA	single stranded deoxyribonucleic acid
SUMO	small ubiquitin-like modifier
SWI/SNF	switch/sucrose nonfermentable
TAP	tandem-affinity purification
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
tRNA	transfer ribonucleic acid
YPD	yeast extract-peptone-dextrose
YPG	yeast extract-peptone-glycerol

Chapter 1

Introduction

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Chapter 1

Introduction

tRNA gene transcription. tRNAs are short, abundant molecules needed during translation for the incorporation of proper amino acids into growing polypeptide chains. Regulating the rate of transcription of tRNA genes is an important method of ensuring suitable levels of these molecules are available for translation[1,2]. RNA polymerase III (RNAPIII) transcribes tRNA genes[reviewed in 3-5]. In addition, the RNAPIII transcriptome includes 5S rRNA genes, the spliceosomal U6 RNA gene, the signal recognition particle 7SL RNA gene, and other small non-coding RNA genes[6].

The rate of tRNA gene transcription is tied to growth control and cell division. Because tRNA molecules are part of the translational machinery, the rate of their transcription directly affects the bulk amount of protein a cell can produce. This protein synthetic capacity is a known determinant of cell growth and proliferation[7-9] making regulation of RNAPIII transcription of intense interest to researchers. Since tRNAs are needed in large amounts during growth, 10-15% of nuclear transcription is dedicated to making these RNA interpreters of the genetic code in cycling cells[10].

tRNA gene expression and cancer. Most cancer cells exhibit high rates of proliferation[reviewed in 11]. Over expression of tRNA genes has been observed in

many types of transformed cell lines and tumour cells[12-14], possibly to support the high level of protein synthesis needed in these cells. Interestingly, it is known that RNAPIII transcription is regulated by the functions of both tumour suppressors (*e.g.* p53 and Rb) and oncogenes (*e.g.* c-myc)[11]. The transforming forms of these proteins up-regulate transcription by RNAPIII.

More recently, it has been suggested that the high rate RNAPIII transcription in cancer cells, in addition to supporting translation, may be more intimately associated to oncogenesis than previously appreciated. Marshall *et al.* reported that elevating the production of a specific methionine tRNA, essential for the initiation of translation, was sufficient by itself, to promote cell proliferation and transformation[15]. This study partly built on the earlier observation that the rate of yeast cell proliferation is highly sensitive to the level of initiator tRNA expression[16,17].

The RNAPIII transcriptional machinery. The transcription of tRNA genes by RNAPIII must be accurate and efficient in order to produce sufficient amounts of needed tRNAs for translation. This requires the co-operation of a wide range of proteins that comprise both the actual polymerase itself and associated transcription factors. The core protein components of the transcriptional machinery, are conserved between yeast and human (Table 1-1).

Initiation of tRNA gene transcription is a sequential process that involves three multi-subunit complexes: RNAPIII, and the multi-subunit transcription factors IIIB (TFIIIB) and IIIC (TFIIIC). These proteins assemble onto tRNA genes in a

stepwise fashion to form a pre-initiation complex (PIC) (Figure 1-1). First, TFIIIC binds to two sequence specific regions of an internal promoter called the *A-box* and *B-box*. Once TFIIIC is bound it directs the binding of another factor, TFIIIB, upstream of the transcriptional start site. Lastly, RNAPIII is recruited by TFIIIB and TFIIIC and positioned correctly at the transcriptional start site to complete the formation of the PIC[reviewed in 18,19]. Importantly, TFIIIB-DNA complexes are extremely stable and capable of initiating multiple rounds of RNAPIII recruitment[20]. Studies in yeast have shown the existence of a process referred to as “facilitated RNAPIII recycling”, where the same transcription factors repeatedly reload the same polymerase. This is significant in achieving the required high rate of transcription of these genes[21,22].

The tRNA transcriptional machinery is modulated by a wide range of regulatory inputs that help ensure that the rate of transcription is appropriate for the demand for tRNAs in protein production[2,23]. Often, it is the case that such inputs include repressive signals from a variety of stress pathways. To illustrate, it is known that RNAPIII transcription is repressed in response to DNA damage, oxidative stress, lack of nutrients, and during membrane stress[1,10,24-26]. It has been proposed that rapid repression of RNAPIII during stress may ensure cell survival by preventing energy from needlessly being spent on tRNA transcription during poor growth conditions[2,9].

Maf1 is a common repressor of tRNA transcription. A common key regulator of RNAPIII transcription, Maf1, represses tRNA gene transcription during cellular

stress[27,28]. Maf1 is conserved from yeast to human and is notable in its ability to integrate inputs from a large variety of stress signals to repress RNAPIII transcription[29,30]. For example, in the budding yeast *Saccharomyces cerevisiae*, a large number of repressive signals all impinge on Maf1 as a repressor of RNAPIII transcription[26,31].

The primary amino acid sequence of Maf1 supplies relatively little information about the function of this repressor, other than it is a small protein (29 to 45 kDa depending on the species) with three conserved sequence boxes, called A, B, and C regions[28] (Figure 1-2). Maf1 proteins also have an acidic tail. *In vitro* RNAPIII transcription experiments using recombinant full-length Maf1 and truncated versions of Maf1 expressed from bacteria revealed that the B and C boxes were vital to the repression[32].

Recent research has added much to our understanding of Maf1 repression of tRNA gene transcription. Maf1 represses RNAPIII by a mechanism that involves the dephosphorylation and nuclear accumulation of Maf1, followed by its physical association with RNAPIII at tRNA genes genome-wide. During unchallenged, normal growth conditions a majority of Maf1 is phosphorylated and found in the cytosol of cells, only a small amount of Maf1 is found in the nucleus and associated with RNAPIII transcribed genes. Then, during repressing conditions, Maf1 is largely dephosphorylated and localized to the nucleus[33,34](Figure 1-3). In addition, repression is by a mechanism that involves Maf1 recruitment to the tRNA genes. Maf1 is known to bind RNAPIII and TFIIIB and specifically inhibit the initiation of transcription by preventing RNAPIII interacting with TFIIIB and

promoters[34,35]. Maf1-mediated repression is associated with decreased association of RNAPIII and TFIIIB with tRNA genes[32].

Recently, x-ray crystallography and cryo-electron microscopic structural studies of Maf1 and RNAPIII were reported[36]. The structure of Maf1 reveals that the previously defined conserved sequence boxes A, B, and C do not correspond to structural modules or defined surface patches, but the hydrophobic core residues of Maf1 is conserved among eukaryotes. From these studies, a mechanism was proposed for Maf1 repression of RNAPIII. In short, Maf1 binds RNAPIII and rearranges a RNAPIII specific sub-complex comprised of three proteins, Rpc82, Rpc34, and Rpc31. This complex is required for transcription initiation. This impairs the recruitment of RNAPIII to a complex of DNA with TFIIIB and thus prevents PIC formation. Maf1 does not seem to impair RNAPIII binding of a DNA-RNA scaffold or inhibit RNA synthesis, suggesting that Maf1 specifically represses transcription initiation from RNAPIII promoters. It was proposed that these data indicate that Maf1 prevents re-initiation by binding RNAPIII during transcriptional elongation. This elicits a rapid repression of RNAPIII transcription of tRNA genes because these genes are short and are transcribed quickly.

However, Maf1 repression may be somewhat more complex than this. Capart *et al.* reported that human Maf1, while being able to inhibit recruitment of TFIIIB and RNAPIII to template, is unable to inhibit facilitated recycling of the polymerase *in vitro* using an immobilized template transcription assay[37]. This contradicts the previously proposed models of Maf1 repression. The reasons

behind this contradiction are unclear. One possibility is that there is an uncharacterized step(s) to Maf1 activation that still needs to be elucidated. This possibility is encouraged by the observation that a Maf1 mutant that is non-phosphorylatable at all six phosphorylation sites results in nuclear accumulation, but not tRNA gene repression. It follows that an unknown, additional activation step, beyond Maf1's dephosphorylation and nuclear localization, is needed for repression [33]. Therefore, it is currently unclear how significant the nuclear localization of Maf1 during stress states is for repression, because there is always some Maf1 found in the nucleus[35]. It is likely that the major contributor to repression is a Maf1 activation step that takes place in the nucleus, and that nuclear accumulation of Maf1 during stress states supports such activation[38].

tRNA genes have a unique set of features that appear to affect cellular physiology independent of translation. The main function of tRNA genes is to provide the template for the production of tRNA molecules because tRNA production is vital for cells to produce protein. Genetic mutation of tRNA genes or hindrance of RNAPIII transcriptional machinery usually compromises fitness. However, research focused in this area from the last decade has revealed unexpected features that make tRNA genes particularly interesting. There is now evidence that these short, scattered genes have the potential to affect the spatial organization of the genome and to drive genome change and evolution[reviewed in 39,40]. Interestingly, these effects on cellular physiology do not seem to depend on the influence of tRNA output on translation. I discuss two of these newly

discovered ‘product independent’ effects below, beginning with condensin-dependent clustering of tRNA genes in budding and fission yeast.

Condensin binding sites coincide with tRNA genes. The conserved condensin complex is required for the condensation of mitotic chromosomes[41-43]. The process by which condensin promotes compacted chromosome structure during mitosis is still being fully elucitated. Although, in general, most models of condensin-mediated condensation involve the interaction between condensin complexes that are bound to scattered loci[44-46].

Two back-to-back reports published in 2008 demonstrated specific enrichement of condensin at tRNA genes in budding yeast[47,48]. Further, these papers argue that condensin may be recruited to these sites by the RNAPIII transcriptional machinery. Surprisingly, a screen of conditional mutant alleles of condensin indicated that condensin has little effect on transcription or tRNA processing, suggesting that enrichment of condensin does not affect tRNA output[48].

First, D’Ambrosio *et al*. found that condensin binding sites overlapped with TFIIIC binding sites[47]. Further, they reported that eliminating TFIIIC binding to tRNA genes, either by a TFIIIC temperature sensitive allele or mutation of a TFIIIC binding site, resulted in reduced association of condensin. Second, Haeusler *at al.* used co-immunoprecipitation experiments to illustrate that there is a direct physical interaction between condensin and TFIIIB and TFIIIC that is not

likely mediated by DNA[48]. This opens the possibility that condensin may be recruited to tRNA genes by one or both of these transcription factors.

Condensin association with tRNA genes may have important functions in mitotic chromosome condensation, although condensin may also function at tRNA genes outside of mitosis. That is, condensin has important functions during interphase. For instance, transcriptional silencing at the budding yeast mating type loci and the *Drosophila Fab7* loci require condensin, as does signaling in fission yeast that is triggered in S-phase by replication interference[49-51]. Clues to understanding the biological significance of condensin enrichment at tRNA genes came from studying a previously known phenomenon of the sub-nuclear clustering of these genes in yeast.

tRNA genes are physically clustered in budding and fission yeast.

Chromatin fibers, which carry tRNA genes, are present as an elaborate three-dimensional structure in the nucleus[52,53]. It has been shown that DNA strands are non-randomly arranged into chromosomal territories and it is evident that these territories are linked to a variety of nuclear processes, such as transcription[54]. Studies using budding and fission yeast have illustrated that tRNA genes are important in overall chromosome architecture in these model organisms.

Although the tRNA genes of budding yeast are scattered evenly throughout the genome, they are physically clustered in the nucleolus[55,56]. This surprising localization depends on both microtubules and the function of condensin[48]. First, interfering with microtubule polymerization, by treating cells with the drug

nocodazole, resulted in tRNA genes that were still clustered together but not localized to the nucleolus, arguing that intact microtubules are required for localizing tRNA genes to the nucleolus. Second, it was shown that conditional inactivation of condensin subunits leads to loss of clustering[48]. Therefore, condensin appears to facilitate gene clustering and microtubules are necessary in nucleolar localization. Lastly, most recent evidence indicates in budding yeast there may actually be two clusters to tRNA genes, one in the nucleolus, as previously noted, and another cluster with centromeres[55]. However, the mechanism of centromeric localization has yet to be determined.

In fission yeast, *S. pombe,* tRNA genes are known to contribute to overall chromosome structure by clustering with the centromeres. Although 50 tRNA genes in fission yeast are encoded at the centromeres, there are more than 100 tRNA genes scattered along the arms of the chromosomes[57,58]. Also, the centromeric localization of tRNA genes on chromosomal arms is mediated by condensin interaction with RNAPIII machinery[57]. In addition, it was clearly shown that RNAPIII transcription has an inhibitory effect on the centromeric localization of tRNA genes. For example, inhibiting RNAPIII promotes tRNA gene localization to centromeres. From this it was speculated, instead of condensin regulating RNAPIII transcription, that transcription was refractory to binding of condensin and thus centromeric localization.

Given the high number of dispersed tRNA genes in both of these species, clustering is likely to have an effect on the spatial organization of a large part of their genomes[55-58]. It is possible that similar genome organization occurs in

higher eukaryotes because of the highly conserved nature of condensin and the RNAPIII transcriptional machinery.

Product independent regulation of tRNA gene transcription. Given that tRNA gene transcription is highly regulated and has product independent effects on cellular physiology, it is feasible that transcription of tRNA genes is regulated to modulate its extra transcriptional outcomes[39]. Below I explore this question as it relates to interference with DNA replication, a phenomenon whereby tRNA genes potentially can be sites of genomic change and drivers of evolution.

DNA replication. Genome duplication is an essential process of proliferating cells and is tightly regulated in order to preserve genomic stability. DNA replication occurs during S-phase of the cell cycle. At the beginning of S-phase replication initiates at specific regions found throughout the genome called replication origins. Once replication is started at the origins, sometimes referred to as “origin firing”, it proceeds in a bi-directional manner, with elongation of newly synthesized DNA proceeding on specialized structures called replication forks[59-61]. The most notable protein components of a replication fork include: 1) the leading and lagging strand DNA polymerases (DNAP), which synthesize the DNA[62-64], and 2) the replicative helicase, MCM(2-7), which separates double standed DNA into single stranded DNA (ssDNA)[65] (Figure 1-4). Once initiated, a replication fork can duplicate several tens of thousands of bases before meeting a converging fork, the converging forks fuse together, and replication terminates.

Impeding a replication fork. A replication fork can encounter barriers or DNA metabolic circumstances that impede its progression. The three most studied cellular events that perturb fork progression are DNA damage, replication inhibitors, and endogenous non-nucleosomal proteins tightly bound to DNA. First, a cell's DNA is under a steady bombardment by DNA damaging agents, which can lead to the formation of various DNA lesions, which are known to block replication forks[66]. Second, treatment of cells with replication inhibitors causes replication forks to slow or stall in their progression either by altering the availability of needed raw materials or by directly inhibiting DNA synthesis[67,68]. Third, certain regions of DNA, which are tightly bound by non-histone proteins, have been shown to be barriers to fork movement[69-71]. Most importantly, any impediment to fork progression must be proficiently overcome to allow the re-establishment of replication because cell survival is dependant upon faithful duplication of the entire genome.

Replication pausing at tRNA genes. During the lifespan of a cell, more than one process will require access to the same region of the genome. For instance, DNA replication and transcription occur on the same DNA template. Further, in eukaryotes the rate of DNA synthesis during replication is at least five times the rate of RNA synthesis by RNAPIII[72,73]. For these reasons, collisions between replication forks and RNAPIII seem unavoidable. It has been shown that tRNA

genes interfere with nuclear DNA replication during normal, unchallenged S-phase[69,71].

This phenomenon was discovered while studying replication in budding yeast[69]. Replication intermediates synthesized during replication can be resolved by neutral-neutral 2D agarose gel electrophoresis and detected by Southern blotting (2D electrophoresis). Desphande and Newlon used 2D electrophoresis to study replication intermediates of a series of plasmids, which contained fragments of the budding yeast chromosome III. Replication fork pause sites, which cause an accumulation of replication intermediates of a certain size, were detected by 2D electrophoresis as an intense region of hybridization along signal arcs. This analysis uncovered a natural, endogenous locus that transiently arrested fork movement (so-called replication fork pausing site). It turned out that the critical element of this locus that was pausing forks was a tRNA gene. Mutations of the plasmid-borne tRNA gene that ablated the assembly of PICs and a temperature sensitive RNAPIII mutant did not pause replication forks indicating that, minimally, the assembly of the PIC is required for the perturbation of fork movement, although the results did not rule out the possibility that active transcription was necessary for fork pausing. These initial experiments plainly showed there was a directionality to the effect tRNA genes had on replication of plasmids because they only cause fork pausing if the direction of transcription was opposite to the movement of the replication fork (Figure 1-5), in order to create head-on collisions between the transcriptional machinery and the replication fork. Taken together, these results supported a model whereby fork

pausing is caused by PIC assembly that is oriented in the opposite direction to a replication fork that copies.

Subsequent work from the Zakian group further demonstrated that replication fork pausing was also observed at tRNA genes located in their endogenous, chromosomal location[71]. The mechanism by which chromosomal tRNA genes were causing fork pausing also required PIC formation. What is more, only tRNA genes oriented to transcribe into the direction of forks that replicate them were seen to cause pausing.

There are sites of the budding yeast genome which are known to bind TFIIIC and not the rest of the RNAPIII transcriptional machinery (known as "extra TFIIIC" or "*ETC"* sites)[74]. It is unknown if these *ETC* sites cause fork pausing, although available evidence suggests that this is unlikely[75].

Replication fork termination. Of late, Fachinetti *et al.* published an interesting paper reporting a new link between replication fork pausing and replication termination[76]. Using genomics approaches, these investigators tested the hypothesis that replication termination occurs at specific chromosomal loci in budding yeast. By using chromatin immunoprecipitation combined with microarray technology (ChIP-chip or ChIP-on-chip approach) and bromodeoxyuridine incorporation they were able to map regions where replication forks met. They concluded that termination almost always occurs at replication pause sites, which includes tRNA genes. The implication is that

replication fork pausing at tRNA genes somehow promotes orderly fusion of replication forks and replication termination.

Replication Fork encounters with tRNA gene PICs. The pausing of forks at tRNA genes is often thought of as replication interference, although the precise mechanism of this interference remains unknown[39]. For example, because pausing has only been measured using population-based assays, it remains unknown if fork interference by individual tRNA genes is relatively constant between cells or highly variable. For example, it is not known if a moderate pausing signal in a 2D electrophoresis experiment reflects moderate pausing in all cells or extremely high pausing in some cells, but no pausing in others.

Further, little is known about the precise molecular interactions or biochemical functions of the proteins of the transcriptional machinery or replication forks that are required for fork pausing. For instance, it is still unclear whether active RNAPIII transcription or just the presence of the PIC at tRNA gene causes the pause[71]. Most commonly, tRNA genes are thought to be barriers to replication because of the tight physical binding of the proteins at these regions. There is highly salt resistant binding of the transcriptional machinery to tRNA genes[77]. The directionality of the pausing could be due to the fact, except for the initial binding of the transcription factors, RNA synthesis occurs on one strand of the template of DNA. In addition, replication occurs on both strands at the same time at the replication fork, but the arrangement of factors is asymmetrical on both strands[78]. This would lead to different proteins interacting in co-directional and head-on

collisions. Still, it is also possible the clustering of tRNA genes together somehow promotes DNA secondary structure that is difficult for replication forks to copy. Finally, it is possible the superhelical tension created in the DNA between converging replication forks and RNAPIII is responsible for the replication perturbation[69].

Replication fork collapse at tRNA genes. Even though little is known about the specific nature of replication fork pausing at tRNA genes, the biological consequences of fork pausing have been elucidated and, most importantly, it is known that fork pausing predisposes replication forks to collapse[79]. Paused forks are unstable DNA structures. Therefore, paused replication forks elicit the activation of signal transduction pathways that direct the stabilization of non-progressing forks[66,80,81]. If the pause persists for long enough, the protein components of the replication fork dissociate from the DNA leading to fork collapse and DNA double-strand breaks[82-84].

The repair mechanisms used by cells to rescue collapsed forks result in chromosomal rearrangements that can negatively affect genome stability[85,86]. There is convincing evidence that tRNA genes are inherently prone to replication-associated instability. For instance, in untreated, normally cycling cells, spontaneous chromosome breakage often occurs at fork blocking tRNA genes[87]. Further, there is an elevation of these breakage events in cells treated with the genotoxic drug hydroxyurea (HU), which depletes the cells of the DNAP substrates deoxyribonucleoside triphosphates (dNTPs) and causes fork

movement to slow. Finally, collapsed forks are repaired by recombination events and there is an increased rate of recombination between active tRNA genes[88]. The physical clustering of tRNA genes may well mediate recombination by enabling homologous tRNA genes to be in close proximity to each other[48].

A chromatin remodeler and a histone variant that are important for DNA double-strand break repair are enriched at tRNA genes. The binding sites of the Snf2-like chromatin remodeler, INO80, correspond to tRNA genes[89]. In budding yeast, INO80 is recruited to DNA double-strand breaks, where it functions to remove nucleosomes, giving DNA repair enzymes access to DNA. INO80 is recruited to double strand breaks by γ–H2A (a phosphorylated form of H2A), which is produced by the DNA damage response machinery[90,91]. Consistently, γ–H2A is also enriched at tRNA genes in budding yeast[92].

Replication-associated proteins modulate fork movement at tRNA genes. After the initial discovery of fork pausing at tRNA genes, further work aimed at understanding how the replication components modulate fork progression at these loci was pursued. Two replication components, Rrm3 and Tof1, were implicated in the control of fork pausing at tRNA genes.

Rrm3 is a member of the conserved Pif1 family of DNA helicases. Rrm3 is a component of the replisome implicated in modulating fork progression at a wide variety of sites in the yeast genome. The Zakian group showed that Rrm3 has a role in promoting fork movement at tRNA genes[71,93]. In *rrm3Δ* cells, forks that move into the front end of tRNA gene PICs pause for an increased period of time

over wild type. Interestingly, it was also seen that forks that travel into co-directionally transcribed tRNA genes pause in the absence of Rrm3. These are genes that no pausing could be detected by 2D electrophoresis in wild type cells. This finding suggested that tRNA genes, which are in the wrong orientation to cause replication pausing in wild-type cells, can still be difficult to replicate and that all tRNA genes present a challenge to replication regardless of their orientation. It also suggests that the pause is not due to superhelical tension because no such tension would result in a fork progressing into the rear end of a PIC. Rrm3, often referred to as a 'sweepase', is proposed to function at the forefront of the fork facilitating the removal the tRNA transcriptional machinery when encountered[94].

Tof1 was also found to be important for fork movement at tRNA genes. Bastia and colleagues found that in *tof1Δ* cells, there was no longer pausing of replication forks at tRNA genes that were known to cause fork pausing in wild-type cells[95]. Therefore, it was concluded that Tof1 helps impose the pause at tRNA genes and likely functions in opposition to Rrm3[95,96]. Seeing as Tof1 has no determined biochemical function, a mechanism by which Tof1 promotes pausing remains unclear.

There is no evidence that suggests that Rrm3 or Tof1 is under any regulation by signaling systems that monitor or control the replication process. Consequently, although Rrm3 and Tof1 might work together to set the rate of replication fork progression through tRNA genes, cells may not be able to control this rate by signaling mechanisms that effect the function of either Rrm3 or Tof1.

So far, studies of this phenomenon have primarily focused on understanding how replication forks resolve an encounter with a blocking tRNA gene (*i.e.* through the function of Rrm3 and Tof1), but it is possible that RNAPIII repression mechanisms exist to diminish fork interference. Specifically, we explored the possibility that perturbation of replication at tRNA genes results in the activation of a replication surveillance system entitled the "replication stress checkpoint" that would elicit repressive signals to RNAPIII.

The replication stress checkpoint. Specific signal transduction modules, collectively referred to as "checkpoints", monitor the status of DNA and respond to defects by activating signaling pathways[66,97-99]. Activation of these pathways results in modulation of cellular processes in order to promote DNA replication fidelity and cell survival[100-102]. DNA structure checkpoint pathways, in general, involve four evolutionarily conserved functional classes of proteins known as sensors, adapters, transducer kinases, and effector targets[79,103,104]. Broadly, sensor proteins first sense abnormal DNA structures and initiate a signaling cascade by the activation of a transducer kinase, often though the use of a non-enzymatic adapter protein. Transducer kinases amplify and relay the signal to effector targets, which then elicit all of the proper responses of that help the cell survive challenges[66,80].

Specifically, there is a checkpoint pathway that monitors replication and responds to any difficulties that may arise; this pathway is named the replication stress checkpoint. This checkpoint in budding yeast is comprised of the sensor

kinase Mec1, the adapter protein Mrc1, the transducer kinase Rad53, and the effector kinase, Dun1[80,84,105](Figure 1-6). All of these yeast proteins have human functional equivalents, many of which have been implicated in human cancer or cancer susceptibility syndromes[106,107]. These proteins modulate cellular processes that support genome stability when replication forks are perturbed, including inducing an arrest of the cell cycle. Cells unable to initiate a checkpoint in response to replication challenges may attempt to proceed through S-phase and into mitosis without properly completed DNA replication, often leading to genomic instability and loss of viability. Indeed, it had been observed that there are increased gross chromosomal rearrangements in mutant yeast cells that lack a functional replication checkpoint[81].

tRNA gene transcription is repressed during replication stress. Building on the knowledge that tRNA genes are naturally sites of replication fork pausing, it was discovered in our lab that budding yeast repress tRNA transcription when cells are experiencing replication stress[108]. Following this discovery, strains with mutations in replication stress checkpoint genes were screened for their ability to inhibit RNAPIII transcription upon HU treatment. It was discovered that cells deficient in replication stress checkpoint signaling were unable to repress transcription, supporting the novel idea replication stress checkpoint proteins control tRNA gene transcription.

Checkpoint repression of tRNA gene transcription seems to be accomplished by a mechanism that requires Maf1 because HU-treated cells *maf1Δ* cells were

unable repress transcription, similar to replication stress checkpoint mutants. In addition, repression by HU is associated with dephosphorylation of Maf1 that required Rad53.

Perhaps, by repressing transcription during HU treatment, cells can minimize the tRNA gene interference of replication allowing already slowed and weakened forks to proceed past these loci unimpeded.

Elevated rates of transcription in replication checkpoint mutants during normal, unchallenged growth. During the course of necessary control experiments on checkpoint control of tRNA gene transcription, a surprising result emerged. It was noticed that mutants of the replication stress checkpoint have induced tRNA gene transcription compared to wild-type cells in untreated, normal growth[108]. Specifically, the tRNA genes were induced in *MEC1*, *MRC1*, and *RAD53* mutants. Transcription was reduced to wild-type levels in a *RAD53*-null mutant by adding back a wild-type version of the protein expressed from a plasmid. Further, the kinase activity of Rad53 is required for this inhibition because adding a kinase-deficient version of Rad53 back to *rad53Δ* cells did not result in reduced transcription. Therefore, we concluded Rad53 is in a pathway that represses transcription in unchallenged proliferating cells.

We propose a model whereby a known signaling pathway constructed of Mec1, Mrc1, and Rad53 contributes to the repression of tRNA genes during normal cycling. While it is known that many genes, including protein encoding genes, are responsive to DNA checkpoint signaling when cells experience

genotoxic stress, this is the first ever indication that the transcription of any gene is controlled by the checkpoint signaling during unchallenged growth.

The suggestion that replication stress checkpoint signaling is functioning during unchallenged growth is now well established experimentally. Mec1 phosphorylates H2A and the Ddc1 subunit of the checkpoint DNA clamp during normal growth[92,109]. Mec1 is also required to replicate some regions of the genome that are classified as “difficult-to-replicate”[110]. Mrc1 is also required for normal timing of replication, as S-phase is slowed in *mrc1Δ* cells[111]. Finally, there is a sub-population of Rad53 that is active during normal cycling[112]. Taken all together, these data indicate that replication stress checkpoint signaling is active during normal proliferation, however, the signaling must be below a certain threshold that is required to restrain cell cycle progression.

We suggest that RNAPIII repression by the replication stress checkpoint may contribute to minimizing the interference to replication of the tRNA genes by removing the fork blocking RNAPIII transcriptional machinery. In addition to the repertoire of known replication associated mechanisms (e.g. Rrm3 and Tof1), this new system adds to the cells ability to overcome replication perturbation at tRNA genes.

Given the high degree of conservation from yeast to human of the RNAPIII transcriptional machinery[5,39,113], the replication fork[114], and the replication stress checkpoint[80], there is much to be gained from studying replication interference by tRNA genes using budding yeast as a model system, where we can take full advantage of the ease of genetic manipulation of this organism.

Research overview. Building upon the preliminary evidence that fork pausing activity of tRNA genes may be regulated by checkpoint signaling, here I further characterize replication stress checkpoint repression of RNAPIII transcription and provide new understanding of the biological significance of this pathway. Also, I report on a series of experiments which illustrate some surprising possible new links between regulatory signaling to tRNA genes and the cell cycle. **Chapter 3** reports the steps of RNAPIII initiation that are targeted by the replication stress checkpoint during replication stress, and the involvement of Maf1 during repression. Repression of transcription in cells experiencing replication stress is associated with loss of RNAPIII and TFIIIB occupancy at tRNA genes and an increase in TFIIIC and Maf1 association. All of this is consistent with the known characteristics of Maf1-mediated repression during other stress conditions[31,115,116]. Of particular importance is data showing that under conditions of elevated RNAPIII activity, cells are sensitive to replication stress. In **Chapter 4** I present studies showing how the protein composition of tRNA genes changes in cells deficient in the replication stress checkpoint. As stated before, it was discovered in our lab that cells lacking replication checkpoint proteins have an elevated rate of transcription. Here, we show that this is correlated with an increase in the occupancy of RNAPIII at tRNA genes and a decrease in the association of condensin, raising the possibility transcription is refractory to condensin loading at these sites in budding yeast. In addition, genetic studies reveal that Maf1 is not part of checkpoint signaling during unchallenged growth.

Chapter 5 builds on data in Chapter 4 by showing RNAPIII occupancy is increased in replication stress checkpoint mutants during normal proliferation. In this chapter, I test the hypothesis that increased association of fork blocking RNAPIII would result in increased fork pausing at tRNA genes. We detected no such increase. These data are indicative of the complexity and lack of understanding of the nature of the fork pause itself and the interactions between the replication fork and transcriptional machinery. Further studies testing the effect of chromatin remodeler INO80, histone chaperone Asf1, histone H2A phosphorylation, and replication proteins Rrm3 and Tof1 on RNAPIII transcription further illustrate the complicated nature of fork pausing. Moreover, increased transcription in *maf1Δ* cells does not confer deleterious consequences on the fitness of cells lacking various replication, checkpoint, or recombination proteins.

Chapter 6 reports preliminary data linking tRNA transcription to cell cycle progression. Activation of the replication stress checkpoint is exclusively sensitive to perturbation of replication forks and therefore, its activity is limited to S-phase of the cell cycle. This, taken together with our lab's discovery that replication stress checkpoint signaling controls tDNA transcription, led us to test if repression of transcription by this replication stress inputs is also exclusive to S-phase. Surprisingly, my results suggest that repression of RNAPIII seems to occur during G2/M, not S-phase as expected. This G2/M repression requires Mrc1. This raises the exciting possibility that replication stress checkpoint signaling is functioning outside of S-phase to modulate fork movement, possibly during the termination of replication. In addition, we report other preliminary data

that suggest that Cdc28, the main yeast cell cyclin dependant kinase (CDK), may be in a signal transduction pathway that also limits RNAPIII transcription. The implications of these findings are discussed in **Chapter 7**, and ideas for future experiments are proposed.

Table 1-1. The core components of the tRNA gene transcriptional apparatus in budding yeast and their human counterparts[5,113].

Table 1-1

tRNA gene transcriptional machinery

TFIIIC

Yeast	*Human*
Tfc1	TFIIIC63
Tfc3	TFIIIC220
Tfc4	TFIIIC102
Tfc6	TFIIIC110
Tfc7	TFIIIC35
Tfc8	TFIIIC90
	Bdp1

TFIIIB

Yeast	*Human*
Spt15	TBP
Brf1	Brf1/Brf2
Bdp1	(see TFIIIC)

RNA polymerase III

Yeast	*Human*
Rpc160	RPC1
Rpc128	RPC2
Rpc82	RPC3
Rpc53	RPC4
Rpc40	RPAC1
Rpc37	RPC5
Rpc34	RPC6
Rpc31	RPC7
Rpc25	RPC8
Rpc19	RPAC2
Rpc17	RPC9
Rpc11	RPC10
Rpb5	RPABC1
Rpb6	RPABC2
Rpc8	RPABC3
Rpb10	RPABC5
Rpb12	RPABC4

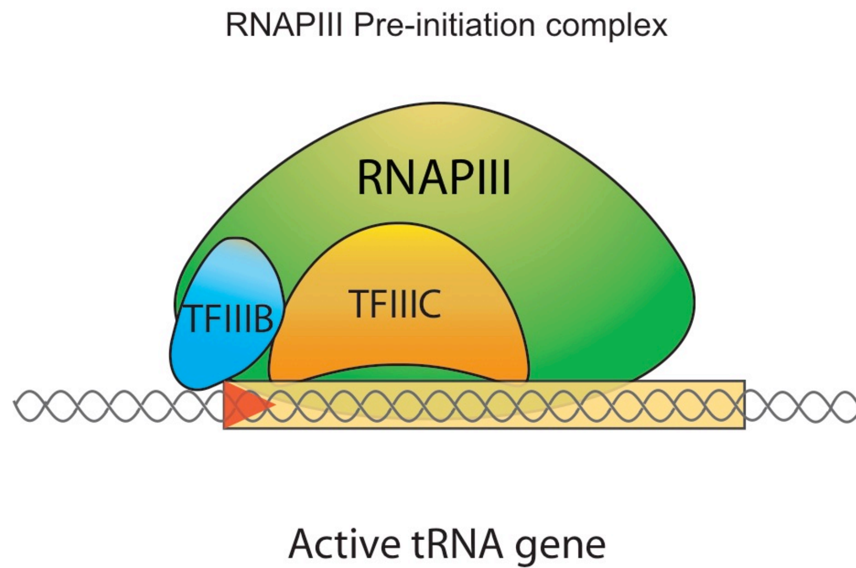

Figure 1-1. The RNAPIII PIC. TFIIIC, TFIIIB and RNAPIII assemble sequentially at the promoters of tRNA genes. Transcription of the tRNA gene (box surrounding DNA) is in the direction of the triangle located at the transcriptional start site.

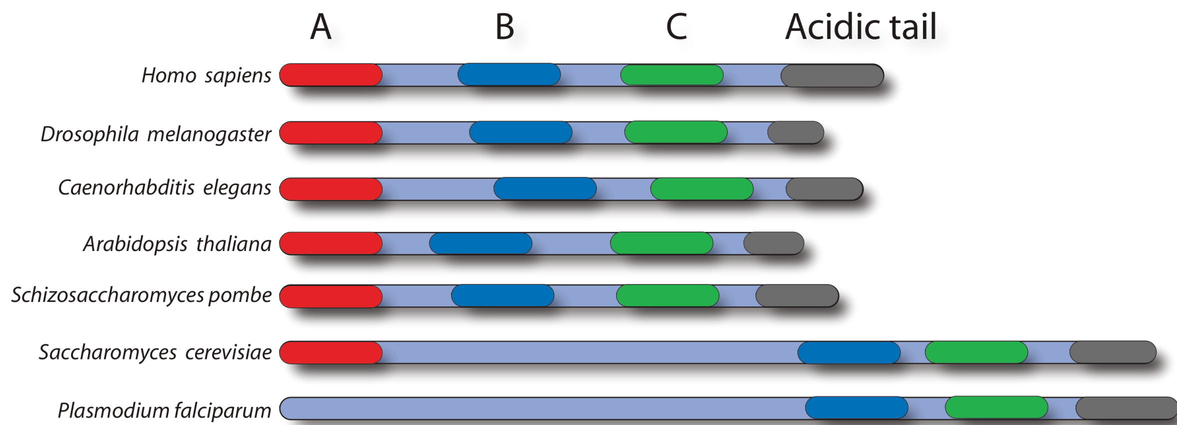

Figure 1-2. Schematic representation of Maf1 and potential orthologs. The primary amino acid sequence alignment of Maf1 proteins from different species[28]. Species is indicated on the left. Conserved domains are shown as ellipses and indicated by the letters “A”, “B”, “C”, and “Acidic tail”.

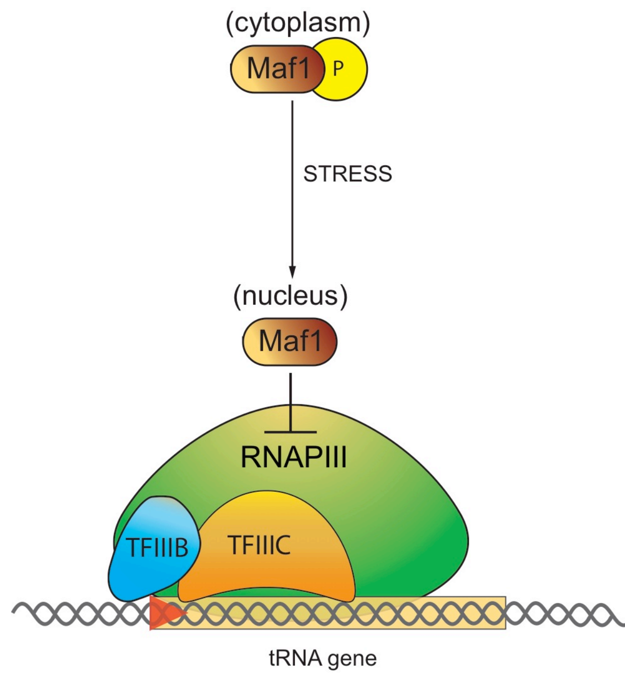

Figure 1-3. Maf1 is a key negative regulator of RNAPIII transcription. Phosphoryation of Maf1 is indicated by the circle with the "P". Upon stress (arrow), Maf1 is dephosphorylated and localizes to the nucleus. Repression is due to recruitment of Maf1 to tRNA genes.

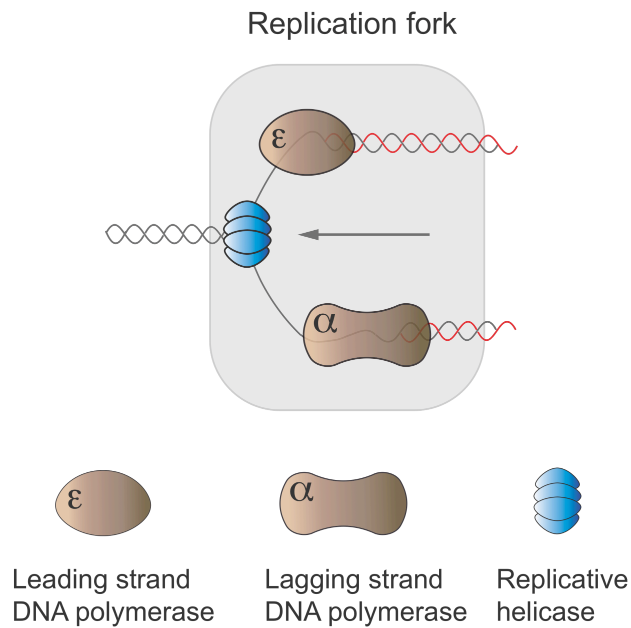

Figure 1-4. Simplified diagram of the replication fork. Replication fork is progressing in the direction of the arrow (to the left). MCM helicase and the DNA polymerases ε and α are shown. Helicase separates double stranded DNA and the polymerases synthesize new DNA in a semi-conservative manner.

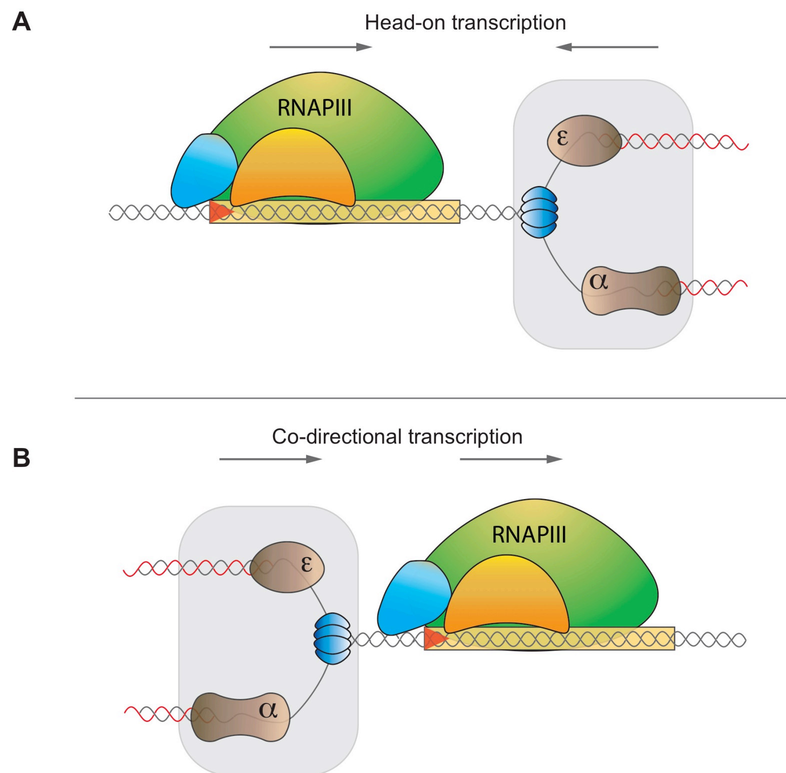

Figure 1-5. Replication interference by tRNA genes. The arrows show the direction of the polymerase movement during nucleic acid synthesis. **A**. PIC formation is such that RNAPIII transcription is in the opposite direction of the movement of the replication fork that copies it. **B**. PIC formation is co-directional with replication.

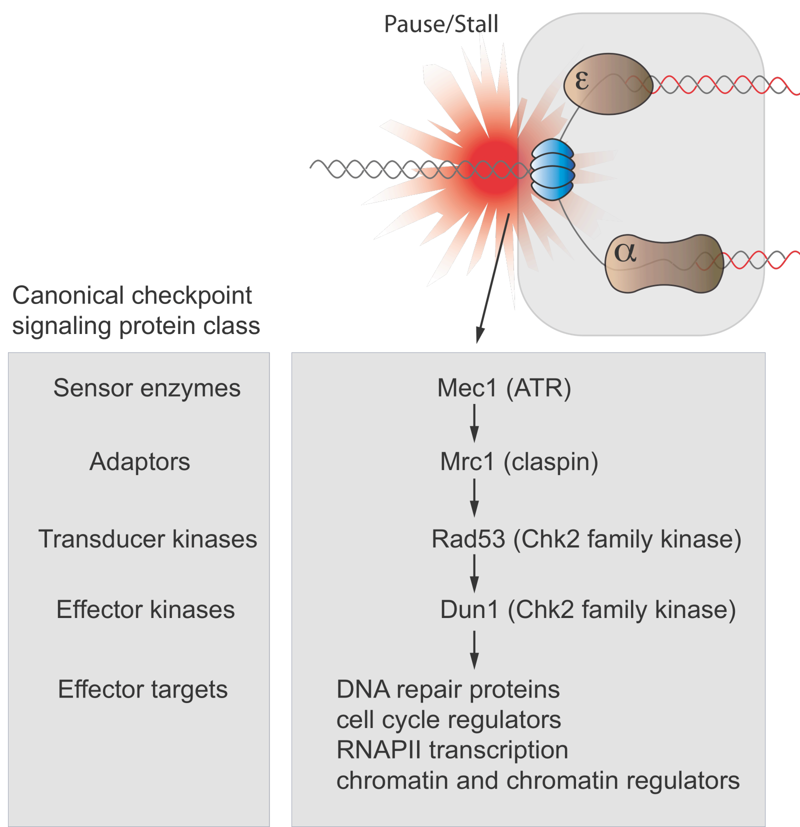

Figure 1-6. The budding yeast replication stress checkpoint. Perturbation of replication fork movement acivates the replication stress checkpoint. The sensor kinase, Mec1, detects a challenge to replication and, through the adaptor protein Mrc1, activates Rad53. Rad53, when active, phosporylates Dun1. This leads to the appropriate responses of the cell to replication disruption. Human orthologs are in brackets.

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Chapter 2

Materials and Methods

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Materials and Methods

Strains, plasmids, and media.

All strains used in this study are listed in Table 2-1. All strains are derivatives of either W303[1] or BY4741[2], unless otherwise indicated. Single deletion mutants, from the *S. cerevisiae* haploid nonessential gene deletion library[3], were confirmed by PCR to be correct using multiple primer sets. Chromosomal mutations were generated by one-step integration using PCR products amplified from previously described plasmids[4-6]. Correct gene deletion and correct addition of sequences encoding epitope tags was verified by PCR using multiple primer sets (flanking target region, internal to the target gene, flanking primer plus primer specific for the replacement marker). Epitope tagging was also confirmed by immunoblotting using primary antibodies that specifically bind epitopes and ensuring that the detected bands migrate at approximately the expected size. Plasmids pRS314 and pMAF1[7] were a generous gift from Ian Willis and were used in spotting assays presented in Fig. 3-11. A lithium acetate procedure was used for plasmid transformation[8]. All media were prepared as described previously and standard genetic methods for transformations were used throughout this thesis[9]. All strains were capable of growth on glycerol and therefore were judged to be competent for respiration.

Treatment of cells in liquid medium.

Before treatment, cultures were grown overnight to early log phase (0.5-2 x 10^7 cells/mL) at 30 °C (unless otherwise indicated) in complete minimal or yeast extract/bactopeptone medium with 2% dextrose[9].

G1 arrest using α-factor. Cultures at 0.5-1 x 10^7 cells/mL were treated with 10 μg/mL α-factor (5 mg/ml stock in 0.1 M HCl) until >90% of cells were unbudded (2.5-3 h depending on experiment). Because tRNA gene transcription is sensitive to nutrient availability, cells were rapidly vacuum filtered from pheromone containing media and re-suspended in conditioned medium (medium from untreated cells grown in parallel without α-factor; cells were re-suspended at the density they had reached during G1 arrest). *BAR1* deletion was used in experiments shown in Figs. 5-1, 5-2, and 5-5 to sensitize cells to α-factor. Therefore, in these experiments, cells were treated with 100 ng/mL of pheromone under the exact same conditions.

G2/M arrest using nocodazole. Cultures at 1 x 10^7 cells/mL were treated with 15 μg/ml nocodazole (5 mg/mL stock in DMSO) for 3 hours[10-12]. Cells were checked by microscopy to ensure >80% of cells were in large-budded ("dumbbell") state.

HU treatment. HU powder was added directly into flasks of cultures at 0.5-1 x 10^7 cells/mL and cells cultured with HU for the length of time indicated.

Treatment with MG132. Cultures of cells at 0.5 x 10^7 cells/mL were treated either with 70 μM MG132 (7 mM stock in DMSO) or 1% DMSO for 1.5 hours[13].

Transcription assays.

The steady state expression level of short-lived primary transcripts of tRNA genes directly reflects ongoing transcription[14-17]. Pre-tRNA transcripts were detected by northern blotting (pre-tRNA^{Leu} and pre-tRNA^{Ser})[17]. U4 snRNA (SNR14) and *SCR1* (7SL RNA) loading controls were also detected by northern blotting[16,17]. Transcripts were detected in 10 μg samples of total RNA isolated from cells that were washed with ice cold water and AE buffer, then frozen at -80 °C before SDS/hot phenol extraction[18]. Sequences of oligonucleotides used for probes for pre-tRNA^{Leu} , pre-tRNA^{Ser} , and U4 are shown in Table 2-2. A DNA probe for *SCR1* in Fig. 6-6 was prepared by random primed labeling of an *SCR1*-specific PCR product (primers F 5'- GTGGGATGGGATACGTTGAGA and R 5'-TAAACCGCCGAAGCGATCA).

Relative expression levels were determined by phosphoimager analysis using GE Healthcare Typhoon 8600 and quantified in ImageQuant TL software; normalization was to U4 snRNA or *SCR1*.

These experiments are based on the assumption that the rate of processing of pre-tRNA transcripts does not change during the course of experiments, which may not necessarily be the case. Therefore, I confirmed many results by ChIP experiments monitoring RNAPIII occupation at tRNA genes. In all cases I tested, transcription assays and ChIP results were in agreement.

Immunoblotting.

Total protein was obtained from cell samples using a trichloroacetic acid-based method[19]. Identical cell equivalents of protein were compared between samples. Actin was used as loading control. Antibodies were as follows: α-Rad53 (yC-19, Santa Cruz #sc-6749), α-actin (Millipore #MAB1501), α-myc (Millipore #9E10), α-HA (Roche #12CA5), α-CBP (Upstate #07-482).

Chromatin immunoprecipitation.

ChIP was performed as described[20]. Yeast strains expressing epitope-tagged target proteins were grown to early log (1.2-1.8 x 10^7 cells/mL). For "HU-treated" ChIP experiments, cultures were split into two and HU was added to half the cells (0.2 M concentration); the other half was left untreated. Both HU and untreated cells were incubated at 30 °C for indicated time. After growth in the absence or presence of HU, cells were subjected to a 'HU washout' procedure prior to formaldehyde cross-linking[21]. Cells were removed from HU-containing medium using vacuum filtration on 0.22 μm polyethersulfone disk (30-60 seconds), release of the vacuum, and immediate re-suspension in the filter using conditioned HU-free medium containing formaldehyde. In all ChIP experiments, cells were formaldehyde cross-linked (final concentration 1%) for 20 minutes at room temperature, harvested, and lysed by beadbeating using a Mini-Beadbeater-16 (Biospec products) for 6 cycles of 3 minutes each at maximum speed with 2 minutes in an ice-bath after each cycle of beadbeating. Chromatin in lysates was sheared using a Branson Sonifier 450 to indicated average size as

monitored by agarose gel electrophoresis. Immunoprecipitation of epitope-tagged targets from the resulting whole cell extracts was performed using 20 μL (~5.0 x 10^6 beads) of protein A-sepharose beads (Amersham) and 2 μg of an antibody that specifically binds to either the HA epitope (12CA5, see Immunoblotting) or the c-myc epitope (9E10, see Immunoblotting). Beads, antibody, and 800 μL extract were incubated for 90 minutes at room temperature with rotation. No antibody and untagged control strain immunoprecipitations were also performed to assess the background due to non-specific binding directly to beads and to ensure that enrichment is epitope-dependent. Following immunoprecipitation, beads were washed for 3 minutes each with FA lysis buffer/0.15 M NaCl (2 times), FA lysis buffer/0.5 M NaCl, ChIP wash buffer, and finally 1X TE, pH 7.5. Washed beads were incubated at 65 °C for 10 minutes in ChIP elution buffer and eluted proteins/cross-linked DNA fragements were boiled for 10 minutes prior to DNA purification. For "input" DNA, 5 μL of pre-immunoprecipitation sample of each cell extract was taken and added to 95 μL ChIP elution buffer, which was then boiled for 10 minutes prior to DNA purification. All solutions have been described[20]. DNA was purified from bulk pull-down of target proteins and pre-immunoprecipitation (input) whole cell extracts using QIAquick PCR purification columns (Qiagen) following the manufacturer's instructions.

DNA samples were either analyzed by semi-quantitative radioactive PCR or real-time PCR[20]. Primers used for ChIP analysis are listed in Table 2-3.

Semi-quantitative radioactive PCR. Primer pairs were developed which amplify different RNAPIII-transcribed genes and an internal region of the *POL1*. 25 μL

PCR reaction contained titrations of DNA to ensure linearity, 12.5 ρmol of each primer, 0.1 mM dNTPs, 2.5 μCi [^{32}P]- α-dCTP, and 1.25 Units of *Taq* DNA polymerase (Invitrogen). PCR products were separated on 13.44% polyacrylamide – 1X TBE gels. PCR products were quantified by phosphorimaging and analyzed using ImageQuant TL software (GE Healthcare). Phosphorimaging was performed using non-saturating conditions for the phosphorimaging plate. Cross-linked fold change (HU-treated compared to untreated or checkpoint mutant compared to wild type) was calculated in two steps. First, for the control and test samples, the quantified intensity of the ChIP PCR product was divided by the intensity of the input DNA PCR product. This yielded values for enrichment. Second, cross-linking fold-change was then obtained by dividing enrichment in test values by enrichment in the matched control samples (untreated cross-linking had a variability of ± 10-15%)

Real-time PCR. Real-time quantitative PCR analysis was performed in 20 μL reactions according to[20] on a Bio-Rad iQ™ 5 iCycler using the PerfeCTa™ SYBR Green SuperMix kit (Quanta BioSciences). RT-PCR reactions were performed in triplicate and quantified as described using the equation POWER(1.9, - $^{Net}C_t$)[20]. Each primer set was independently optimized for primer efficiency.

Note that in most experiments, two sub-units of each multi-protein complex were analyzed in independent experiments. TFIIIB being the exception, sub-units of the same protein complexes have remarkably similar patterns of cross-linking, as expected.

Flow cytometry.

Cellular DNA content was determined by flow cytometry as described[19]. Briefly, cells were strained with propridium iodide, sonicated, and analyzed using a FACScan flow cytometer (Becton-Dickinson).

Spotting assays.

For experiments in Figs. 3-10, 3-11, and 5-4, yeast strains were grown to log phase (2-4 x 10^7 cells/mL) at 30 °C. 10^7 cells were harvested, washed, and re-suspended in 1 mL of sterile water. Ten-fold serial dilutions (10^7, 10^6, 10^5, 10^4, and 10^3 cells/mL) were made from this suspension and 5μL aliquots from these dilutions were spotted onto plates. Glucose and glycerol containing plates were incubated at 30 °C for 2 and 5 days respectively before they were photographed.

For the plasmid complementation experiment in Fig. 3-11A, *MAF1 trp1Δ* and *maf1Δ trpΔ* strains were transformed with a low-copy *TRP1* vector that was empty (pRS314) or expressed Maf1-myc under control of it endogenous promoter (pMAF1). These strains were grown in trp^-/dextrose liquid synthetic medium, and then spotted on increasing concentrations of HU in rich medium containing glycerol as the carbon source. Plasmid retention under these conditions was confirmed by plating assays, and we demonstrated by immunoblotting that Maf1-myc is expressed at the same level regardless of the location of the cognate gene (data not shown, performed by V. Nguyen). BY4741

and *maf1Δ* strains also lacking *TRP1* were grown up in trp^{+}/dextrose liquid synthetic medium before spotting. Spotting in Fig. 3-11A was performed by D. Hockman.

RNA isolation and analysis of *RPC160*, *RPC82,* and *POL1* message abundance.

Total RNA was isolated by hot phenol extraction[18] from cells described in Fig. 6-4. cDNA was generated from isolated RNA using Quanta qScript cDNA superMIx and subjected to real-time PCR on a BioRad iCycler. *RPC160*, *RPC82*, and *POL1* expression was normalized to an internal region of *ACT1*. Primer sequences used in real-time PCR are listed in Table 2-4.

Table 2-1. Yeast strains used in this study.

Strain	Relevant Genotype	Source
W303 and derivitives		
Y300 (Wild type = W303)	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15*	[1]
Y301	Y300 *rad53-21*	[22]
maf1Δ	Y300 *maf1Δ*	This study
rad53-21 maf1Δ	Y301 *maf1Δ*	This study
RPC160-HA	Y300 *3HA-RPC160::HIS5MX6*	This study
RPC82-HA	Y300 *3HA-RPC82::HIS5MX6*	This study
RPC160-HA mrc1Δ	Y300 *3HA-RPC160::HIS5MX6 mrc1Δ::URA3*	This study
RPC82-HA mrc1Δ	Y300 *3HA-RPC82::HIS5MX6 mrc1Δ::URA3*	This study
RPC160-HA slx5Δ	Y300 *3HA-RPC160::HIS5MX6 slx5Δ::KanMX6*	This study
RPC160-HA slx8Δ	Y300 *3HA-RPC160::HIS5MX6 slx8Δ::KanMX6*	This study
TFC1-myc	Y300 *myc13-TFC1::KanMX6*	This study
TFC6-HA	Y300 *3HA-TFC6::KanMX6*	This study
TFC1-myc mrc1Δ	Y300 *myc13-TFC1::KanMX6 mrc1Δ::URA3*	This study
TFC6-HA mrc1Δ	Y300 *3HA-TFC6::KanMX6 mrc1Δ::URA3*	This study
SMC2-myc	Y300 *myc13-SMC2::KanMX6*	This study
SMC4-myc	Y300 *myc13-SMC4::KanMX6*	This study
SMC2-myc mrc1Δ	Y300 *myc13-SMC2::KanMX6 mrc1Δ::URA3*	This study
SMC4-myc mrc1Δ	Y300 *myc13-SMC4::KanMX6 mrc1Δ::URA3*	This study
POL2-myc	Y300 *myc13-POL2::KanMX6*	[23]
POL2-myc bar1Δ	Y300 *myc13-POL2::KanMX6 bar1Δ::LEU2*	This study
POL2-myc rrm3Δ	Y300 *myc13-POL2::KanMX6 rrm3Δ::NAT*	This study
POL2-myc maf1Δ	Y300 *myc13-POL2::KanMX6 maf1Δ::NAT*	This study
POL2-myc mec1-100	Y300 *myc13-POL2::KanMX6 mec1-100::LEU2(HIS)*	[23]
MCM7-myc	Y300 *myc13-MCM7::URA3*	[23]
MCM7-myc bar1Δ	Y300 *myc13-MCM7::URA3 bar1Δ::LEU2*	This study
MCM7-myc rrm3Δ	Y300 *myc13-MCM7::URA3 rrm3Δ::NAT*	This study
MCM7-myc maf1Δ	Y300 *myc13-MCM7::URA3 maf1Δ::NAT*	This study
W34b	Y300 *hta1-s129a hta2-s129a*	[24]
RAD52-myc	Y300 *myc13-RAD52::KanMX6*	This study
RAD52-myc bar1Δ	Y300 *myc13-RAD52::KanMX6 bar1Δ::LEU2*	This study
RAD52-myc rrm3Δ bar1Δ	Y300 *myc13-RAD52::KanMX6 rrm3Δ::NAT bar1Δ::LEU2*	This study
RAD52-myc mrc1Δ	Y300 *myc13-RAD52::KanMX6 mrc1Δ::NAT*	This study
RAD52-myc mrc1Δ bar1Δ	Y300 *myc13-RAD52::KanMX6 mrc1Δ::NAT bar1Δ::LEU2*	This study
pdr5Δ	Y300 *pdr5Δ::KanMX6*	This study
pdr5Δ mrc1Δ	Y300 *pdr5Δ::KanMX6 mrc1Δ::URA3*	This study
RPC160-HA pdr5Δ	Y300 *3HA-RPC160::HIS5MX6 pdr5Δ::KanMX6*	This study

BY4741 and derivitives		
BY4741	MAT**a** *his3Δ1 leu2Δ0 mat15Δ0 ura3Δ0*	[2]
RPC160-HA	BY4741 *3HA-RPC160::HIS5MX6*	This study
RPC82-HA	BY4741 *3HA-RPC82::HIS5MX6*	This study
BRF1-HA	BY4741 *3HA-BRF1::HIS5MX6*	This study
TBP-HA	BY4741 *3HA-SPT15::HIS5MX6*	This study
TFC1-myc	BY4741 *myc13-TFC1::KanMX6*	This study
TFC6-HA	BY4741 *3HA-TFC6::HIS5MX6*	This study
MAF1-myc	BY4741 *myc13-MAF1::KanMX6*	This study
trp1Δ	BY4741 *trp1Δ::HIS5MX6*	[3]
maf1Δ trp1Δ	BY4741 *maf1Δ::KanMX6 trp1Δ::HIS5MX6*	This study
maf1Δ pRS314	BY4741 *maf1Δ::KanMX6 trp1Δ::HIS5MX6* carrying [pRS314]	This study
maf1Δ pMAF1	BY4741 *maf1Δ::KanMX6 trp1Δ::HIS5MX6* carrying [pMAF1]	This study
vma2Δ	BY4741 *vma2Δ::KanMX6*	[3]
rrm3Δ	BY4741 *rrm3Δ::NAT*	This study
tof1Δ	BY4741 *tof1Δ::KanMX6*	[3]
rrm3Δ tof1Δ	BY4741 *rrm3Δ::NAT tof1Δ::KanMX6*	This study
sgs1Δ	BY4741 *sgs1Δ::KanMX6*	[3]
srs2Δ	BY4741 *srs2Δ::KanMX6*	[3]
sgs1Δ maf1Δ	BY4741 *sgs1Δ::KanMX6 maf1Δ::NAT*	This study
srs2Δ maf1Δ	BY4741 *srs2Δ::KanMX6 maf1Δ::NAT*	This study
arp8Δ	BY4741 *arp8Δ::KanMX6*	[3]
asf1Δ	BY4741 *asf1Δ::NAT*	[21]
rtt109Δ	BY4741 *rtt109Δ::KanMX6*	[3]
RPC37-TAP	BY4741 *RPC37-TAP::HIS*	[25]
RPC53-TAP	BY4741 *RPC53-TAP::HIS*	[25]
RPC128-TAP	BY4741 *RPC128-TAP::HIS*	[25]
15DaubΔ and derivitives		
15DaubΔ	MAT**a** *ade1 his2 leu2 trp1 ura3 Δns bar1Δ*	[26]
cdc28-13	15DaubΔ *cdc28-13*	[27]

[a]Plasmid described in[28]
[b]Plasmid described in[7]

Table 2-2. Oligonucleotides used for probes in Northern blotting.

Target transcript	**Sequence**	**Source**
pre-tRNA^{Leu3}	5'- CCAAACAACCACTTATTTGTTGA -3'	[17]
pre-tRNA^{Ser}	5'- GTGCCATTTCGATTTGAAA -3'	[17]
U4 snRNA	5'- CCATGAGGAGACGGTCTGG -3'	[16,17]

Table 2-3. Oligonucleotides used for ChIP.

Oligonucleotide	Sequence	Source
tI(AAU)E2 F	5’- CATCTACACGAAGGGATGGG -3’	This study
tI(AAU)E3 R	5’- GCCAGTACTAATGTTCTCCGC -3’	This study
tF(GAA)P2 F	5’- GACGCTTGGACCATTTATAAAGCAC -3’	[16]
tF(GAA)P2 R	5’- CCATAAGAGAAGGAGCAGTCAAGTTCA -3’	[16]
tL(CAA)G3 F	5’- ATGCGTATATATTGGCGCTCC -3’	This study
tL(CAA)G3 R	5’- TTGCCTGAAATGCTCGTTCT -3’	This study
POL1 +2489 F	5’- TGCACCAGTTAATTCTAAAAAGGCA -3’	[16]
POL1 +2717 R	5’- AAAACACCCTGATCCACCTCTGAA -3’	[16]
SCR1 -151 F	5’- CCCTCGGTATTCTTTTAACA -3’	This study
SCR1 +11 R	5’- CAGAAAGCCATTACAGCCTA -3’	This study
tK(CUU)G1 F	5’- ATACAATGTGGCTCGGCTACAAATC -3’	[16]
tK(CUU)G1 R	5’- TGGGACTCTTAGAAGGGAAATAGCTCT -3’	[16]
tA(AGC)F F	5’- AAGATTTCAACAAACGTTCGGG -3’	This study
tA(AGC)F R	5’- AAAGATTGTACGGGAAATGGAAAAT -3’	This study
tS(CGA)C F	5’- TGGATCTGTGAAAAAACGCCT -3’	This study
tS(CGA)C R	5’- CTGGTTGTAAATGTGCTAAGTCTGTGA -3’	This study
ARS607 F	5’- CACATTATTCGGCACAGTAGGTA -3’	This study
ARS607 R	5’- TTCTGCACAAGGCTTTTTCT -3’	This study
2800bp from *ARS607* F	5’- TCAGTCAAGGGCTCGTTCT -3’	This study
2800 bp from *ARS607* R	5’- TGCCCTGACTAGTTTGCG -3’	This study
Upstream *tA(AGC)F* F	5’- GTAAAAGCGCGGAAACAT -3’	This study
Upstream *tA(AGC)F* R	5’- TCCAAACAGAAATGATACAGGT -3’	This study
Downstream *tA(AGC)F* F	5’- ATTTTCCATTTCCCGTACAATC -3’	This study
Downstream *tA(AGC)F* R	5’- GGAGTGTGAATGGTGTGAGTG -3’	This study
HIS2 +514 F	5’- TCTCCTGTCTTACTTCCAATC -3’	[16]
HIS2 +829 R	5’- GTGAAGCTACAGGAACTCCG -3’	[16]

Table 2-4. Oligonucleotides used for gene expression (RT-PCR)

Oligonucleotide	Sequence
RPC160 F	5'- AGATTGTGAGCTGCTTGGTA -3'
RPC160 R	5'- TGAACCTGGTAGCATCGC -3'
RPC82 F	5'- GTGTAAACGACGAAGAGGAG -3'
RPC82 R	5'- CTTCAAATCGGACAACGG -3'
POL1 F	5'- GACAAAATGAAGAAAATGCTGAT -3'
POL1 R	5'- TAATAACCTTGGTAAAACACCGTG -3'
ACT1 F	5'- GCTCAATCCAAGAGAGG -3'
ACT1 R	5'- CCAAGGCGACGTAACATAG -3'

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Chapter 3

Steps of RNA polymerase III transcription targeted by the replication checkpoint during replication stress and involvement of Maf1 in repression

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Chapter 3

Steps of RNA polymerase III transcription targeted by the replication checkpoint during replication stress and involvement of Maf1 in repression

Introduction

In higher eukaryotes, a variety of proteins have important functions in directly inhibiting RNAPIII transcription and thus restraining cell growth, including tumour supressors[1-3]. Budding yeast use functionally similar mechanisms to repress this type of transcription[4]. For example, the Maf1 protein integrates many stress signals that repress RNAPIII transcription in yeast[5]. Cells lacking Maf1 are viable, but do not fully repress RNAPIII in response to an assortment of poor growth conditions. This suggests many signaling pathways associated with these stress states all impinge on Maf1 to inhibit transcription. This makes Maf1 a critical integrator of these stress pathways functioning to repress tRNA genes[6,7]. Further, *maf1Δ* cells have an elevated rate of tRNA gene transcription compared to wild type cells during unchallenged growth, indicating that Maf1 limits RNAPIII activity even during normal proliferation[8,9].

Another stress condition, replication stress, has recently been discovered to cause repression of RNAPIII transcription of tRNA genes[10]. Further, this repression requires the replication stress checkpoint. The most employed technique used to induce replication stress and activate the replication stress checkpoint is to treat the cells with the genotoxic drug HU. At a high concentration (0.2M), HU reversibly inhibits the enzyme ribonucleotide reductase

(RNR), which is responsible for the production and accumulation of the deoxynucleotide triphosphates (dNTPs) during S-phase. Without the necessary dNTP substrates, DNA polymerases slow in their progression and eventually may stall. HU treatment elicits a global activation of the replication stress checkpoint ultimately leading to the activation of Rad53 and Dun1. These members of the Chk2 family of protein kinases, when activated, prevent cell-cycle progression, stabilize paused replication forks, and block further replication initiation at unfired origins[11-13]. Cells treated with HU experience what is termed "replication stress" because they are having difficulty replicating their genome.

It is currently unclear if Maf1 functions to signal replication stress to tRNA genes. This is feasible because, in the absence of Maf1, cells are unable to fully repress RNAPIII transcription upon HU treatment. However, this does not exclude the possibility that Maf1 functions independently of the replication stress checkpoint pathway during replication stress. Here we use genetic experiments to distinguish between these possibilities. We conclude that, based on our results, Maf1 is functioning in the same pathway as Rad53 to repress tRNA gene transcription[10].

How Maf1 exerts its influence on the RNAPIII transcriptional machinery during repression has been an important area of research. One key mechanistic question is whether Maf1 inhibits the RNAPIII system by proximal association with RNAPIII transcribed genes. Previous studies have shown Maf1 is recruited to tRNA genes during nutrient limitation. Genome-wide ChIP-chip experiments, performed by two groups, mapped the loci of Maf1 enrichment in formaldehyde

cross-linked cells[14,15]. These studies found only a weak association of Maf1 with RNAPIII genes in unchallenged growth. Importantly, Maf1 association at these sites drastically increases during repressing conditions. At the same time, the RNAPIII occupancy at tRNA genes decreases during repression, which requires Maf1. This suggests that the increased association of Maf1 with RNAPIII genes and a concurrent loss of RNAPIII occupancy enables Maf1-dependant repression during nutrient starvation.

Here, using ChIP experiments, we assess the steps of RNAPIII transcription that are targeted during replication stress. We monitored the association of six subunits of the RNAPIII transcription machinery during treatment with HU. We find that during replication stress, RNAPIII and TFIIIB subunits decrease in occupancy at a tRNA gene. At the same time, TFIIIC subunits increase in their occupancy at this locus.

We also tested if the association of Maf1 increases at RNAPIII genes during HU treatment. We report an HU-correlated increase in cross-linking of Maf1 to RNAPIII transcribed genes. We suggest that replication perturbation in cells exposed to HU triggers the canonical mechanism of RNAPIII repression by Maf1.

maf1Δ cells have very high RNAPIII activity compared to wild type when grown on media containing the non-fermentable carbon source glycerol[16] and we report that *maf1Δ* cells are sensitive to HU when grown on glycerol containing medium. From this, we suggest that elevated RNAPIII activity can decrease fitness in cells experiencing replication stress.

Results

Replication stress triggers repression of the tRNA genes by replication stress checkpoint signaling.

First, I began by establishing HU treatment protocol for strong induction of replication stress. Experiments show that HU powder added directly to growth medium (to 0.2 M final concentration) and culture of cells for two hours causes early S-phase arrest and Rad53 hyper-phosphorylation in mixed populations of cells (Fig. 3-1A and B). This indicates these cells are experiencing replication stress and have activated replication stress checkpoint signaling. As well, I performed Northern blotting of total RNA extracted from HU treated and untreated wild type cells using two probes that specifically hybridize to short-lived pre-tRNA species (Fig. 3-1C and D). The abundance of these pre-tRNA transcripts is indicative of the ongoing transcription of these genes[17-20]. U4 snRNA was used as a loading control. I found the abundance of both a pre-leucine and a pre-serine tRNA transcript decreases upon HU treatment (Fig. 3-1C, compare HU treated pre-tRNA levels with untreated). This implies tRNA gene transcription is repressed in cells treated with HU in this manner. Quantifying the levels of pre-tRNA transcripts revealed that HU treatment resulted in tRNA transcription being repressed to 20-40% of untreated transcription (Fig. 3-1D). The remainder of experiments will employ this protocol for HU treatment.

I found that the replication stress checkpoint is required for full repression of transcription during HU treatment[10]. When cells lack functional Rad53, they fail to

fully repress transcription upon HU treatment. Cells lacking Rad53 are inviable so we used a strain harboring a temperature sensitive allele of *RAD53*, *rad53-21*[21]. To illustrate this point, growth of this strain at 37 °C results in inactivation of the Rad53-21 protein. These cells were treated with HU at the restrictive temperature and I monitored tRNA gene transcription by Northern blotting (Fig. 3-2A and B). Both pre-Leucine and pre-Serine tRNA species were probed. Percent of untreated transcription for each species was determined in two steps. First, the intensity of the pre-tRNA species band was normalized to the U4 band intensity to give a normalized abundance of each species. Then, this normalized abundance in HU-treated samples was divided by non-treated samples normalized abundance and expressed as a percent change from untreated. I found that *rad53-21* cells treated with HU exhibited tRNA gene transcription that was similar to untreated cells (Fig. 3-2A, compare *rad53^{ts}* +HU to *rad53^{ts}* -HU). Quantitation of three independent experiments confirmed this finding as HU treated *rad53-21* cells had tRNA gene transcription that was 100-120% of untreated cells (Fig. 3-2B). It is evident that inactivation Rad53 results in dampening of repression seen during replication stress.

Maf1 and Rad53 are in the same pathway leading to RNAPIII repression during replication stress.

Maf1, like Rad53, is needed for full repression of tRNA gene transcription during HU treatment[10] (Fig. 3-2C and D). To test the possibility that active replication stress checkpoint signaling impinges on Maf1 during replication

stress, I determined whether Maf1 and Rad53 are part of the same pathway leading to HU repression. I monitored the effect of *MAF1* deletion on the blockage of repression in *rad53-21* cells. *rad53-21 maf1Δ* double mutants were treated with HU, and Northern blotting was used to determine if the blockage of repression is additive or non-additive to the effect of the *rad53-21* single mutant (Fig. 3-3 and 3-12). I tested cells both at the permissive (30 °C) and the non-permissive (37 °C) temperatures for the mutant *RAD53* allele. Blockage of repression in the *rad53-21* strain is greater at the restrictive temperature than the permissive (Fig. 3-3), consistent with the inactivation of the mutant protein at elevated temperatures. Further, the dampening of repression was not greater in the *rad53-21 maf1Δ* double mutant at either temperature than the *rad53-21* mutation, by itself. Therefore, Maf1 is in a pathway that includes Rad53, which functions to repress tRNA gene transcription when cells are exposed to HU.

Transcription steps targeted by the replication stress checkpoint during replication stress.

The ChIP approach is a powerful method for directly observing the association of RNAPIII transcriptional machinery and regulatory factors at *in vivo* sites[22-24]. For example, evidence from studies of Maf1 repression during other stresses suggest an increased association of Maf1 with tRNA genes during repression and a decrease in the association of RNAPIII at these loci[14,15]. I used ChIP analysis to monitor RNAPIII transcription proteins and Maf1 association with tRNA genes during HU treatment.

I engineered yeast strains that expressed either HA or c-myc epitope tagged versions of six RNAPIII transcription factors from their endogenous gene location[25] (RNAPIII subunits Rpc160-HA and Rpc82-HA, TFIIIB sub-units TBP-HA and Brf1-HA, and TFIIIC sub-units Tfc1-myc and Tfc6-HA). Next, I optimized a ChIP protocol initially developed in the Struhl lab to ensure that ChIP data obtained is of the highest possible quality[26]. In particular, sonication conditions were optimized to achieve an average DNA fragment size that was less than 500 base pairs (Fig. 3-4A). This is particularity important because tRNA genes are relatively short (around 100-200 base pairs) and larger fragments than this could result in unacceptably high background. Additionally, we tested the immunoprecipitation conditions to optimize target protein depletion from ChIP whole cell extracts . We concluded that using 2 μg of anti-HA antibody and 10 μl protein A sepharose beads (~5.0 x 10^6 beads) added to HA epitope extracts resulted in an IP efficiency of about 30-50%, depending on the target (Fig. 3-4B, C and D). Whereas, 2 μg anti-myc antibody and the same amount of protein A sepharose beads added to Tfc1-myc resulted in about 80% of target being removed from supernatant. These are acceptable IP efficiencies for these ChIP experiments, as it is known that formaldehyde cross-linking interferes with immunoprecipitation through an unknown mechanism. No band migrating at the position of any of these tagged transcription proteins was detected in protein extracts prepared from wild type cells and protein A beads alone are not responsible for target immunodepletion (Fig. 3-4B-D, untagged and No anti-body).

As anticipated[19,27,28], in actively proliferating cells, cross-linking of Rpc160-HA is highly elevated at two tRNA genes, *tI(AAU)E2* and *tA(AGC)F*, over the RNA polymerase II transcribed gene *POL1*, consistent with the high level of tRNA gene transcription in these cells (Fig.3-5A). What is more, this enrichment of tDNA depends on the presence of the HA epitope and the addition of anti-HA antibody, indicating enrichment is not due to non-specific background binding during immunoprecipitation.

Rpc160-HA and Rpc82-HA cells were treated with HU and processed according to the ChIP protocol with one notable exception. HU contains a chemical group that may react with formaldehyde. The possibility the HU quenching formaldehyde cross-linking was raised by Stephen Bell and colleagues, based on the chemistry of formaldehyde cross-linking, the chemical structure of HU, and their own ChIP data[29]. To ensure that the putative formaldehyde-HU reaction was not out competing DNA-transcription protein cross-linking, we removed HU containing media by rapid vacuum filtration and immediately re-suspended cells in conditioned media containing formaldehyde. This approach has been validated for analysis of protein association with the RNA polymerase II-transcribed DNA damage response genes[30].

Cross-linked DNA purified from Rpc160-HA and Rpc82-HA immunoprecipitate was probed by quantitative PCR using primers for the *tI(AAU)E2* gene. Change in occupancy during HU treatment was determined by normalizing to occupancy in untreated cells and expressed as fold-change from untreated. Treatment with HU results in loss of association of RNAPIII sub-units to about 40% of untreated

(Fig. 3-5B and C). This percent decrease correlates well with the decrease in RNAPIII transcription with HU, which is also about 30-40% of untreated in this strain background.

A similar ChIP approach was used to monitor the change in TFIIIB and TFIIIC occupancy during replication stress. Cross-linking of TFIIIB sub-units, TBP-HA and Brf1-HA, also decreased at the same *tI(AAU)E2* gene (Fig. 3-6). In contrast, TFIIIC sub-units Tfc1-myc and Tfc6-HA, increased in their association at *tI(AAU)E2* upon HU treatment (Fig. 3-7). This is consistent with published evidence that under repressive conditions due to nutrient limitation, TFIIIC occupancy increases at tRNA genes. It has been proposed that because of the presence of TFIIIC binding sites within the transcribed region of a tRNA gene, TFIIIC may be removed by elongating RNAPIII during active transcription (after initiation) and further, repression is coupled to an increase in TFIIIC binding, due to the lack of TFIIIC removal by RNAPIII[19,27].

Bulk expression of tRNA gene transcriptional proteins seems to be unaffected by HU treatment ensuring that effects on cross-linking during replication stress are not due to changes in protein abundance (Fig. 3-8).

Maf1 association with RNAPIII transcribed genes increases during replication stress.

I next measured changes in association of Maf1 fused to the c-myc epitope with RNAPIII transcribed genes upon treatment with HU. Using the same HU treatment and ChIP protocol as before, I probed Maf1-myc cross-linked DNA by

radioactive PCR. I tested three tRNA genes and *SCR1,* the longest RNAPIII transcribed gene, which is often studied while monitoring Maf1 association. Consistently, during HU treatment, in concert with changes in transcription protein occupancy, cross-linking of Maf1 to genes transcribed by RNAPIII increases about 2.5- to 3-fold compared to cross-linking in untreated cells (Fig. 3-9). This increase is not due to an increase in abundance of Maf1 during this condition (Fig. 3-9B)

RNAPIII activity senstitizes cells to replication stress.

Maf1 is required for repression of tRNA gene transcription during replication stress. It is reasonable to hypothesize that elevated rates of RNAPIII transcription in cells lacking Maf1 could be associated with increased sensitivity to HU. It has been reported that *maf1Δ* cells are slightly sensitive to 20 mM HU in a competitive growth assay in liquid culture[31]. Our results indicated that when spotted on rich solid media with glucose as a carbon source, *maf1Δ* cells grow similarly to wild type cells when exposed to HU (Fig. 3-10A). It may be that growth on rich media, with the favored carbon source glucose, confers advantages to cells that veil the effect of HU on *maf1Δ* cells. To uncover such an effect, we spotted cells on media containing an alternative carbon source, glycerol. Although yeast cells can use glycerol for the production of energy, its non-fermentable nature makes it a poor carbon source and cells using glycerol experience energy limitation[16]. When *maf1Δ* cells are cultured in medium containing glycerol they show very high transcription compared to wild type cells

(Fig. 3-10B, compare lanes 3 and 4). This high transcription is not due to differences in cell-cycle profiles between wild type and *maf1Δ* cells (Fig. 3-10C). At normal culturing temperatures, *maf1Δ* cells are slightly sensitive to growth using glycerol (Fig. 3-11A and B, compare *MAF1* and *maf1Δ* cells on YPGlycerol with no HU). Additionally, *maf1Δ* cell growth is inhibited further by HU in glycerol containing medium (Fig 3-11A and B). This sensitivity to HU is complemented by the addition of Maf1-myc expressed from a plasmid[32]. Lastly, we conclude that HU sensitivity of *maf1Δ* cells grown using glycerol is not due to an indirect effect of abnormal general metabolism because deleting *VMA2* gene, which encodes for a vacuolar H+ -ATPase and not known to affect tRNA gene transcription[33], confers sensitivity to glycerol but, not a sensitivity to HU (Fig. 3-11B). Taken together, these results suggest that abnormally high tRNA gene transcription sensitizes cells to the challenged posed to replication by HU treatment.

Discussion

We have shown that Maf1, a common negative regulator of RNAPIII, is critical in the repression of tRNA gene transcription in response to replication perturbation by the addition of HU.

Maf1 functions by transducing active replication stress checkpoint signals into repressive RNAPIII signals during HU treatment. First, we knew previously that Maf1 is needed for HU repression (Fig. 3-2C and D). Further, I showed here that deletion of *MAF1* did not lead to a greater blockage of HU repression when combined with *rad53-21* mutation (Fig. 3-3). In addition, other work has shown

Maf1 dephosphoryltion during replication stress requires Rad53 and Rad53 is activated normally upon HU treatment in *maf1Δ* cells[10]. All of this evidence taken together leads us to propose that Maf1 functions downstream of Rad53 during replication stress conditions (and in all probability, upstream proteins of the replication stress checkpoint). It is not surprising that inhibition of RNAPIII during HU treatment requires Maf1 since numerous other cellular stress pathways impinge on Maf1 to repress tRNA gene transcription[6].

Next, using ChIP, we determined that inhibition of RNAPIII during replication stress is associated with a loss of occupancy of polymerase at the template (Fig.3-5). Further, we found that HU treatment is correlated with a loss of TFIIIB occupancy at tRNA genes (Fig. 3-6) and an increase in the occupancy of TFIIIC (Fig. 3-7). PIC formation may be targeted by active checkpoint regulation since HU repression is associated with PIC disassembly. Also, repression involves a mechanism that includes recruitment of Maf1 to tRNA genes because Maf1 association with tRNA genes increases upon treatment with HU (Fig. 3-9). This ChIP analysis of cross-linking to tRNA genes during repression elicited by HU has all the hallmarks of repression during other conditions of stress by Maf1[14,15].

Lastly, we found that under conditions of heightened RNAPIII activity *maf1Δ* cells are sensitive to HU treatment (Fig. 3-11). This sensitivity is suppressed by expression of *MAF1* from a plasmid.

Maf1 is a critical player in the replication stress checkpoint control of tRNA gene transcription. However, how activated Rad53 ultimately leads to dephosphorylation of Maf1[10] under these conditions at this point remains unclear.

One possibility is that Rad53 activates a phosphatase of Maf1. Phosphatases are known to be significant for repression of tRNA transcription during nutrient limitation. For that reason, we propose that a phosphatase of Maf1 could be important for replication stress activation of Maf1. One possible candidate is Pph3, a phosphatase that has been linked to replication stress control[34] and physically associates with a non-catalytic regulator sub-unit, Psy2, that co-purifies with Rpc25, a sub-unit of RNAPIII[35]. In contrast, the possibility still exists that active Rad53 inactivates a kinase of Maf1. In this scenario, the phosphorylation state of Maf1 would be in constant flux, being phosphorylated and dephoshorylated. During unstressed growth, the overall balance of Maf1 phosphorylation would favour phosphorylated and inactive Maf1. Inactivation of a Maf1 kinase by Rad53 during replication stress would alter the balance, resulting in more dephoshporylated and repressing Maf1. An example of such a kinase would be casein kinase II (CK2) which is known to directly regulate RNAPIII and phosphorylate Maf1[36,37].

Decreases in tRNA gene association of RNAPIII sub-units in cells experiencing replication stress are well correlated with quantified repression of RNAPIII transcription. Both tRNA gene transcription and RNAPIII occupancy at template during HU treatment are about 30-40% of untreated. This may indicate Maf1-dependant repression during replication stress is not by a mechanism that holds a significant fraction of inactive RNAPIII at transcription sites. However, we cannot rule out the possibility that Maf1 interaction with RNAPIII at tRNA genes somehow causes a conformational change in the structure of RNAPIII that

results in decreased formaldehyde cross-linking and repression without complete removal from the DNA.

TFIIIB dissociation from tRNA genes during HU may be useful for maintaining the repressed state. Previous *in vitro* experiments have shown that Maf1 can interact with TFIIIB sub-units (likely Brf1) and possibly prevent TFIIIB from forming new PIC complexes at tRNA gene promoters during prolonged repression[9,32]. Although, during acute repression, TFIIIB association remains unchanged[15]. This suggests that Maf1's ability to prevent the assembly of TFIIIB-DNA complexes may be a characteristic of extended repression, like the HU treatment protocol used here.

Maf1-dependant repression does not directly target TFIIIC occupancy during replication stress. Rather, increased TFIIIC cross-linking is likely a consequence of the repression of RNAPIII, preventing transcribing RNAPIII from removing TFIIIC from its binding sites. It may be advantageous for Maf1 not to target TFIIIC because binding of TFIIIC to promoters is the first step of PIC formation. TFIIIC association during this stress might allow for the rapid assembly of transcription complexes following the removal of HU and permit quick re-establishment of RNAPIII transcription[19]. This would diminish any lag in tRNA gene transcription following eradication of replication stress.

It is unknown if increased Maf1 association with RNAPIII genes during replication stress is transient in nature leading to dissociation of RNAPIII, or if Maf1 becomes stably bound to these sites in the course of disengaging the polymerase. If Maf1-mediated replication stress repression is similar to other

stress states, it seems likely that Maf1 binds to RNAPIII and prevents re-initiation[38]. Increased Maf1 cross-linking to RNAPIII genes may represent capture of Maf1 briefly associating with RNAPIII genes before termination of the last round of transcription. Additional work will be needed to test these models of repression associated with HU treatment.

My results reveal a paradox: How does the cross-linking of Maf1 increase at tRNA genes while the cross-linking of RNAPIII decreases at the same time when these proteins are likely physically interacting during repressing conditions? Based on my results, it is possible that Maf1 is also interacting with either proteins bound at tRNA genes or tDNA during HU treatment. This possibility is encouraged by evidence showing that Maf1 binds and targets TFIIIB during the course of repression during some poor growth conditions[9,32].

Replication interference by tRNA genes in *maf1Δ* cells during replication stress on glycerol containing media may decrease fitness because of the added burden to replication of elevated transcription at tRNA genes. In cells treated with HU, repression of RNAPIII transcription could reduce the possibility that a weakened fork paused at a tRNA gene will collapse. This suggestion is partly based on studies of chromosomal stability reported from the Weinart lab. His group reported that a fragment of around 4000 base pairs from chromosome VII of budding yeast shows chromosomal instability during unchallenged growth[39]. The site of instability contains two tRNA genes that pause replication forks. Instability is further increased in replication stress checkpoint mutants, which we know to have elevated tRNA transcription. In our speculative model, the increase in

instability at this site may be due to a failure to repress fork-blocking transcription. Our data that cells deficient in Maf1 are sensitive to HU supports the proposal that replication stress checkpoint control of tRNA gene transcription increases genome stability. Direct testing for gross chromosomal changes in cells under these conditions however has not been performed. Until such data are available, we cannot rule out the possibility that elevated tRNA gene transcription during growth on glycerol and HU decrease fitness because of aberrant changes in translation or general metabolism.

Based on our observations, we suggest the following speculative model of Maf1-mediated repression during replication stress (Fig. 1-13). During normal growth conditions, PIC assembly leads to active transcription of tRNA genes. Only a very small portion of Maf1 is associated with tRNA genes, which slightly restricts RNAPIII activity[8,9,15]. Upon addition of HU, Maf1 is dephosphorylated by a mechanism dependent on active replication stress checkpoint signaling. This enables Maf1 recruitment to tRNA genes and underlies the observed increase in association of Maf1 during HU treatment. Transcriptional repression is at the level of reduced RNAPIII occupancy at the DNA.

We propose that Maf1 repression during replication stress includes Maf1 recruitment to tRNA genes where it may physically interact with RNAPIII[38]. This Maf1-RNAPIII interaction causes the dissociation of the polymerase (and presumably Maf1) from the template. However, after two hours of HU exposure, there is still increased Maf1 cross-linking to tRNA genes over untreated samples. This may indicate that Maf1 repression during replication stress is a dynamic

process. After one RNAPIII complex is repressed at a tRNA gene, another RNAPIII PIC may attempt to assemble at this gene. Maf1 then represses this new RNAPIII complex. Therefore, Maf1 is constantly being recruited to tRNA genes to repress new RNAPIII transcriptional machineries attempting to assemble at these sites, accounting for the observed increase in Maf1 association at these sites during HU treatment.

TFIIIB is also targeted during replication stress as its occupancy is decreased by HU. TFIIIC occupancy significantly increases possibly due to a lack of removal by elongating RNAPIII.

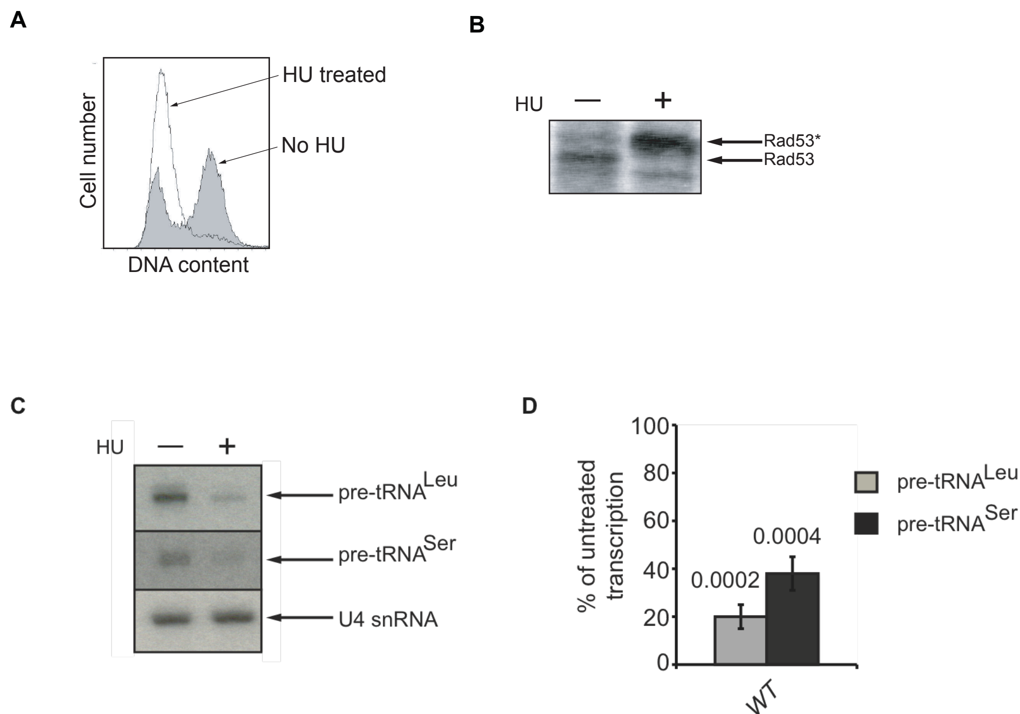


Figure 3-1. RNAPIII transcription is repressed in cells experiencing replication stress. A. Wild type cells were grown to early log phase and either not treated with HU (No HU) or treated with 0.2 M HU for two hours (HU treated). DNA content was analyzed by flow cytometry following staining of DNA with propidium iodide. **B.** Following the HU treatment described in A, total protein from HU treated (+) and untreated (-) cells was extracted and subjected to western blotting using α-Rad53 primary anti-body. Hypo-phosphorylated Rad53 is indicated by "Rad53" and hyper-phosphorylated Rad53 is indicated by "Rad53*". **C.** Northern blot of total RNA from wild type cells either HU treated (+) or left untreated (-) as above. Probes specifically hybridize to either pre-Leucine or pre-Serine tRNA species and abundance of these species was normalized to snRNA U4 recovery. **D.** Quantification of three independent experiments in HU-treated cells using phosphoimaging and ImageQuant TL software. Transcription is represented as percentage of untreated transcription. Error bars represent +/- s.d. from the mean for three independent experiments.

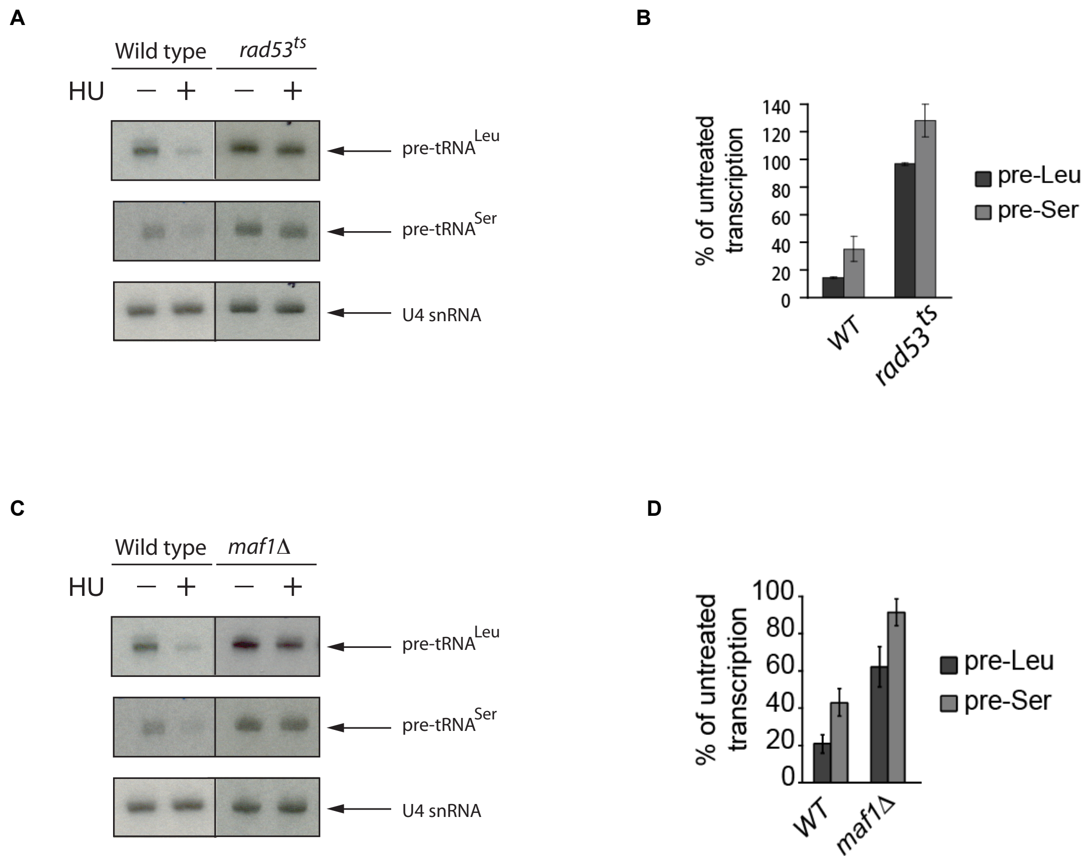


Figure 3-2. Dampening of RNAPIII repression during replication stress in *rad53-21* and *maf1Δ* cells. A. Northern blot of total RNA extracted from HU treated (+) or untreated (-) wild type and *rad53-21* cells cultured at restrictive temperature, 37 °C. **B.** Quantification of three independent experiments, such as shown in A. Transcription of each tRNA gene in HU-treated cells is represented as percent change from untreated transcription for each strain. Error bars indicate +/- s.d. from the mean. **C.** Northern blot, similar to A, using wild type and *maf1Δ* cells. **D.** Quantification of three independent experiments in C, same as previous quantitations.

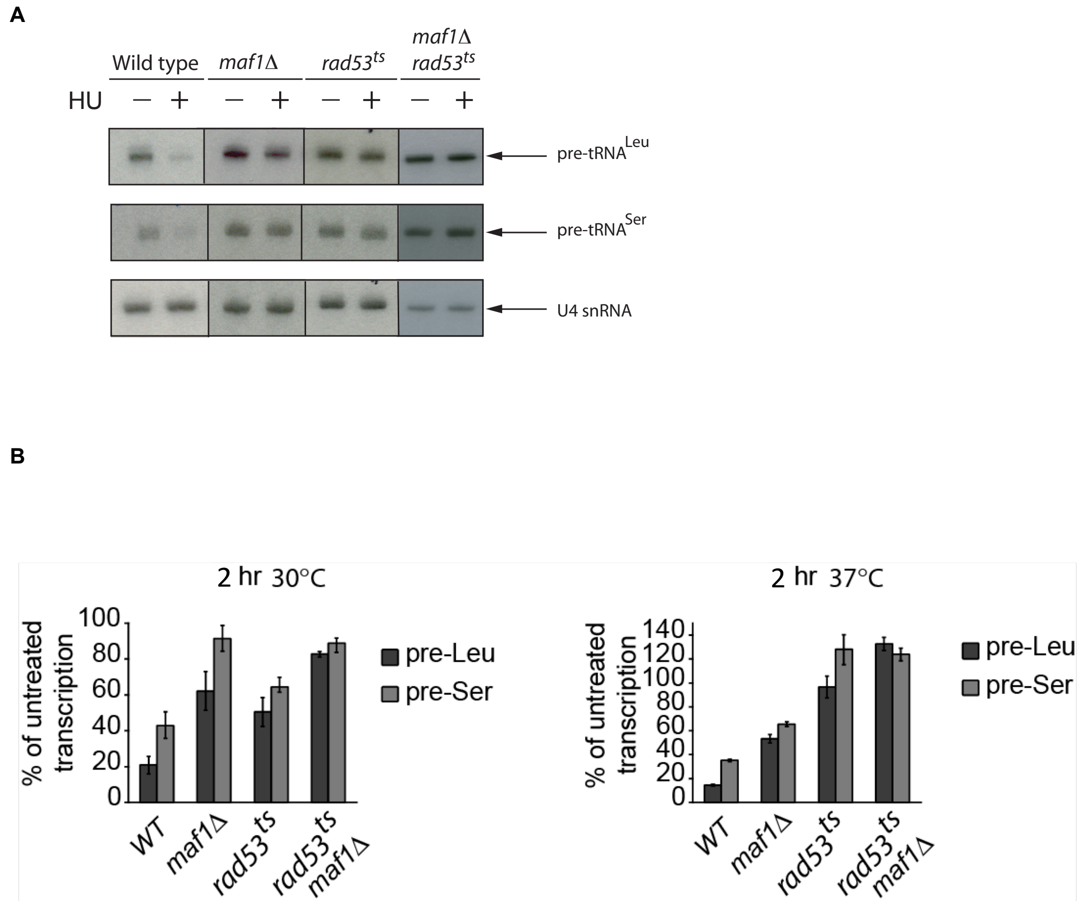


Figure 3-3. Maf1 conveys replication stress signals to the tRNA genes during replication stress. A. Northern blot monitoring pre-tRNA abundance from HU treated (+) or untreated (-) in wild type, *maf1Δ*, *rad53-21,* and *rad53-21 maf1Δ* cells cultured at 37 °C. Data from Fig. 3-2A and 3-2C is included here. **B.** Quantitation of three independent experiments of cells cultured either at permissive temperature (30 °C) and restrictive temperature (37 °C) for the *rad53-21* allele. Treatment effects are expressed as a percentage of untreated transcription.

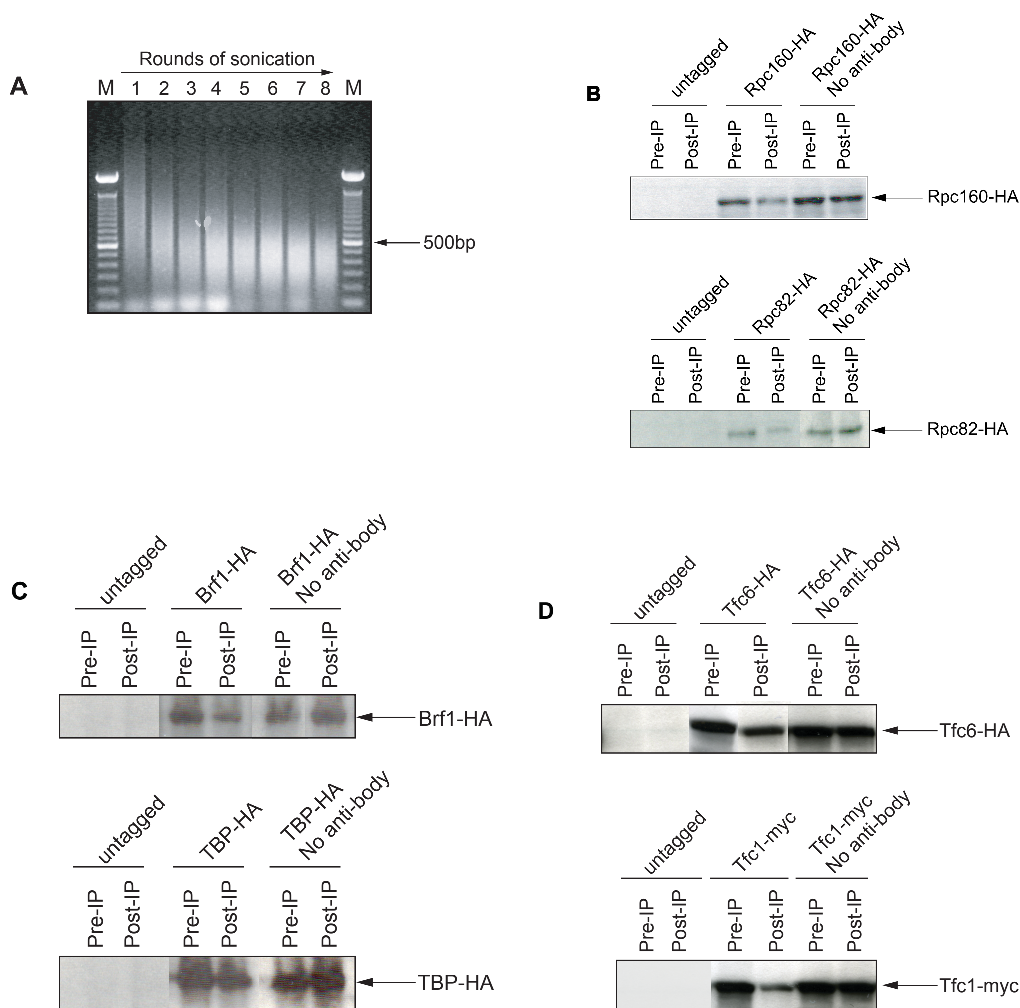


Figure 3-4. Validation of sonication protocol and determination of immunoprecipitation efficiency. A. Validation of the ChIP sonication protocol. Whole cell extracts were prepared according to [26] with minor modification. Extracts were sonicated for 1 to 8 rounds of 20 seconds each, and samples of extract were taken after each round (lanes 1-8). After formaldehyde crosslinks were reversed by boiling, purified DNA was loaded on a 1% agarose gel and stained with ethidium bromide. After the eighth round of sonication the DNA was sheared into fragments that are, on average, less than 500 bp in length. M, DNA size ladder. **B-D.** Immunodepletion for six epitope tagged transcription proteins is determined by immunoblotting. Samples of whole cell extract, prepared as in A, were taken before (pre-IP) and after chromatin immunoprecipitation (Post-IP) of cell extracts for six target proteins. Three IPs for each factor were performed: one in the absence of the epitope tag and the presence of anti-epitope antibody (untagged), one in the presence of epitope and antibody (target-epitope), and one in the presence of epitope and the absence of antibody (No antibody).

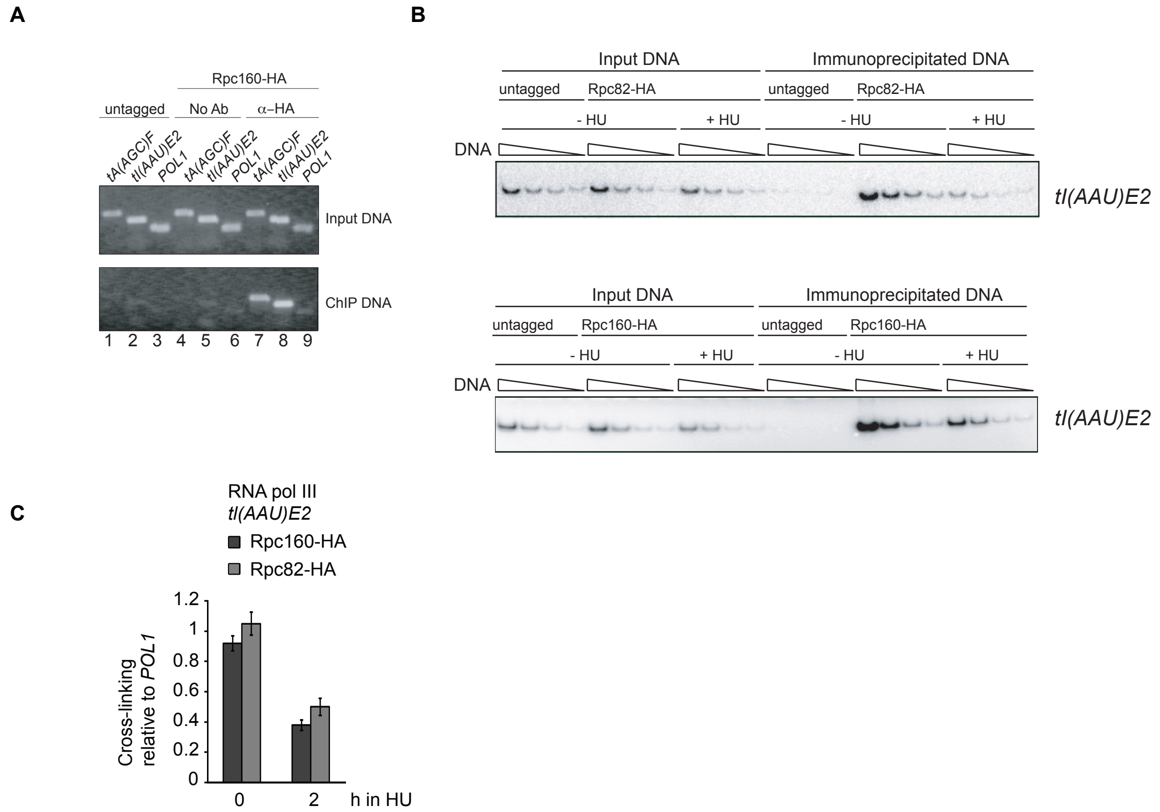
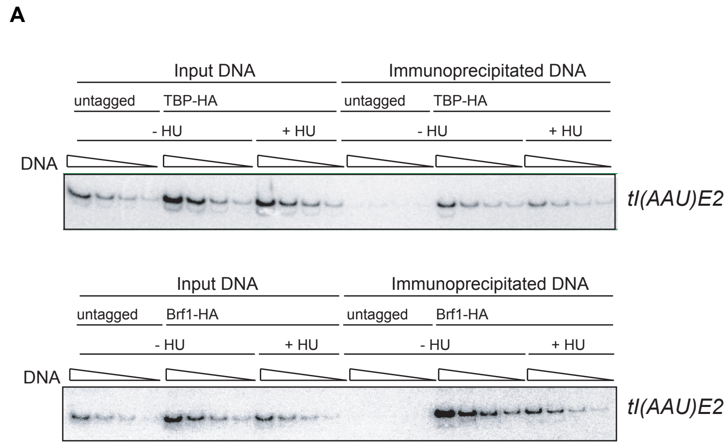


Figure 3-5. RNAPIII is enriched at tRNA genes in proliferating cells and its occupancy decreases during replication stress. A. Raw PCR data from a representative ChIP experiment of Rpc160-HA in normally cycling cells. Cross-linked DNA purified from Rpc160-HA immunoprecipitate was probed by PCR. PCR products were run on a 1% agarose gel and stained with ethidium bromide. Two tRNA genes, *tI(AAU)E2* and *tA(AGC)F*, and a negative control gene, *POL1*, were tested. Rpc160-HA is enriched at tRNA genes over *POL1* (compare lanes 7 and 8 to 9). Enrichment of DNA by IP depends on anti-HA antibody and presence of the epitope (compare lanes 7 and 8 with lanes 1,2,4,and 5). **B.** I engineered separate strains expressing HA-tagged versions of RNAPIII subunits Rpc160 and Rpc82. These strains were not treated with HU (- HU) or treated with 0.2 M HU (+HU) for two hours prior to cross-linking with formaldehyde and CHIP using α-HA antibody. Immunoprecipitated DNA was probed by quantitative radioactive PCR using primers that specifically amplified the *tI(AAU)E2* gene. 2-fold serial dilutions of input and ChIP DNA was added to the PCR reactions to ensure linearity of PCR. **C.** Quantitation of ChIP analysis of Rpc160-HA and Rpc82-HA occupancy relative to untreated cells. 0 hours in HU is sample of cells taken immediately after the addition of HU. 2 hours represents cells HU treated cells for two hours. Values in the graph are averages of three PCR quantitations +/- s.d. for a single ChIP experiment.

A



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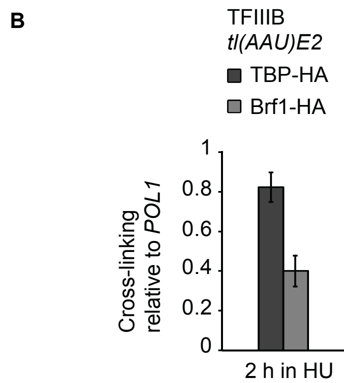
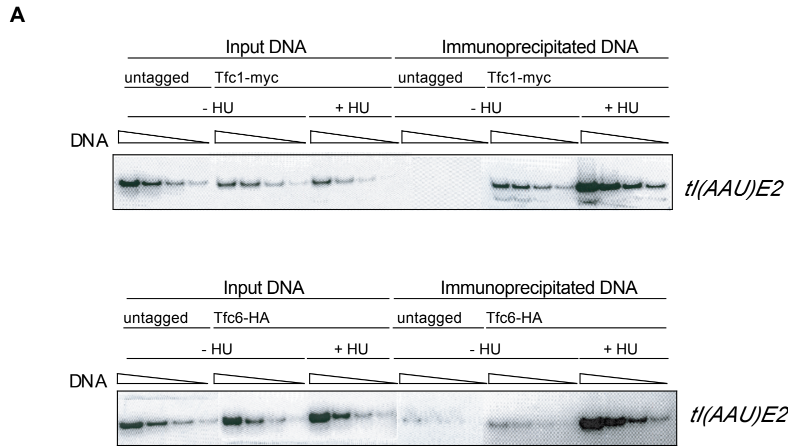


Figure 3-6. TFIIIB decreases in occupancy during replication stress. A. Raw quantitative radioactive PCR data from a representative TBP-HA and Brf1-HA ChIP experiment. Cells were either treated with 0.2 M HU for two hours (+ HU) or left untreated (-HU). Target association was tested at *tI(AAU)E2* by ChIP. **B.** Quantitation of ChIP analysis of TBP-HA and Brf1-HA occupancy relative to untreated cells. Values in the graph are averages of three PCR quantitations +/- s.d. for a single experiment. Note that TBP-HA dissociation from *t(IAAU)E2* may be masked by its cross-linking to the nearby *SLO1* gene.

A



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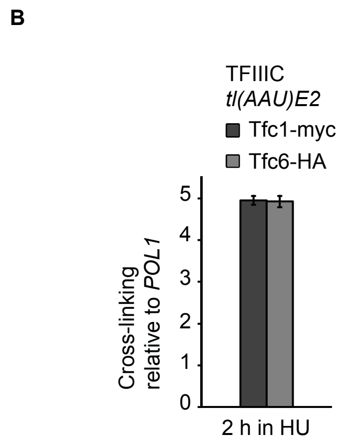


Figure 3-7. TFIIIC occupancy increases during replication stress. A. Raw quantitative radioactive PCR data from a representative Tfc1-myc and Tfc6-HA ChIP experiment. **B.** Quantitation of ChIP analysis of Tfc1-myc and Tfc6-HA occupancy. Values in the graph are averages of three PCR quantitations +/- s.d. for a single experiment.

A

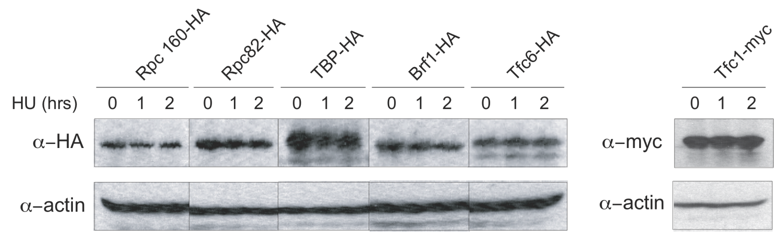


Figure 3-8. Steady-state expression of subunits of RNAPIII, TFIIIB, and TFIIIC during replication stress. Expression of epitope tagged sub-units of transcription proteins in cells treated with HU for 0, 1, or 2 hours assayed by immunoblotting.

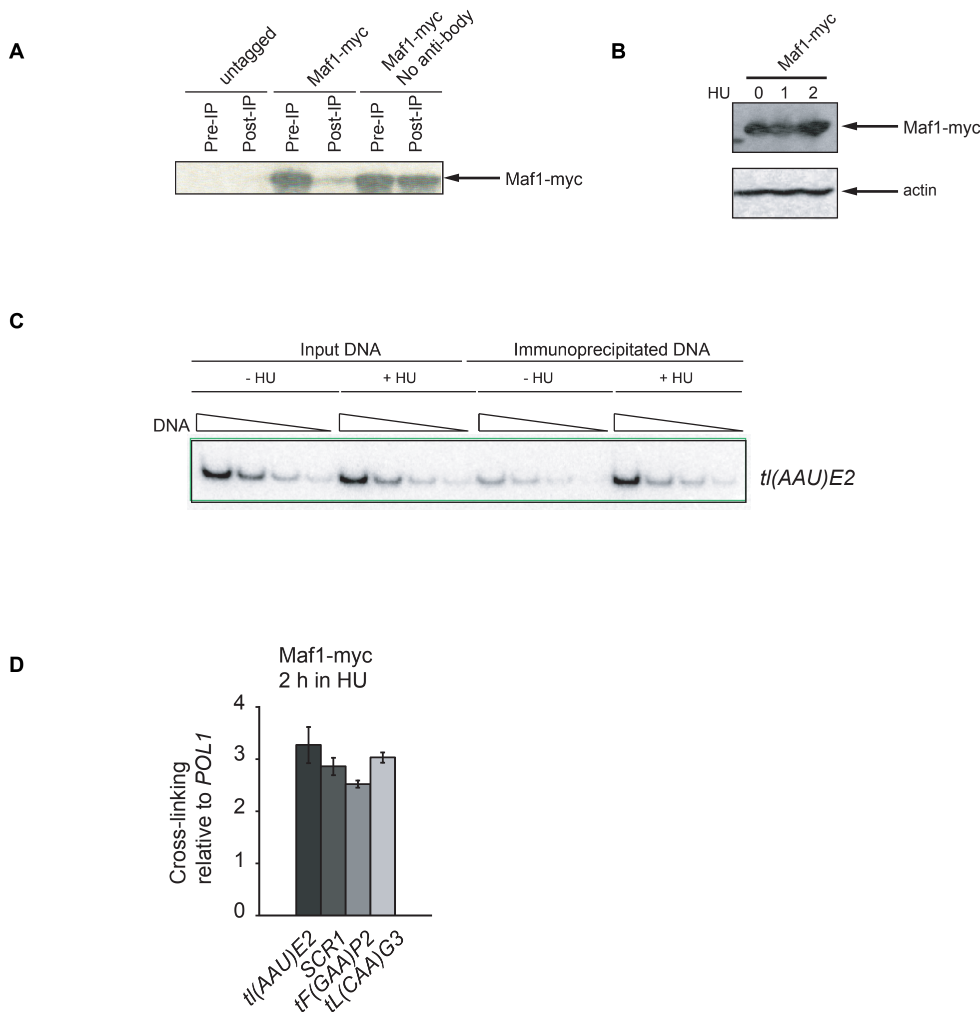


Figure 3-9. Maf1 association increases at RNAPIII transcribed genes. A. Immunodepletion of Maf1-myc using an anti-myc antibody. Samples of ChIP whole cell extract were taken pre- and post-IP. Maf1-myc immunodepletion monitored in three immunoprecipitations as in Fig. 3-4B through D. Immunoblots were performed to determine the level of Maf1-myc in each extract. **B.** Expression of Maf1-myc remains constant during replication stress as monitored by immunoblotting. **C.** Maf1-myc expressing cells were not treated (-HU) or treated with 0.2 M HU for two hours (+ HU). Following formaldehyde cross-linking of these cells, chromatin immunoprecipitation was performed using anti-myc antibody. Cross-linked DNA purified from immunoprecipitates was probed by quantitative radioactive PCR using primers for *tI(AAU)E2.* **D.** Quantitation of ChIP analysis of Maf1-myc occupancy during HU treatment relative to untreated cells at four RNAPIII transcribed genes: the tRNA genes *tI(AAU)E2, tF(GAA)P2, tL(CAA)G3* and *SCR1*. Association of Maf1-myc in cells treated with HU is compared to untreated. Values in the graph are averages of three PCR quantitations +/- s.d. for a single experiment.

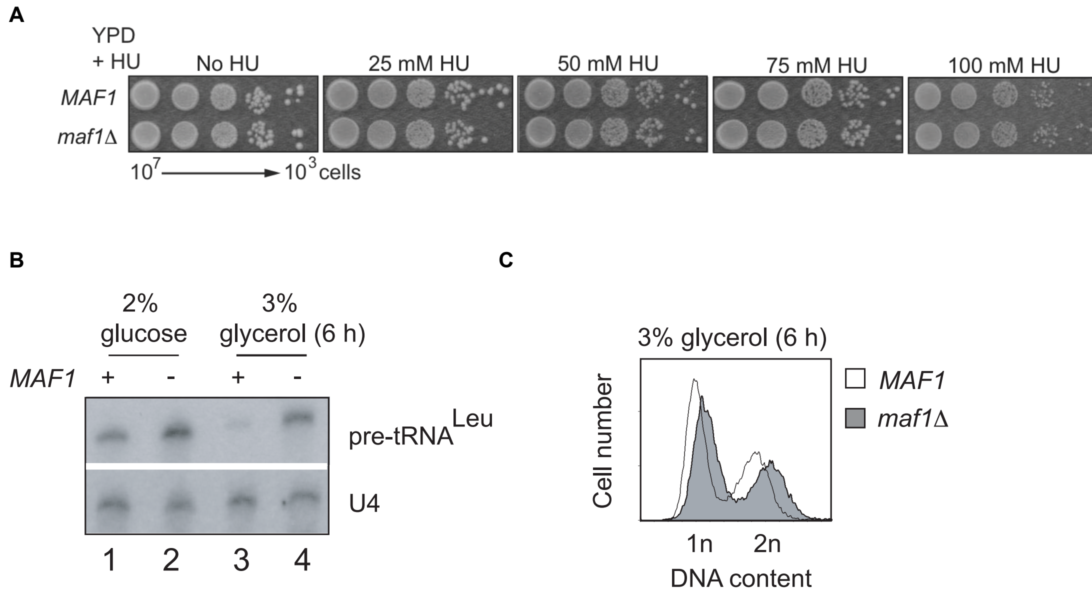
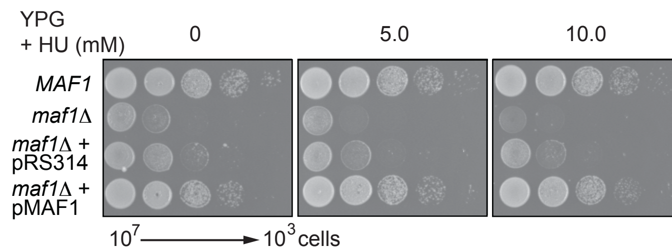


Figure 3-10. Deletion of *MAF1* does not confer HU sensitivity on rich media and results in very high tRNA gene transcription in cells using glycerol. A. *MAF1* deletion does not confer HU sensitivity during growth on rich media. Wild type and *MAF1* deleted cells were grown to exponential phase in rich medium. Serial 10-fold dilutions of these cells were spotted onto plates with 2% glucose and various concentrations of HU. Plates were incubated at 30 °C for three days. **B.** *maf1Δ* cells have high transcription during growth on glycerol when compared to wild type. Cells with Maf1 (+) or lacking Maf1 (-) were grown to early log and the cultures were split into two and the cells collected by centrifugation. Half the cells were re-suspended in medium with 2% glucose and the other half of the cells were re-suspended in medium with 3% glycerol. These cells were then incubated for 6 hours at 30 °C and total RNA extracted and analyzed by Northern blotting. **C.** *MAF1* deletion does not affect the cell cycle profile of glycerol-grown cells. DNA content was analyzed by flow cytometry after DNA staining by propridium iodide.

A



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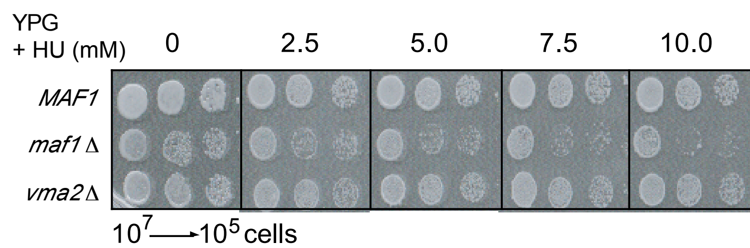

Figure 3-11. Conditions of increased RNAPIII transcription result in sensitivity to replication stress. A. *MAF1, maf1Δ, maf1Δ* + control plasmid, and *maf1Δ* + *MAF1* plasmid cells were grown to exponential phase and 10-fold serial dilutions were spotted onto plates with 3% glycerol and various concentrations of HU. Plates were incubated at 30 °C for five days. Note that empty plasmid confers some resistance to HU. The reason for this is unkown. **B.** The glycerol sensitivity of *vma2Δ* cells is not associated with sensitivity to HU. Dilutions of indicated strains were spotted on YPglycerol (3%) plates, as before, and incubated five days at 30 °C.

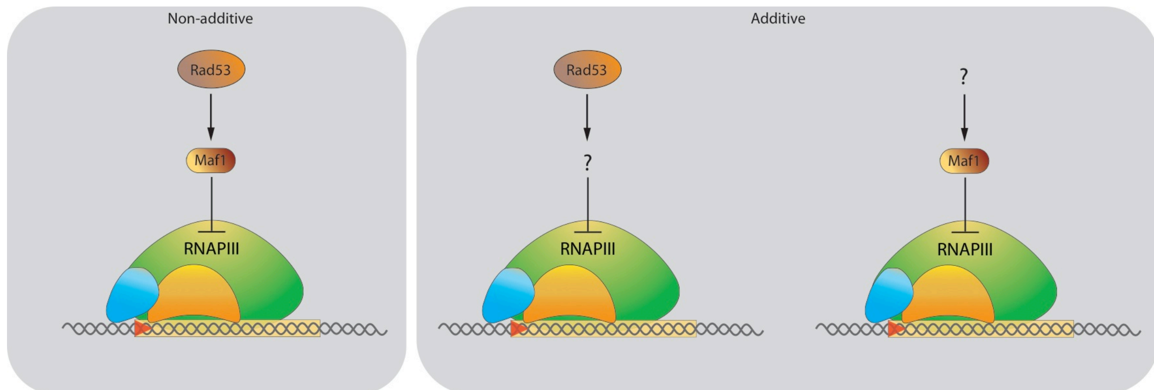

Figure 3-12. Genetic method to determine if Maf1 signals replication stress to tRNA genes. To determine if Maf1 is part of the replication stress signal to tRNA genes during HU treatment a genetic approach was used. If *maf1Δ rad53-21* double mutant blockage of repression is equal to the single mutants (non-additive effects), then Maf1 and Rad53 are likely in the same linear pathway (left panel). Conversely, if the *maf1Δrad53-21* blockage is greater than the single mutants (additive effects), then Maf1 and Rad53 are likely in different pathways leading to RNAPIII repression (right panel).

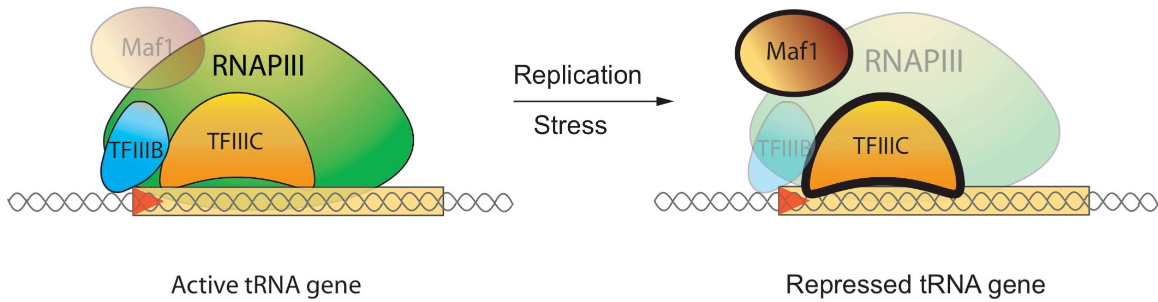

Figure 3-13. Relative transcription protein association in active and replication stress checkpoint repressed tRNA genes[40-42]. Thick lines represent increased association, transparency represents decreased association.

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Chapter 4

Insights into the control of tRNA gene transcription by the replication stress checkpoint during normal proliferation

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Chapter 4

Insights into the control of tRNA gene transcription by the replication stress checkpoint during normal proliferation

Introduction

tRNA genes are unusual loci of the yeast genome because of their ability to interfere with replication during normal S-phase[1-3]. Possibly to minimize the disruption to replication, the replication stress checkpoint restrains tRNA gene transcription during normal growth and proliferation[4,5]. This surprising discovery came while studying RNAPIII transcription in response to DNA damage (induced by treatment with methylmethanesulfonate) or to HU exposure. In a course of control experiments looking at RNAPIII activity in genotoxin untreated samples, it was noticed that mutant yeast cells, which are unable to propagate replication stress signals, have an increased rate of tRNA transcription compared to wild type cells when left untreated. Specifically, cells with inactivating mutations in *MEC1, MRC1,* and *RAD53* all have elevated tRNA gene transcription during normal, unchallenged growth. Careful analysis of these cells illustrated that replication stress checkpoint limited tRNA gene transcription during standard laboratory growing conditions[5].

Little is known about replication stress checkpoint repression of tRNA gene transcription during normal proliferation because this regulation was discovered so recently. For example, it is possible this repression involves activation of Maf1, similar to checkpoint repression during HU treatment. However, It is unclear whether Maf1 is involved in RNAPIII repression during normal growth

although deletion of *MAF1* leads to elevated transcription in cycling cells[6,7]. Nevertheless, little is reported in the literature about the specific reasons why *maf1Δ* cells have elevated tRNA gene transcription[8].

Further, much about the mechanism of replication stress checkpoint repression of tRNA gene transcription during normal proliferation is unclear. If repression during normal proliferation is like repression during HU then probably repression is due to dissociation of RNAPIII from template DNA. But, this needs to be tested experimentally because RNAPIII inhibition may include repression of transcription while holding the polymerase at the tRNA genes[9], similar to RNA polymerase II pausing seen in metazoans[10,11].

Lastly, many protein complexes are found enriched at tRNA genes in standard laboratory growing conditions[12-16]. One such complex is condensin[13,14], a complex required for mitotic genome condensation[17-19]. Condensin is one of three multi-sub-unit complexes in yeast that contain SMC (**s**tructural **m**aintenance of **c**hromosomes) proteins. This complex is constructed of two SMC proteins, Smc2 and Smc4, which are targeted to the chromosomes by regulatory non-SMC sub-units[reviewed in 20] (Fig. 4-1). Most models of condensin binding to eukaryotic chromosomes involve direct interactions with DNA because yeast SMC complexes are known to bind DNA in a non-sequence specific manner.

However, condensin binding sites *in vivo* coincide with specific DNA sequences: the tRNA genes[13,14]. The exact mechanism for condensin enrichment at tRNA genes is still under investigation, but there is evidence suggesting that

recruitment of condensin is due to physical interaction with the RNAPIII transcriptional machinery at these loci[14].

In this work, I report similarities and differences between replication stress checkpoint control of tRNA gene transcription during normal growth and replication stress. First, I present evidence that suggests that Maf1 is not involved in replication stress checkpoint restraint of transcription during unchallenged growth. This implies that during good growth conditions, the replication stress pathway targets RNAPIII transcription in a way that is independent of Maf1.

Next, in order to test if dissociation of RNAPIII from DNA is part of the repression mechanism during normal cycling, I tested RNAPIII occupancy at tRNA genes in wild type and *mrc1Δ* strains and found increased RNAPIII occupancy at tRNA genes in *mrc1Δ* cells in untreated, normally growing conditions. This indicates that association of the polymerase with template DNA is the step of transcription modulated by the stress checkpoint. In contrast to this, I report that there is very little difference in the occupancy of TFIIIC at tRNA genes in *mrc1Δ* compared to wild type cells in standard growth conditions.

Finally, knowing that in fission yeast RNAPIII transcription may be incompatible with condensin association at tRNA genes[21,22], I hypothesized here that increased tRNA gene transcription in replication stress checkpoint mutants may lead to decreased condensin association. Consistent with this hypothesis, *mrc1Δ* cells have decreased association of condensin at tRNA genes. This may have significant physiological consequences for cells lacking the replication

stress checkpoint because of the importance condensin has in tRNA gene mediated genome organization[14,21].

Results

Maf1 does not receive inputs from replication stress checkpoints during normal cycling.

As previously mentioned, deletion of *MAF1* leads to increased tRNA gene transcription during normal growth[6,7]. Based previous results, I tested for Maf1 involvement in the control of transcription by the replication stress checkpoint during unchallenged proliferation. To accomplish this, I used a genetic approach to determine if Maf1 and Rad53 were part of the same pathway leading to limitation of RNAPIII activity during this good growth condition. Similar to previous experiments, I determined the effect of *MAF1* deletion in the *rad53-21* strain by Northern blotting (Fig. 4-2). I cultured wild type, *maf1Δ, rad53-21, and rad53-21 maf1Δ* cells at both 30 °C and 37 °C to early log phase and then extracted total RNA. Northern blotting was used to determine the abundance of unstable pre-processed leucine tRNA to measure the transcription of tRNA genes[23,24]. In contrast to results obtained for cells treated with HU, I determined that Maf1 and Rad53 are likely not in the same pathway because deletion of *MAF1* results in additional elevation of transcription beyond that associated with Rad53 inactivation (i.e. greater induction of transcription in double mutant over the two single mutants alone) (Fig.4-2B and C). In addition, It appears that *rad53-21 maf1Δ* are slower growing than either single mutant; it is possible that the

highly elevated transcription of the double mutant decreases fitness in comparison to single mutants (Fig. 4-2D).

Deletion of *MAF1* does not confer defects in cell-cycle progression during normal growth. We arrested both wild type and *maf1Δ* cells in G1 with the mating pheromone α-factor and released these strains synchronously into S-phase by removing the α-factor from the medium. I followed the cell-cycle progression of these cells by taking samples of cells and staining the DNA with propridium iodide and analyzing DNA content by flow cytometry (Fig. 4-3). At every time point that we analyzed, the cell-cycle profile of the *maf1Δ* strain was very similar to the wild type, suggesting that *maf1Δ* cells do not have a global defect in cell cycle progression.

Deletion of *MRC1* results in increased RNAPIII occupancy at tRNA genes during both replication stress and unchallenged growth.

Mrc1 is a non-enzymatic adaptor protein in the replication stress checkpoint pathway. Mrc1 travels along chromatin with the replication fork[25]. Once a block to replication is encountered, Mec1 kinase is recruited to the obstructed fork where it physically interacts with and phosphorylates Mrc1[26]. This sets up a physical platform at the stalled fork where Rad53 is recruited and activated by Mec1 phosphorylation[27,28].

Cells lacking Mrc1 are viable, but are unable to propagate replication stress checkpoint signals[29] and we have found *mrc1Δ* cells have elevated tRNA gene

transcription[5]. Here, I used *MRC1* deletion as a way to disrupt replication stress checkpoint in normally cycling cells while maintaining viability of the cells.

I used ChIP to measure association of RNAPIII with tRNA genes in wild type and *mrc1Δ* cells. To begin, Rpc160-HA, Rpc82-HA, Rpc160-HA *mrc1Δ*, and Rpc82-HA *mrc1Δ* cells were treated with 0.2 M HU, for two hours. This was followed by formaldehyde cross-linking and processing of cells by the ChIP procedure. Using both quantitative radioactive PCR and real-time PCR, I probed the DNA cross-linked to RNAPIII sub-units purified from immunoprecipitates. Wild type cross-linking was arbitrarily set to one and *mrc1Δ* cross-linking was compared to wild type as a fold-change. There is a 2-3 fold increase in RNAPIII sub-unit cross-linking at *tF(GAA)P2* and *tK(CUU)G1* tRNA genes in *mrc1Δ* (Fig. 4-4) under HU treatment, implying that Mrc1 is involved in signaling that leads to dissociation of RNAPIII during replication stress. These data add evidence to our model that during replication stress, repression by the replication stress checkpoint is by dissociation of RNAPIII from tRNA genes.

Next, I assayed RNAPIII cross-linking in wild type and *mrc1Δ* during normal growth and compared this to HU treated cells. The same strains as before were grown to early log and cells were harvested for ChIP processing and cross-linking quantitation, as done previously. Similar to HU treated cells, *mrc1Δ* cells experiencing good growth conditions have greater RNAPIII sub-unit cross-linking to tRNA genes than wild type (Fig. 4-5A and B). This is in harmony with the elevated tRNA gene transcription seen in these cells.

Increased Rpc160-HA cross-linking in *mrc1Δ* was not due to an increase in the cellular abundance of this protein. By immunoblotting, it is clear the level of Rpc160-HA in wild type and *mrc1Δ* cells is similar (Fig. 4-5C). Additionally, *mrc1Δ* cells have a similar cell-cycle profile to wild type (Fig. 4-5D). Therefore, differences in cross-linking in *mrc1Δ* cells are not due to cell-cycle defects. All of this taken together implies that during unchallenged growth, replication stress signaling represses RNAPIII transcription by targeting RNAPIII association with DNA, similar to cells treated with HU.

Deletion of *MRC1* results in little change in TFIIIC occupancy at tRNA genes during normal proliferation.

Repression of RNAPIII transcription is often correlated with changes in the association of transcription factors[30,31]. To determine if repression by the replication stress checkpoint during normal, unchallenged growth is correlated with a increase in TFIIIC occupancy, I tested cross-linking of two epitope tagged TFIIIC sub-units, Tfc1-myc and Tfc6-HA, to tRNA genes in wild type and *mrc1Δ* cells. I grew wild type and *mrc1Δ* strains expressing epitope-fused sub-units to early log phase and collected and processed these cells according to our standard ChIP procedure. I ascertained cross-linking of both Tfc1-myc and Tfc6-HA to four tRNA genes: *tK(CUU)G1, tA(AGC)F, tS(CGA)C,* and *tF(GAA)P2.* Optimization of PCR conditions used to probe these genes was performed to ensure PCR was in linear range and PCR efficiency was high. Neither of these TFIIIC sub-units differed to a great extent in occupancy at any of these genes

(Fig. 4-6). This is in contrast to the situation in cells treated with HU to induce replication stress: under these conditions, TFIIIC occupancy increased at tRNA genes during repression. Here, elevated tRNA gene transcription in the *mrc1Δ* strains does not affect TFIIIC occupancy at these loci.

mrc1Δ cells have decreased association of condensin with tRNA genes during unchallenged growth.

Lastly, I ascertained whether or not condensin association with tRNA genes differed in *mrc1Δ* cells compared to wild type. First, I created yeast strains that expressed myc tagged sub-units of condensin, Smc2-myc and Smc4-myc, in both a wild type and *mrc1Δ* backgrounds. Then, using ChIP, I determined the level of cross-linking of these sub-units to tRNA genes. Both Smc2-myc and Smc4-myc were enriched at tRNA genes compared to *POL1* negative control region (Fig. 4-7A). These enrichments depended on the use of the anti-myc antibody in the immunoprecipitation. Further, at three tRNA genes, cross-linking of both Smc2-myc and Smc4-myc is decreased in *mrc1Δ* (Fig. 4-7B and C).

Discussion

Our genetic analysis of *MAF1* and *RAD53* mutants suggested that replication stress triggered by HU treatment results in Rad53-dependant activation of Maf1. Knowing that compromising either the replication stress checkpoint or Maf1 leads to elevated RNAPIII transcription in normal proliferation led us to test if a similar relationship existed between Maf1 and Rad53 in the absence of a exogenous

replication inhibitor. In contrast to previous findings, I report here that Maf1 does not contribute to RNAPIII repression by the replication stress checkpoint during normal growth.

I determined by ChIP how the protein occupancy of tRNA genes differs in *mrc1Δ* cells compared to wild type cells. Both during replication stress and during unchallenged proliferation RNAPIII occupancy at tRNA genes is greater in *mrc1Δ* strains than wild type. From this, I suggest the regulation of transcription during normal growth is similar to regulation during replication stress in that repression is due to dissociation of RNAPIII from template.

Repression by Maf1 often results in an increase in TFIIIC occupancy at tRNA genes[32,33] (also see chapter 3). Because Maf1 does not seem to be involved in signaling to tRNA genes during normal cycling, I tested if TFIIIC occupancy changes when *MRC1* is deleted. Even though transcription is high and RNAPIII occupancy is increased in *mrc1Δ* cells, very little difference in TFIIIC occupancy is detected in this strain compared to wild type cells during good laboratory growth conditions, dissimilar to RNAPIII regulation by active checkpoint signaling during replication stress.

Lastly, condensin enrichment at tRNA genes is important detrminant of the three-dimensional arrangement of the yeast genome[14,21]. As reported in fission yeast, high tRNA transcription may compromise condensin association with these genes[21,22]. Interestingly, I find evidence that condensin association with tRNA genes is decreased in cells lacking Mrc1.

My initial results suggest that the target for RNAPIII repression during normal growth and proliferation is association of the polymerase with the tRNA genes. Likely, this dismantling of the RNAPIII-template DNA complex is in response to Mrc1 function (and presumably the rest of replication stress checkpoint signaling). The mechanism of checkpoint dependant dissociation of RNAPIII from DNA during normal cycling is unknown. Our data, thus far, suggest that Maf1 is not an important contributor of replication stress checkpoint restraint of tRNA gene transcription during normal growth.

Consequently, this raises the possibility the replication stress pathway controls a step in transcription not regulated by Maf1. A possible Maf1-independent target of repression is RNAPIII “facilitated recycling”, where the same RNAPIII is re-loaded and transcribes the same tRNA gene it has transcribed previously[34,35]. Polymerase re-cycling must involve significant coupling between transcription termination and initiation so that the RNAPIII could be passed back and re-loaded at the transcriptional start site after the completion of each round of transcription. The Willis lab suggests that re-cycling of the polymerase is not subject to Maf1 repression *in vitro*[36]*.* Thus, it is possible that the replication stress checkpoint is negatively effecting polymerase re-cycling to repress transcription during normal growth.

Another possible mechanism of repression by the replication stress checkpoint could involve chromatin re-organization. Cells use a number of strategies to regulate chromatin structure to make DNA available for various processes, such as transcription. One such strategy involves the action of “chromatin remodelers”,

which facilitate chromatin “opening” by modulating nucleosome position or composition[37].

The most abundant ATP-dependent chromatin remodeler of yeast, RSC, is found at tRNA genes[15]. RSC is a member of the SWI/SNF-family chromatin remodeling complexes and RSC repositions and ejects nucleosomes *in vitro*[38]. Studies have demonstrated tRNA genes are generally deficient of nucleosomes[39-41]. It is suggested that RSC may help keep tRNA genes devoid of nucleosomes because inactivation of the main catalytic subunit of RSC makes these loci susceptible to assembly into chromatin, and causes a general loss of RNAPIII occupancy[15,42]. Also, there is evidence that RSC is involved in RNAPIII repression during nutritional stress[43]. It could be active replication stress checkpoint signaling impinges on the acitivity of RSC and leads to chromatin reorganization that affects RNAPIII loading at tRNA genes or the stable association of the polymerase with these loci.

From the available evidence combined, I suggest the following model in which the signaling proteins of Mec1, Mrc1, and Rad53 constitute the core of a signaling system that is required for repression of tRNA gene transcription during normal cycling in wild type cells. Then, if these cells are treated with the genotoxin HU, further perturbation of fork movement leads to full engagement of the checkpoint signaling system. In this context, the common RNAPIII repressor, Maf1, functions to transduce replication stress checkpoint activation to repressive signals, which impinge on the tRNA genes (Fig. 4-8).

The fact that Rad53 and Mrc1 are essential for the repression of tRNA gene transcription during normal growth does not exclude the possibility of a direct contribution from Mec1. For instance, Mec1 might directly regulate a chromatin remodeler or modulate the activity of a transcription factor, in addition to triggering phosphorylation of Rad53 through the function of Mrc1.

TFIIIC occupancy in wild type cells increases during HU treatment, which is consistent with Maf1-dependant repression reported in the literature[32,33]. Therefore, TFIIIC occupancy not changing in untreated *mrc1Δ* cells is in agreement with the finding that Maf1 is not involved in replication interference repression during normal growth.

It is interesting that deletion of *MRC1* results in little change in TFIIIC occupancy, but a significant decrease in condensin association because this contradicts common models of condensin loading at tRNA genes. D'Ambrosio *et al.* proposed that TFIIIC binding sites constitute a chromosomal feature that is important for productive condensin association in both interphase and mitosis[13]. Using ChIP-chip, they show that condensin binding sites coincide with TFIIIC binding sites. Further, inactivation of TFIIIC or mutation of the TFIIIC promoter binding site results in decreased condensin association. From this, they propose a TFIIIC centered condensin loading pathway. Based on this proposal, we would predict that the little differences in TFIIIC occupancy in *mrc1Δ* cells compared to wild type would result in little change in condensin association, if TFIIIC were indeed responsible for condensin loading during normal cycling. One possible reason for this discrepancy may be technical. In all of the experiments that

D'Ambrosio *et al.* performed, the cells were arrested in cell cycle progression using either α-factor, HU, or the microtubule polymerization inhibitor nocodazole. All of these agents, in addition to cell cycle arrest, lead to a moderate to severe repression of tRNA gene transcription (and possibly increased occupancy of TFIIIC at tRNA genes) (See Chapter 3 and 6). Therefore, repression of RNAPIII in the course of their experiments may be confounding the results.

In this same paper[13], these authours report that another complex, the cohesin loader Scc2/4, is found at tRNA genes and is required for full association of condensin, in addition to TFIIIC. It may be that replication stress signaling modulates Scc2/4 in a way that promotes condensin recruitment to these loci. In addition, Haeusler *et al.* have reported a physical interaction of TFIIIB and condensin, which does not depend on DNA, and suggested TFIIIB is also important in recruitment of condensin to tRNA genes[14]. Disruption of replication stress checkpoint in normally cycling cells could result in modulation of TFIIIB occupancy, and therefore decreased condensin association.

I propose that the inactivation of the replication stress checkpoint may result in changes in protein complex association with tRNA genes (Fig.4-9). Specifically, in association with increased tRNA gene transcription, *mrc1Δ* cells have increased RNAPIII occupancy at DNA and decreased association of condensin.

Decreased condensin association at these sites could have a number of physiological effects on cells deficient in replication stress checkpoint signaling. Condensin has ATP-dependant DNA compaction activity that is proposed to function during mitosis[17,18]. If the replication stress pathway is linked to the

decrease in association of condensin in cycling cells, there is the prospect that inactivation of replication stress checkpoint results in defects in DNA condensation. Condensation is critical to the proper segregation of newly replicated sister chromatids in preparation for cell division[44,45]. Misregulation of this process may lead to genomic instability in replication stress checkpoint mutants during anaphase as improperly condensed sister chromatids are pulled apart.

Condensin also has important functions during interphase. tRNA genes are physically clustered in the nucleolus in budding yeast[46]. Work in the Engelke laboratory has shown that condensin is required for the clustering of tRNA genes[14]. They clearly showed using microscopic techniques that inactivation of five temperature sensitive mutants of condensin sub-units resulted in disruption of clustering. Because these genomically dispersed tRNA genes are clustered in the nucleolus in wild type cells, it is inferred these loci have significant effects on overall three-dimensional genome organization. We speculate the decrease in condensin association in replication stress checkpoint mutants may result in a decrease in clustering of tRNA genes and have an effect on genome architecture. This speculation builds on a number of observations. RNAPIII transcription is known to have an inhibitory effect on the clustering of tRNA genes at the centromeres of fission yeast[21]. Decreasing RNAPIII transcription by mutation of TFIIIC sub-unit, Sfc3, enhances centromeric association of these genes. It was further shown that the blockage of normal localization is linked to a decrease in the association of condensin with dispersed tRNA genes. This

suggests the condensin complex may be released from tRNA genes during RNAPIII transcription and that transcription can inhibit the clustering of these genes[21,22].

Finally, there is a dose dependant relationship between replication fork pausing and RNAPIII transcription at tRNA genes[1]. Disruption of replication stress signaling, which results in increased tRNA gene transcription and an increase in occupancy of fork-blocking RNAPIII, may result in greater fork pausing at tRNA genes and possibly increased propensity for replication fork collapse. During unchallenged proliferation, replication stress signaling may reduce the likelihood of fork collapse by rearranging the nucleo-protein complex to a configuration that allows replication forks to progress past these genes[5] (i.e. repression of RNAPIII). This mechanism would be important for maintaining genomic stability as collapse of forks is repaired by mechanisms that result in gross chromosomal rearrangements.

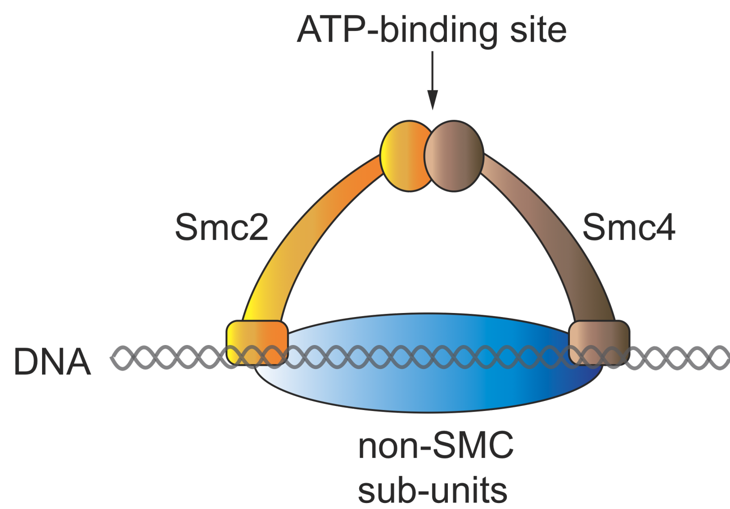

Figure 4-1. Simplified schematic of condensin. Condensin is a multi-subunit complex involved in DNA compaction. SMC sub-units are targeted to DNA by non-SMC sub-units. Smc2 and Smc4 interact to form an ATP-binding site.

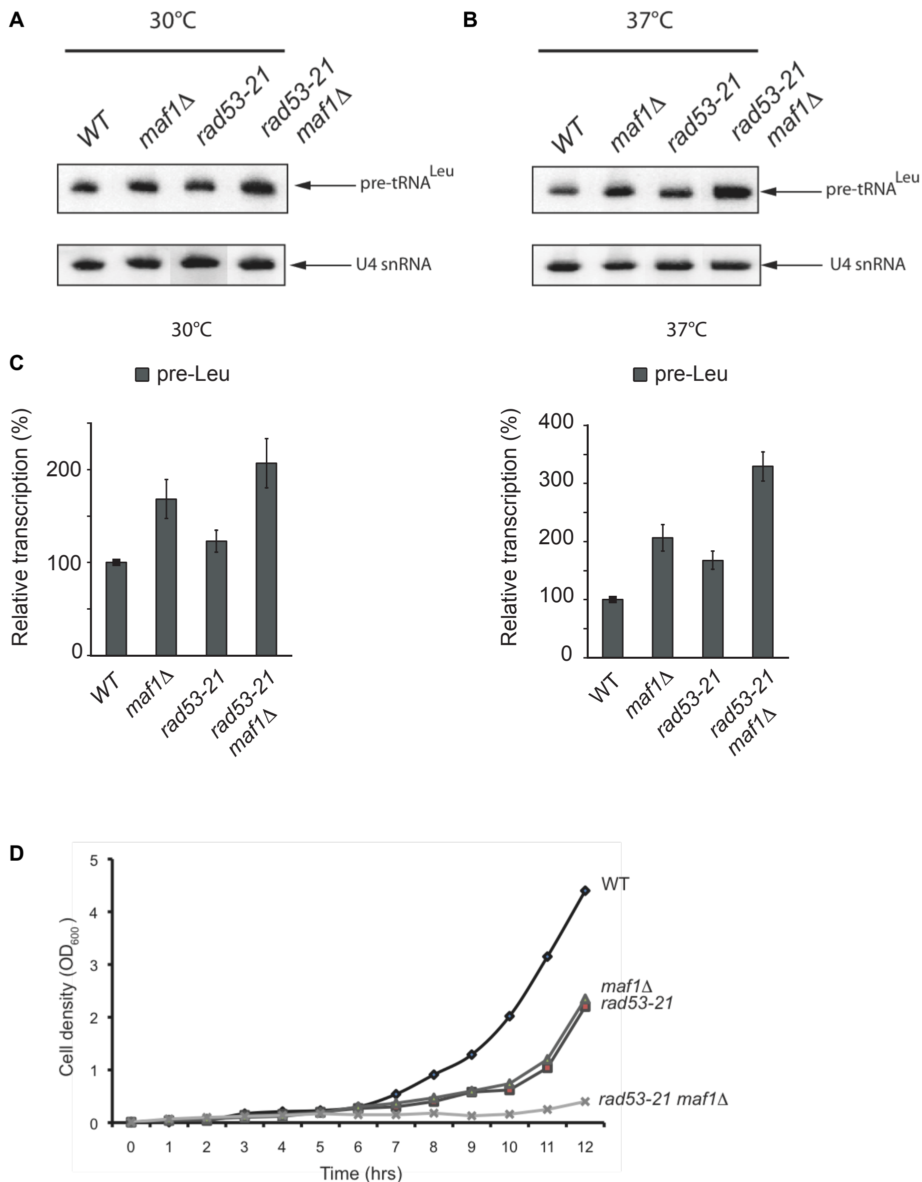

Figure 4-2. Maf1 does not mediate replication stress checkpoint control of tRNA gene transcription during normal cycling. A. Wild type, *maf1Δ, rad53-21, and rad53-21 maf1Δ* cells were grown to early log phase at 30 °C. Total RNA was extracted and probed by Northern blotting. **B.** Same as in A, except cells cultured at 37 °C. **C.** Quantification of A and B, using phosphoimaging and ImageQuant TL software. Error bars represent s.d. of a triplicate experiment. **D.** *rad53-21 maf1Δ* are slower growing than either single mutant. Wild type, *maf1Δ, rad53-21, and rad53-21 maf1Δ* cells were grown to early log phase overnight and seeded in fresh media at OD_{600} 0.05. Cell densities were taken every hour by spectrophotometer.

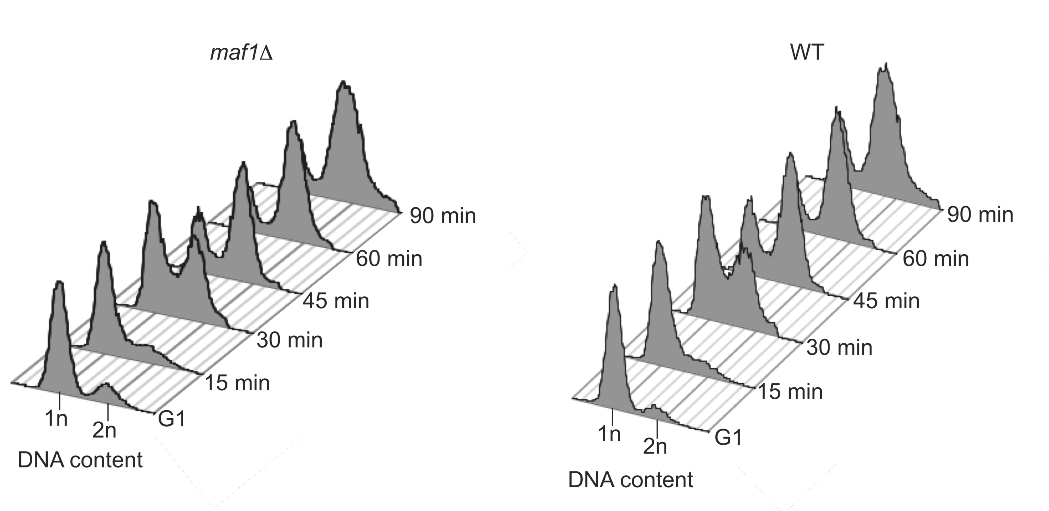

Figure 4-3. Deletion of *MAF1* does not alter cell-cycle progression following G1 arrest and release. *maf1Δ* and WT cells were arrested in G1 using α-factor added to the medium (G1). Cells were then released synchronously from arrest by removing cells from α-factor containing medium by vacuum filtration and re-suspending cells in pre-conditioned media. Samples of cells were taken at the indicated time points following re-suspension and subjected to analysis by flow cytometry using propridium iodide to stain DNA.

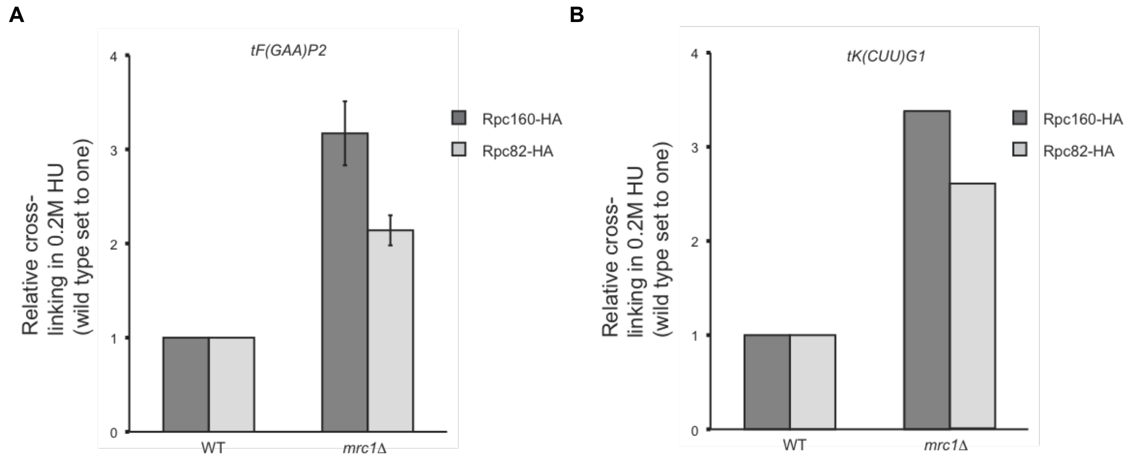

Figure 4-4. Occupancy of RNAPIII at tRNA genes is increased in *mrc1Δ* cells compared to wild type during HU treatment. A. Formaldehyde cross-linking of Rpc160-HA and Rpc82-HA to *tF(GAA)P2* during replication stress is analyzed by ChIP in wild type and *mrc1Δ* cells. Cells were treated with 0.2 M HU for two hours prior to formaldehyde cross-linking. Chromatin immunoprecipitate is probed by quantitative radioactive PCR. Cross-linking of Rpc160-HA and Rpc82-HA in *mrc1Δ* is compared to wild type cross-linking, which is arbitrarily set to one. Error bars represent quantitation of three independent PCR reactions +/- s.d. for a single ChIP experiment. **B.** Comparison of Rpc160-HA and Rpc82-HA cross-linking to *tK(CUU)G1* in wild type and *mrc1Δ* strains during the HU treatment as above. Chromatin immunoprecipitate is probed by quantitative real-time PCR analysis. *mrc1Δ* cross-linking is compared to wild type, as above.

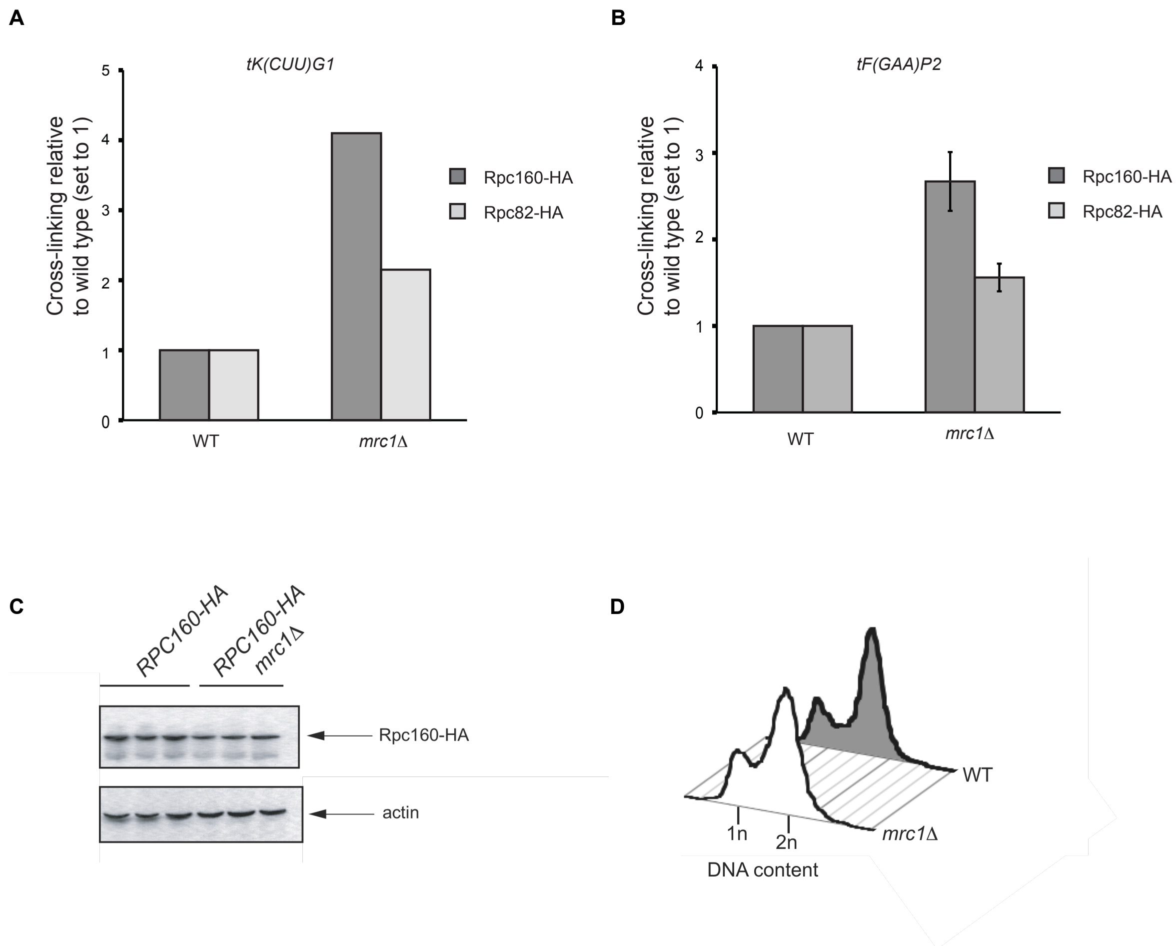

Figure 4-5. Occupancy of RNAPIII at tRNA genes increases in *mrc1Δ* cells during normal, unchallenged proliferation. A. Cross-linking of Rpc160-HA and Rpc82-HA to *tF(GAA)P2* during normal growth in wild type and *mrc1Δ.* Cells grown to early log phase growth and subjected to ChIP analysis. Cross-linking was quantified by quantitative radioactive PCR analysis. Cross-linking of transcription proteins in *mrc1Δ* is normalized to wild type. Error bars represent quantitation of three independent PCR reactions +/- s.d. for a single ChIP experiment. **B.** Comparison of Rpc160-HA and Rpc82-HA cross-linking to *tK(CUU)G1* in wild type and *mrc1Δ* strains during early log growth. Chromatin immunoprecipitate is probe by quantitative real-time PCR analysis. Cross-linking in *mrc1Δ* cells is compared to wild type as before. **C.** Increase in Rpc160-HA occupancy at tRNA genes is not due to an increase in abundance of this protein in *mrc1Δ* cells. Rpc160-HA abundance assayed by immunoblotting in wild type and *mrc1Δ* cells cultured under the same unchallenged growth conditions in A and B. **D.** Cell cycle profiles of wild type and *mrc1Δ* strains are similar. DNA content of cycling cells is analyzed by flow cytometry.

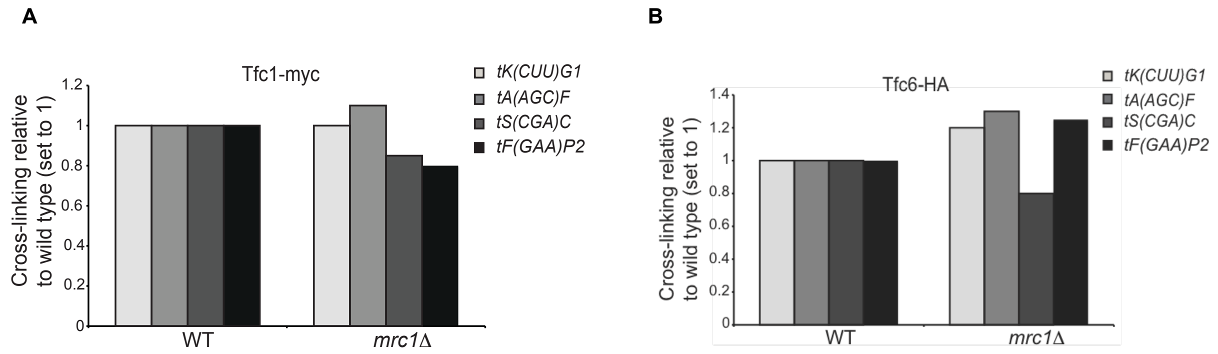

Figure 4-6. TFIIIC occupancy at tRNA genes does not change in *mrc1Δ* cells. A and B. Tfc1-myc and Tfc6-HA cross-linking at four tRNA genes, indicated in the legend, is compared in wild type and *mrc1Δ.* Cross-linking is determined by ChIP analysis. Quantitation of DNA precipitated with TFIIIC sub-units was by quantitative real-time PCR analysis. Wild type sub-unit association is arbitrarily set to 1 and *mrc1Δ* association is compared to wild type.

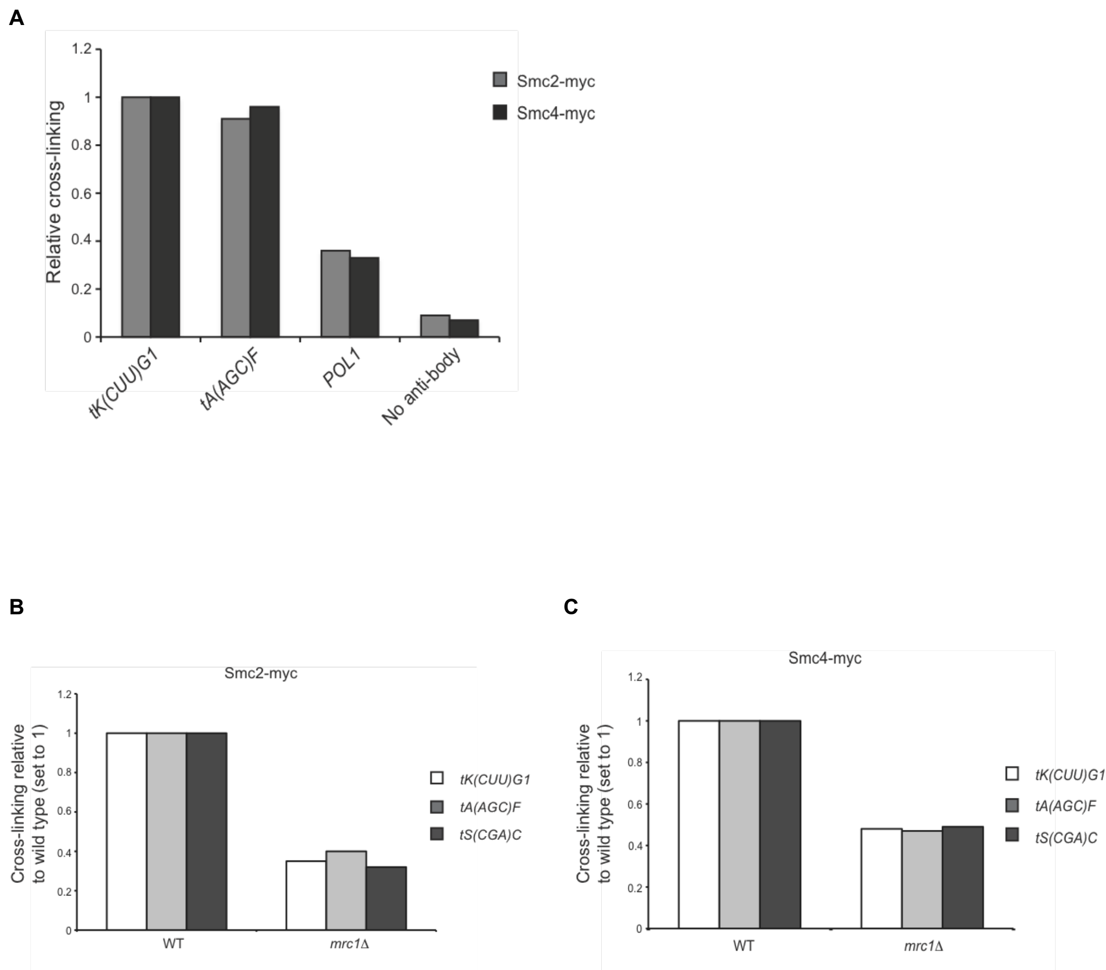

Figure 4-7. Condensin association with tRNA genes may decrease in *mrc1Δ* cells. A. Association of condensin sub-units is elevated at tRNA genes. Relative cross-linking is determined using ChIP procedure with or without anti-myc anti-body. Cross-linking at *tK(CUU)G1* is arbitrarily set to one and cross-linking at *tA(AGC)F, POL1,* and No anit-body is compared to *tK(CUU)G1.* **B and C.** Association of c-myc epitope tagged sub-units of condensin (Smc2-myc and Smc4-myc) at three tRNA genes is compared in wild type and *mrc1Δ.* Cross-linking is determined by ChIP analysis. Quantitation of immunoprecipitated DNA was performed as before.

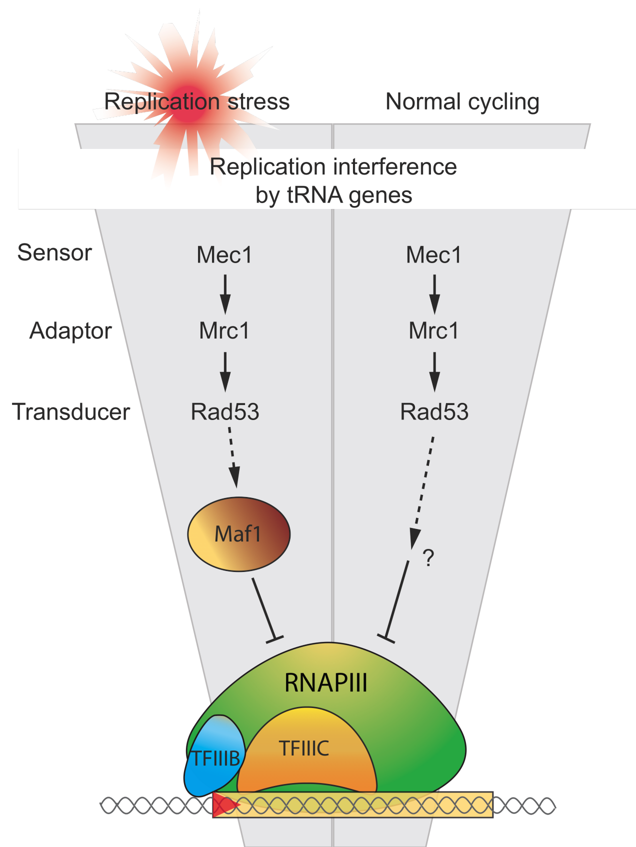

Figure 4-8. Restraint of tRNA gene transcription by replication stress checkpoint signaling involves Maf1 during replication stress but not during normal proliferation. Checkpoint proteins are represented in pathways according to dependencies found in previous literature. Dashed lines signify signaling steps that have yet to be fully characterized.

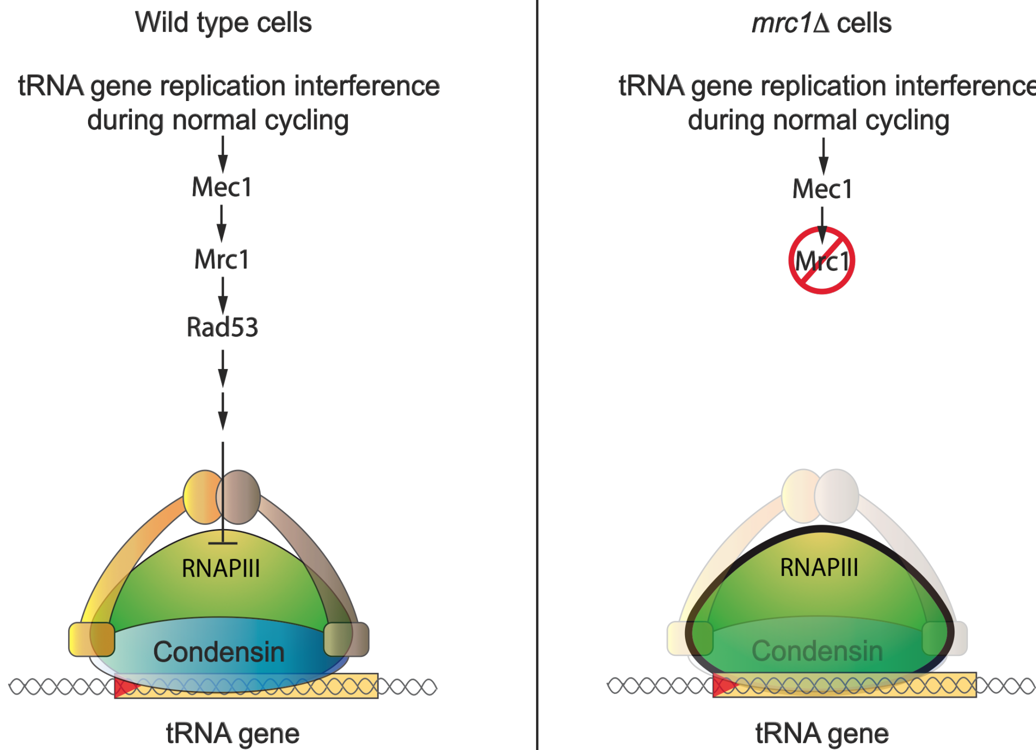

Figure 4-9. Changes in molecular composition of tRNA genes in cells lacking replication stress checkpoint during normal growth. Cells lacking the replication adaptor protein Mrc1 (crossed circle) are unable to propagate checkpoint signals (right panel). These cells have increased occupancy of RNAPIII at tRNA genes, consistent with their elevated rate of transcription. Also, condensin association decreases in *mrc1Δ* cells. Thick lines represent increased complex association and transparency represents decreased complex association.

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Chapter 5

Studies of replication and chromatin modification as related to the regulation of tRNA gene transcription

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Preamble

In this chapter, I report on two topics: 1) replication interference by tRNA genes in cells with elevated tRNA gene transcription and 2) the function of replication associated proteins and chromatin modification in the regulation of tRNA gene transcription.

Introduction

Replication represents a potentially hazardous event in the life of a cell. Accurate DNA duplication during S-phase is vital for cell division so that both the mother and daughter cell can acquire one complete and undamaged copy of the genome. Many events happening inside and outside of the cell can negatively affect the stability of a cell's genome by interfering with the replication process. For example, replication fork pausing is detected at many sites dispersed throughout the budding yeast genome during normal S-phase. Included in these sites are centromes, 35S rRNA genes, silent mating type loci, and tRNA genes[1-4]. Studies have shown that active tRNA genes work as replication fork barriers because the RNAPIII PIC interferes with progression of the replication fork[2,3].

Rrm3 is a member of the Pif1 family of DNA helicases. Rrm3 is known to be an important protein for the modulation of fork pausing at tRNA genes, even though the mechanism by which Rrm3 supports fork progression through active

tRNA genes is unclear. It has been proposed that Rrm3 is associated with replication forks and removes fork blocking non-nucleosomal protein complexes[3,5,6]. As a result, fork pausing at tRNA genes is exacerbated in *rrm3Δ* cells as detected by 2D electrophoresis. The idea that Rrm3 protects against fork interference that leads to genome instability is indirectly supported by the finding that deletion of *RRM3* is synthetically lethal with mutations that inactivate replication stress checkpoint function, including *rad53Δ* and *mrc1Δ* [5].

Admire *et al.* reported that tRNA genes are sites commonly linked to the induction of chromosomal rearrangements in the absence of any exogenous stress[7]. This spontaneous instability increases in *rrm3Δ* cells, *mec1Δ* cells, and in cells treated with HU, which suggests that replication fork pausing at these sites may be mechanistically linked to genomic rearrangements. This data taken together indicate that chromosome breaks at tRNA genes are: 1) caused by replication defects at these sites and 2) are the central source of genomic instability at these sites.

In the previous chapter of this thesis, I showed that there is greater occupancy of RNAPIII at tRNA genes in *mrc1Δ* cells over wild type during normal growth[8]. To extend these results, I used ChIP experiments to monitor replication protein association with tRNA genes. It is reasonable to expect that elevated RNAPIII transcription, which results in greater occupancy of the fork blocking RNAPIII, will translate into greater replication fork pausing at these loci. This greater fork pausing at tRNA genes may be detected as increased cross-linking of replication fork proteins at these sites. However, I found that there is no increase in fork

protein association with tRNA genes in cells with elevated tRNA gene transcription. I will discuss the possible implications of these results for our understanding of replication pausing at tRNA genes. This discussion will highlight a controversy that currently exists in the literature on replication fork interference by tRNA genes. Lastly, I am going to discuss one possible scenario that this result may be consistent with.

In addition to this, I explored the function of replication proteins in regulating tRNA gene transcription. Specifically, I screened strains lacking the replication proteins Rrm3 and Tof1, both of which are implicated in replication fork control at tRNA genes[3,9]. In addition, I measured tRNA gene transcription in mutants lacking DNA helicases shown to be important for replication fork stalling, Sgs1 and Srs2[10,11].

Finally, *ASF1, RTT109, ARP8* and *HTA* genes are all involved in chromatin modification. Asf1 and Rtt109 are required for histone H3 lysine 56 acetylation[12,13], Arp8 is a sub-unit of the INO80 chromatin modifier[14], and *HTA* mutants are defective in the production of γ-H2A[15-17]. I tested mutants of these genes for their involvement in regulating RNAPIII transcription of tRNA genes as well.

None of the mutants I tested exhibited defects in repression of transcription. This is particularly interesting in the case of *RRM3* mutation because it is predicted to impact transcription based on the most accepted model of Rrm3's function at tRNA genes[5,18-20]. Collectively, these findings indicate that: 1) replication stress checkpoint control of tRNA gene transcription does not impact

on replication fork pausing at tRNA genes, at least as detected by replication fork protein cross-linking to these sites and 2) so far, there is no evidence RNAPIII transcription is restrained by the function of replication associated proteins or chromatin modification during replication stress and normal proliferation.

Results

Monitoring replication by determining the association of replication proteins with DNA: ChIP protocol development

During S-phase of the cell cycle, replication proteins are assembled at origins of replication and once replication is initiated, the proteins travel along strands of DNA with the replication fork [21-23]. ChIP assays have been used to study replication by monitoring association of replication proteins with regions of DNA as proxy for fork movement[10,24]. Low temperatures (19 °C) were used to capture replication proteins at forks. To ascertain if I was able to use ChIP to measure association of replication proteins with tRNA genes, I arrested cells in G1 using the yeast mating pheromone α-factor and synchronously released them into S-phase at 19 °C (Fig. 5-1). A ChIP protocol[25] was used to monitor replication protein association with DNA with one notable exception; DNA shearing was not as extensive as before. For replication protein ChIP, I used 3 rounds of 20-second sonication to obtain chromatin fragments about 1 kb in size (Fig. 3-4A). This sonication protocol provided more robust results presumably because replication fork proteins are difficult to capture at any particular small locus owing to the speed at which they travel along the DNA. The longer

fragment size increases the likelihood of cross-linking a fork protein in the vicinity of a small locus such as a tRNA gene, yielding a more reliable PCR signal[10,24].

I used two strains that expressed c-myc epitope tagged replication proteins for ChIP experiments, Pol2-myc and Mcm7-myc. Pol2 is a sub-unit of the leading strand DNA polymerase, polε, and Mcm7 is a sub-unit of the replicative helicase, MCM2-7. Flow cytometry analysis indicated that replication initiated between 20 and 40 minutes after G1 release and was completed by 80 min for both of these strains (Fig. 5-1A). I probed purified DNA from the ChIP procedure by real-time PCR using primers that amplified the efficient early firing replication origin *ARS607*[26,27], *tA(AGC)*F, a known fork blocking tRNA gene[3] that pauses forks initiated from *ARS607*, and a region that is approximately half way in between the origin and *tA(AGC)F*, +2800 bp from *ARS607* (Fig. 5-1B). First, I arrested Pol2-myc cells in G1 and released them, taking samples at G1, and 20, 40, 60, and 80 minutes post release for ChIP processing (Fig.5-1C). Cross-linking of Pol2-myc increased dramatically at all loci between 20 and 40 minutes after release, which correlates to initiation of replication suggested by flow cytometry. By 80 minutes the cross-linking of Pol2-myc had returned to levels seen during G1 arrest. It is noted that cross-linking of Pol2-myc is more efficient at *ARS607* versus the other two loci. This probably is due to the temporal dynamics of cross-linking at the origin versus the other regions[28]. Pol2-myc is assembled and sits poised at *ARS607* for a period of time before origin firing, which allows formaldehyde cross-linking to be greater here versus the non-origin regions[21].

Similarly, Mcm7-myc cross-linking to these regions was determined under the same experimental conditions. Mcm7-myc cross-linking peaked at these regions at 40 minutes post G1 release and returned to pre-replication levels by 80 min (Fig. 5-1D). This data shows convincingly that ChIP analysis is useful for monitoring association of Pol2-myc and Mcm7-myc with *tA(AGC)F*.

Replication protein association with *tA(AGC)F* is not greater in cells with elevated RNAPIII transcription in cycling cells.

I studied the association of Pol2-myc and Mcm7-myc with *tA(AGC)F* in cycling cells. In particular, I tested if *rrm3Δ*, *mec1-100* and *maf1Δ* cells have increased cross-linking of these replication proteins at *tA(AGC)F*. *mec1-100* is a mutant allele of *MEC1* that is deficient in replication stress checkpoint function but still maintains viability[29].

I tested Pol2-myc cross-linking to regions immediately upstream and downstream of *tA(AGC)F* in wild-type, *rrm3Δ*, *maf1Δ*, and *mec1-100* cells grown to early log (Fig. 5-2A). Primers were also designed to monitor Pol2-myc cross-linking to two RNA polymerase II transcribed genes as negative control regions, *HIS2* and *POL1*. There is an increase in Pol2-myc association at *tA(AGC)F* in *rrm3Δ* cells over wild type in harmony with the increase fork pausing seen in this mutant by 2D electrophoresis[3]. However, there is no increase in Pol2-myc cross-linking in either *mec1-100* or *maf1Δ* strains.

Mcm7-myc cross-linking at *tA(AGC)F* was similar to Pol2-myc. I found that cross-linking of Mcm7-myc increased in *rrm3Δ* cells at *tA(AGC)F*, but increased

tRNA gene transcription in *maf1Δ* cells did not lead to increased Mcm7-myc cross-linking to *tA(AGC)F*, indicating that replication fork pausing at this gene, at least detected by ChIP, is not increased in cells with elevated RNAPIII transcription of tRNA genes.

Rrm3 and Tof1 do not contribute to regulation of tRNA gene transcription during normal growth or replication stress.

Rrm3 is proposed to function at tRNA genes by removing the fork blocking RNAPIII transcriptional machinery from the DNA using its helicase activity[5,18,19]. If this were the case, then cells lacking Rrm3 would be predicted to have elevated rates of transcription compared to wild type, similar to replication stress checkpoint mutants, because Rrm3 would no longer be displacing the transcriptional machinery during S-phase. I tested this hypothesis by Northern blotting.

I cultured wild type and *rrm3Δ* cells and took samples from early log (optical denisity at 600 nm of 0.5,1, and 3) to four days growth (optical density at 600 nm ≥ 15). Total RNA was extracted and subjected to Northern blotting using a probe specific for a short-lived leucine pre-tRNA species to assay tRNA gene transcription. U4 snRNA was probed as a loading control. At all cell densities tested, tRNA gene transcription in the *rrm3Δ* cells was similar to wild type (Fig. 5-3A). Further, to assess if Rrm3 is involved in signaling to tRNA genes during replication stress, I treated wild type and *rrm3Δ* cells with HU or left cells untreated and monitored tRNA transcription by Northern blotting analysis of total

RNA, as before. Cells lacking Rrm3 are able to repress RNAPIII transcription in response to HU treatment as well as wild type cells (Fig. 5-3B, WT-lanes 1 and 3 compared to *rrm3Δ*- lanes 2 and 4).

Tof1 also has also been shown to affect replication progression at tRNA genes, although Tof1 is proposed to function in opposition to Rrm3. *tof1Δ* cells no longer display detectable replication pausing at tRNA genes that are acknowledged fork blockers in wild type cells[9]. Therefore, it seems that Tof1 is required for fork pausing to occur. I also tested *tof1Δ* cells for their ability to repress transcription during normal growth conditions and during replication stress by similar Northern blotting procedure. I found that transcription in *tof1Δ* cells during exponential growth (optical density of 600 nm at 0.5 and 1) was very similar to wild type (Fig. 5-3C). Also, I determined that *tof1Δ* cells are able to repress transcription upon HU treatment, even in the absence of Rrm3 (Fig. 5-3D). The data shown here illustrate that neither Rrm3 nor Tof1 affects transcription to any significant degree under conditions studied.

DNA helicases Sgs1 and Srs2 are not required for repression of tRNA gene transcription during replication stress.

In addition to Rrm3, other DNA helicases are known to be important for genomic stability. Sgs1 of budding yeast is a member of the 3'-5' RecQ family of helicases[30,31]. Yeast strains lacking Sgs1 exhibit an assortment of DNA defects, including hyper-recombination, chromosome loss, and genomic rearrangements[32,33]. Importantly, Sgs1 functions in stabilizing the association of

the protein components with HU-stalled replication forks, and prevents fork collapse[10,24]. Furthermore, *sgs1Δ* cells are partially defective in replication stress checkpoint activation[34]. First, I tested if Sgs1 is involved in replication stress signaling to tRNA genes by either treating wild type and *sgs1Δ* strains with HU or leaving them untreated and determining these cell's ability to repress RNAPIII transcription by Northern blotting. Samples of cells were taken after 1 and 2 hours of HU exposure. I found that Sgs1 is not involved in signaling repression to tRNA gene during replication stress because *sgs1Δ* cells were fully able to repress transcription upon HU treatment (Fig. 5-4A). Further, it was evident that there was little change in transcription in *sgs1Δ* cells versus wild type during exponential growth.

Srs2 is another 3'-5' DNA helicase of *S. cerevisiae*[35]. Previous studies had determined that Srs2 functions during replication as well. Srs2 physically interacts with a DNA polymerase during normal replication and *srs2Δ* cells display a faster rate of replication fork progression than wild type[36]. Further, Srs2 has been shown to inhibit recombination at stalled replication forks by disrupting recombination protein-DNA interactions at non-progressing forks[37]. Similar to Sgs1, Srs2 has also been implicated in replication stress checkpoint activation[11].

Therefore, it was reasonable to test if Srs2 is involved in replication stress associated repression of tRNA transcription. However, I found that *srs2Δ* cells repress tRNA transcription during normal growth conditions and in response to HU to the same extent as wild type cells (Fig. 5-4B).

Lastly, deletion of *MAF1* in addition to either *SGS1* or *SRS2* deletion does not confer significant additional sensitivity to growth on HU-containing medium beyond that observed for cells lacking *SGS1* or *SRS2* alone (Fig. 5-4C). These results are consistent with these helicases not being involved in the replication stress signaling that leads to repression of tRNA gene transcription.

Histone H2A phosphorylation, INO80 chromatin remodeling, and histone H3 lysine 56 acetylation are not required for repression of tRNA gene transcription during replication stress.

Chromatin modification is a significant part of many signaling pathways in eukaryotes. Recent research has illustrated a number of approaches that cells use to modulate chromatin structure to initiate signals or respond to certain events. Histone chaperones, histone modifiers, and chromatin remodeling machines are used to orchestrate these processes.

Phosphorylation of histone H2A (H2A, H2AX in metazoans) is a widespread chromatin mark that cells use to respond to genotoxic stress[15,38]. This phosphoylation of H2A (referred to as γ-H2A in budding yeast) occurs as a result of replication fork perturbation in a Mec1-dependant manner[39]. Importantly, sites of γ-H2A enrichment have been mapped to tRNA genes[16]. This raises the possibility that phosphorylation of H2A is needed for replication stress checkpoint control of RNAPIII transcription. We obtained a mutant yeast strain that had a serine to alanine mutation at the phosphorylation site of H2A and an isogenic wild type strain[17]. I tested these cells for inhibition of RNAPIII during replication

stress by the same HU treatment and Northern blotting analysis used before. I found that γ-H2A is not part of the signaling that leads to repression of tRNA transcription because this mutant supports the same level of transcription as wild type cells during normal proliferation, and represses transcription as well as wild type in HU-treated cells (Fig. 5-5A).

We next turned our attention to the chromatin remodeller INO80. INO80 is a conserved complex that is recruited to DNA double strand breaks in response to phosphorylation of H2A by Mec1[40,41]. INO80 serves to remove the core histones, including γ-H2A, near the break making the DNA available to DNA repair proteins. Mutation of the INO80 specific sub-units Arp8 or Nhp10 results in deficiencies in checkpoint activation[42]. In addition, INO80 is known to function at stalled replication forks and this protein is enriched at tRNA genes[14], making this complex a suitable candidate to be involved in repression of RNAPIII transcription due to replication interference.

I used deletion of *ARP8* to inactivate INO80 function[14] and tested the capacity of these cells to repress tRNA transcription during replication stress. I subjected wild type and *arp8Δ* cells to the same HU treatment protocol used before and monitored RNAPIII transcription of tRNA genes by Northern blotting of total RNA. However, I found that INO80 function is not necessary for inhibition of tRNA transcription during HU exposure or normal proliferation (Fig. 5-5B).

To finish, I investigated the involvement of histone H3 lysine 56 (H3K56) acetylation in repression of RNAPIII transcription. H3K56 is a chromatin mark that has been linked to DNA replication and checkpoint activation in cells

experiencing replication stress[43]. Rtt109 is a lysine histone acetyltransferase that acetylates histone H3[13] and the histone chaperone Asf1 interacts with Rtt109 and is required for this process[12]. The absence of either Asf1 or Rtt109 abolishes H3K56 acetylation in yeast cells[12,13]. *asf1Δ* and *rtt109Δ* cells are sensitive to HU[44] and H3K56 acetylation is important for stability of stalled replication forks[45].

I tested both *asf1Δ* and *rtt109Δ* cells for their ability to repress tRNA gene transcription to determine if these proteins are linked to RNAPIII. I used cells that had been treated for 1 hour with 0.2 M HU, or left untreated. H3K56 acetylation seems unlikely to contribute to the restraint of transcription in HU treated or unchallenged, normally growing cells because *asf1Δ* and *rtt109Δ* cells repressed tRNA transcription at close to wild type levels in both of these environmental conditions (Fig. 5-5C).

Rad52 is not detected at tRNA genes either in cycling or S-phase cells.

Many recombination pathways in yeast require the conserved Rad52 protein[46,47]. Rad52 functions at stalled or collapsed replication forks as a mediator between stabilized replication forks and recombinational repair of collapsed forks. Rad52 is recruited to sites of double-strand breaks, such as those formed by collapse of replication forks, where it forms “Rad52 foci” as observed by immunofluorescence microscopy. Rad52 activity at stalled replication forks displaces the single-stranded DNA binding protein, replication protein A (RPA), and exchanges it for the homologous recombination protein Rad51, which allows for the initiation of recombination[48]. Interestingly, deletion of

RAD52 resulted in suppression of recombination between active tRNA genes[49]. Further, Rad52 foci are observed in replication stress checkpoint mutants that are arrested in HU[50].

I tested the hypothesis that Rad52 is recruited to tRNA genes[50]. One possibility is that greater replication fork collapse at these sites results from an inability to repress RNAPIII transcription in replication stress checkpoint mutants. I began by constructing strains that expressed Rad52-myc in a wild type and *mrc1Δ* background. Exponentially growing Rad52-myc and Rad52-myc *mrc1Δ* cells were formaldehyde treated and subjected to processing according to our ChIP protocol. I tested the association of Rad52-myc with two tRNA genes, *tA(AGC)F* and *tS(CGA)C*, and *HIS2* as a negative control region. Real-time PCR signal from immunoprecipitates using Rad52-myc strains was only about 2-3 fold greater than untagged signal using all primers (Fig. 5-6A). Therefore, very little Rad52-myc cross-linked to any of these regions in either wild type or *mrc1Δ* cells. Furthermore, cells lacking Mrc1 did not yield any greater amount of tRNA gene containing fragments in chromatin immunoprecipitates.

It might be that in order to detect Rad52-myc cross-linking to tRNA genes that cells need to be synchronously replicating DNA in S-phase. To determine of this is the case, I arrested Rad52-myc cells in G1 with α-factor and released these cells into S-phase and took cells at 15, 30, 45, and 60 minutes after release. Flow cytometry illustrated that wild type, *mrc1Δ* and *rrm3Δ* cells had all initiated DNA replication by 60 minutes after release (Fig. 5-6B). Similar to before, I could detect very little Rad52-myc cross-linking to *tA(AGC)F* in cells synchronously

replicating DNA in wild type, *mrc1Δ*, and *rrm3Δ* strains. These results suggest that very little Rad52-myc is associated with tRNA genes, even in replication stress checkpoint deficient and *rrm3Δ* cells. However, we cannot rule out the possibility that the DNA secondary structure at tRNA genes or the structure of Rad52-myc is somehow not amenable to Rad52 cross-linking in any of the genomic backgrounds we used.

Discussion

Here, we present the first ChIP studies of replication fork pausing at tRNA genes in mutant cells with elevated RNAPIII transcription.

I used formaldehyde cross-linking of two replication fork proteins as a proxy measurement for replication fork pausing at tRNA genes. We first determined that we could monitor replication by determining cross-linking of Pol2-myc and Mcm7-myc to regions of DNA on chromosome VI, which included a fork-blocking tRNA gene, *tA(AGC)F*[3]. However, further work revealed that neither Pol2-myc or Mcm7-myc association is increased at *tA(AGC)F* in cells that have increased transcription over wild type.

Next, we report that Rrm3 and Tof1 have no effect on RNAPIII transcription of tRNA genes during normal growth and replication stress, even though these proteins affect replication fork pausing at these sites[3,9]. Likewise, DNA helicases Sgs1 and Srs2, which function in maintaining stability of stalled replication forks and thereby promote genomic stability[10,24,37], are not required for repression of tRNA transcription in unchallenged and replication stressed cells.

Additionally, I tested a number of chromatin modifications that were plausibly involved in signaling pathways that lead to RNAPIII repression. I found that γ-H2A, INO80 remodeling, and H3K56 acetylation had little effect on the repression of tRNA transcription in HU treated and untreated cells. And lastly, no evidence was found to support the hypothesis that Rad52 is enriched at tRNA genes.

Previously, we have shown that cells lacking the replication stress checkpoint exhibit increased occupancy of RNAPIII at tRNA genes (see chapter 4). From this we hypothesized that increased occupancy of the replication fork blocking RNAPIII at tRNA genes might lead to increased replication fork pausing at these sites. Our ChIP analysis suggest that this might not be the case because *MEC1* or *MAF1* mutation does not lead to increased replication fork association with tRNA genes.

Our results illustrate the complexity of replication fork pausing at a tRNA gene, which has been borne out in recent literature. Two groups have used ChIP experiments combined with microarray technology (or ChIP-chip approach) to monitor replication protein association genome-wide. Azvolisnksy *et al.* used Pol2-myc cross-linking to DNA in unsynchronized budding yeast cells to monitor replication progression[51]. Similar to the approach that I took, peaks of enrichment of Pol2-myc were thought to correspond to loci of replication interference. These authours reported that fork pausing naturally occurs at some tRNA genes that are transcribed in the same direction that they are replicated (i.e. these tRNA genes are in the wrong orientation to block replication as detected by 2D electrophoresis). Furthermore, some well-known fork pausing tRNA genes do not

have Pol2-myc association that is greater than background, indicating these genes are not fork blockers as detected by this method.

GINS (**G**o, **I**ich, **N**ii, **S**an) has been recently discovered as a component of the replication fork. Sekedat *et al*. monitored replication using the cross-linking of a sub-unit of the GINS complex in cells synchronously progressing through S-phase[52]. These results illustrated enrichment of GINS at every tRNA gene in the yeast genome. Therefore, these results support a model in which all active tRNA genes are difficult to replicate.

Hence, there are discrepancies between fork pausing tRNA gene measurements obtained by ChIP-chip studies using Pol2-myc cross-linking, ChIP-chip studies using GINS cross-linking, and 2D electrophoresis[53]. Specifically, fork-blocking tRNA genes, as determined by 2D electrophoresis, do not seem to interfere with replication when studied by ChIP Pol2-myc association and vice versa. Further, every single tRNA gene perturbs replication when investigated by GINS cross-linking but, only a sub-set of tRNA genes are revealed as fork-blockers using Pol2-myc as proxy for replication progression. The reasons for these discrepancies are unknown and this makes it difficult to develop persuasive models of replication fork perturbation by tRNA genes that we can then test experimentally.

However, the experiments presented here raise some interesting possibilities. The latest studies have shown that tRNA genes alone might not be a significant site of recombination under normal conditions[54,55], perhaps because mechanisms exist to control replication fork pausing at these sites and keep fork collapse to a

minimum. However, perturbation of replication by mutation of replication stress checkpoint proteins or HU exposure increases the probability that tRNA genes are a source of recombinogenic lesion[7,53,56].

Based on my results, it could be that replication perturbation by tRNA genes in replication stress checkpoint deficient cells somehow promotes genomic instability without causing increased fork pausing (Fig. 5-7). Indeed, it has been shown that there is no correlation between the amount of fork pausing and the amount of recombination in budding and fission yeast[54,55]. Therefore, even though replication fork pausing is not increased in *mec1-100* cells, replication associated instability at tRNA genes might be increased by producing unstable DNA structures or an inability to maintain certain replication proteins at the replication fork.

My results also have an interesting implication for the most commonly accepted models of Rrm3 function. Rrm3, the so-called ‘sweepase’, is proposed to function at the advancing face of the replication fork, removing RNAPIII transcriptional machinery when a replication fork encounters it[3,5]. Thus, we would predict its function to have a negative effect on transcription of tRNAs. However, my results, and the results of others[54], show that Rrm3 has little effect on tRNA gene transcription. As a consequence, we suggest that Rrm3 promotes replication progression at tRNA genes in a way that does not affect tRNA transcription, possibly without displacing the RNAPIII PIC. Further, Rrm3 is not functioning in a pathway leading to repression of RNAPIII, like the replication stress checkpoint, to promote replication progression.

In addition, I tested the possibility that γ-H2A and H3K56 are needed for inhibition of RNAPIII in response to replication interference. One of the important questions relating to replication checkpoint repression of RNAPIII transcription remains; what is the mechanism of initiation of checkpoint signaling during normal proliferation?[53] In the case of HU treatment, checkpoint-signaling initiation has been well-characterized[57,58]. Mec1 senses excess single-stranded DNA (ssDNA) bound by RPA, formed by the uncoupling of the progression of DNA polymerases and the replication helicase at the replication fork (Fig. 5-8). HU, by limiting the availability of dNTPs, inhibits the progression of the DNA polymerases without affecting the movement of the replication helicase. Therefore, an uncoupling between replicative helicase and DNA polymerase movement during HU treatment generates about 100 base pairs of excess ssDNA. At the replication fork, RPA binds to ssDNA and stabilizes it, including superfluous ssDNA produced during HU exposure. This excess ssDNA formed during HU treatment is the abnormal DNA structure that leads to the recruitment and activation of Mec1[58].

On the other hand, replication stress checkpoint signaling during normal S-phase is initiated by an unknown mechansim. Although they might be signal initiation marks in other contexts, our results show that γ-H2A and H3K56 are not chromatin modifications that are involved in replication stress checkpoint repression of RNAPIII transcription.

One evident possibility is that perturbation of replication by tRNA genes during normal S-phase also results in the formation of excess ssDNA coated by RPA. In

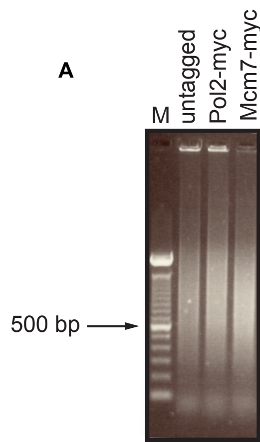
this possibility, tRNA genes would be blocking the progression of the DNA polymerases but not interfering with the progression of the helicase, similar to HU treated cells. Testing mutant alleles of RPA subunits for their ability to repress tRNA gene transcription during unchallenged proliferation would be a way to explore such a model.

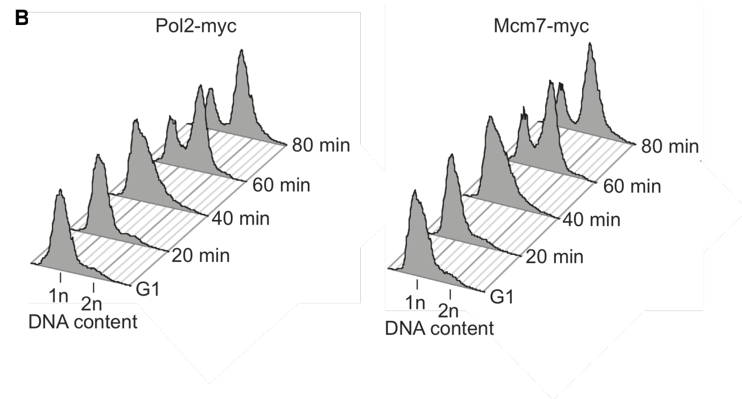
Further, INO80 chromatin remodeling is also not involved in repression. Taken together, these data suggest that none of these methods of chromatin modification is an important part of replication stress checkpoint signaling to tRNA genes.

When replication fork progression is stalled at a specific genomic site and the fork stall is not overcome, the fork will eventually collapse. Rad52 is a homologous recombination protein that acts at collapsed forks to aid in the repair of double-strand breaks induced by collapsed replication forks[48]. I did not find any evidence for Rad52 cross-linking to tRNA genes in wild type, *mrc1Δ*, or *rrm3Δ* cells, possibly suggesting that there is not a significant amount of fork collapse at these loci in these mutants or that homologous recombination is not a significant method cells use to overcome replication fork collapse at tRNA genes.

Based on previous studies of replication checkpoint signalling, genome stability control, and fork pausing at tRNA genes, I developed straightforward predictions about the functions of replication and chromatin proteins, and two chromatin marks, on transcriptional regulation of the tRNA genes. I also hypothesized that artificial elevation of tRNA gene transcription would result in higher cross-linking of replication proteins to tRNA genes. None of my evidence

implicates Rrm3, Tof1, Sgs1, Srs2, INO80, γ-H2A or H3K56ac in the control of tRNA gene transcription. Future working models of tRNA gene regulation by the replication stress checkpoint must be constrained by these results, particularly the unexpected finding that the helicase Rrm3 does not control tRNA gene transcription.


A
M
untagged
Pol2-myc
Mcm7-myc
500 bp


B
Pol2-myc
80 min
60 min
40 min
20 min
G1
1n
2n
DNA content
Mcm7-myc
80 min
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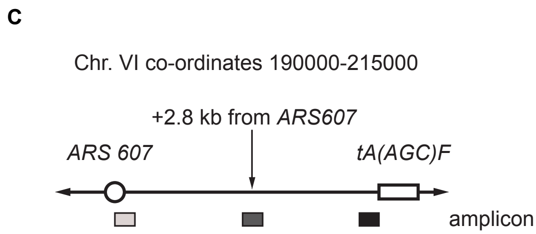
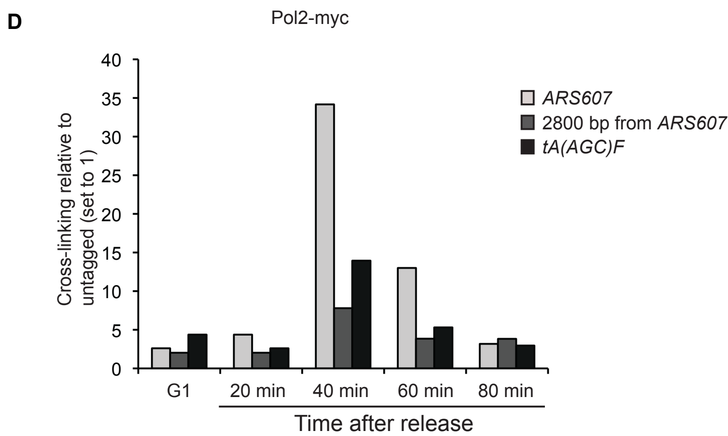
G1
1n
2n
DNA content


C
Chr. VI co-ordinates 190000-215000
+2.8 kb from ARS607
ARS 607
tA(AGC)F
amplicon


D
Pol2-myc
Cross-linking relative to untagged (set to 1)
ARS607
2800 bp from ARS607
tA(AGC)F
G1
20 min
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60 min
80 min
Time after release

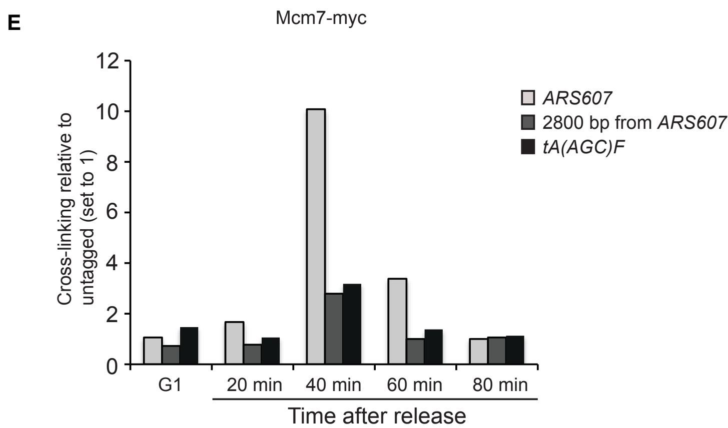
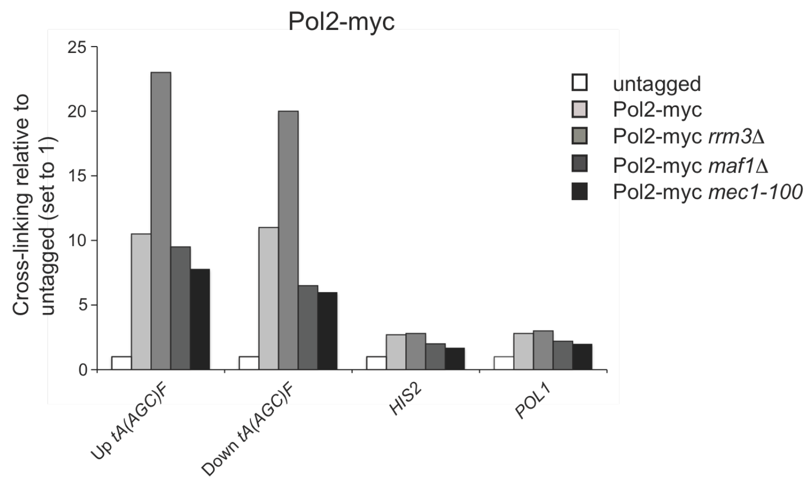

E
Mcm7-myc
Cross-linking relative to untagged (set to 1)
ARS607
2800 bp from ARS607
tA(AGC)F
G1
20 min
40 min
60 min
80 min
Time after release

Figure 5-1. ChIP analysis enables the determination of replication protein association with *tA(AGC)F*. A. Three rounds of 20 second sonication burst results in chromatin fragments of average size of 1 kb. ChIP extracts from untagged, Pol2-myc and Mcm7-myc cells were sonicated for 3 rounds of sonication, 20 seconds in length. Formaldehyde cross-links were reversed and DNA purified from extracts. DNA was run on a 1% agarose gel and stained with ethidium bromide. **B.** Flow cytometry analysis reveals that S-phase is completed by 80 minutes after release from synchronization at 19°C. Pol2-myc and Mcm7-myc cells were arrested in G1 by α-factor treatment and released into S-phase by removal of mating pheromone in medium by vacuum filtration and re-suspension of cells in pre-conditioned medium. *BAR1* deletion was used to increase strain sensitivity to α-factor. Samples were taken before and after release at indicated time points and DNA content was measured by flow cytometry. **C.** Diagram of regions if Chromosome VI that were amplified by primer pairs used in ChIP anaysis of replication protein cross-linking. **D.** DNA polymerase cross-linking increases as cells progress through S-phase and decreases when cells enter G2/M. Primers amplify an early-firing origin, *ARS607,* +2.8 kb from *ARS607,* and the known fork pausing *tA(AGC)F* (at +5.6 kb from *ARS607*). Relative association of Pol2-myc with theses regions is determined by ChIP. Height of the bars represents fold-change of real-time PCR signal over signal from IP performed using an untagged strain. **E.** Replicative helicase cross-linking at these sites is similar to DNA polymerase cross-linking as cells proceed through S-phase and into G2/M. ChIP analysis of Mcm7-myc cross-linking determined as described in B.

A



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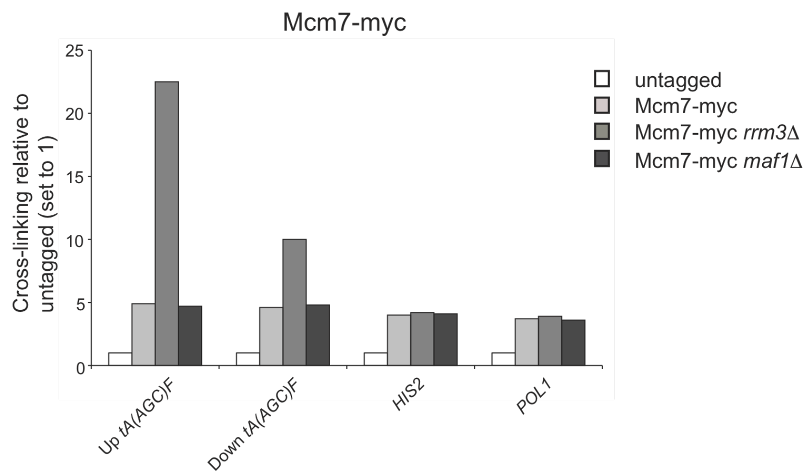


Figure 5-2. Increased tRNA gene transcription does not lead to greater cross-linking of replication proteins to a known fork pausing tRNA gene. A. Pol2-myc association is increased at *tA(AGC)F* in *rrm3Δ* cells, but not *mec1-100* or *maf1Δ* in cycling cells. Cells were grown to early log, treated with formaldehyde, and harvested for ChIP analysis. Pol2-myc cross-linking was determined for regions immediately upstream and downstream of *tA(AGC)F* and two RNA polymerase II transcribed genes, *HIS2* and *POL1*. Strains used were untagged wild-type, Pol2-myc, Pol2-myc *rrm3Δ*, Pol2-myc *maf1Δ*, and Pol2-myc *mec1-100*. Real-time PCR signals from Pol2-myc strains were normalized to signal from untagged cells, which was set to one. **B.** Mcm7-myc association at *tA(AGC)F* is also increased in *rrm3Δ* cells, but not in *maf1Δ* cells. Mcm7-myc cross-linking to upstream and downstream *tA(AGC)F, HIS2,* and *POL1* is monitored in early log growth by ChIP. Strains used were untagged, Mcm7-myc, Mcm7-myc *rrm3Δ*, and Mcm7-myc *maf1Δ*. Quantitation as in A.

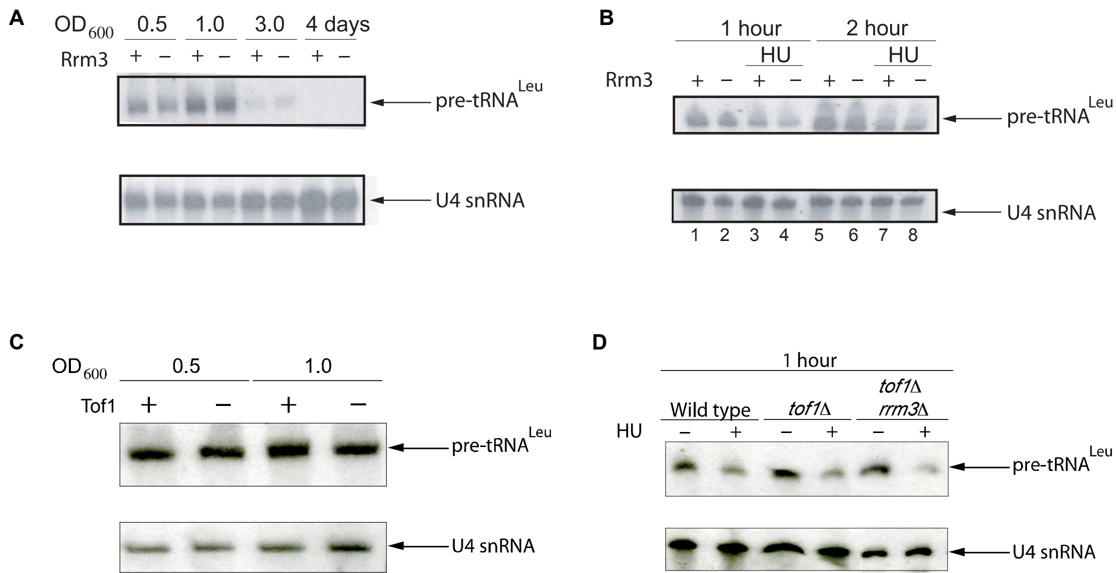


Figure 5-3. Rrm3 and Tof1 are not required for the repression of tRNA gene transcription either during normal growth or replication stress. A. *rrm3Δ* cells have similar tRNA gene transcription to wild type during unchallenged proliferation. Wild type and *rrm3Δ* cells were grown in rich medium and cell samples were taken at optical density at 600 nm of 0.5,1,3, and after 4 days of growth ($OD_{600} \geq 15$). Northern blotting of total RNA was performed using probes that hybridize to leucine pre-tRNA species and U4 snRNA as a loading control. **B.** *rrm3Δ* cells repress tRNA transcription during treatment with HU similar to wild type. Wild type and *rrm3Δ* cells were grown to early log and either treated with 0.2M HU of left untreated. Cell samples were taken at 1 and 2 hours and total RNA extracted and analyzed by Northern blotting. **C.** *TOF1* deletion does not affect transcription during early log. Wild type and *tof1Δ* cells were grown to optical density at 600 nm of 0.5 and 1. Cell samples were taken and total RNA was extracted for Northern blotting, as before. **D.** Tof1 is not required for down-regulation of tRNA transcription upon HU treatment. Wild type, *tof1Δ*, and *tof1Δ rrm3Δ* cells were grown to early log and either treated with 0.2M HU of left untreated. Cell samples were taken after 2 hours HU exposure and total RNA extracted and analyzed by Northern blotting, as before.

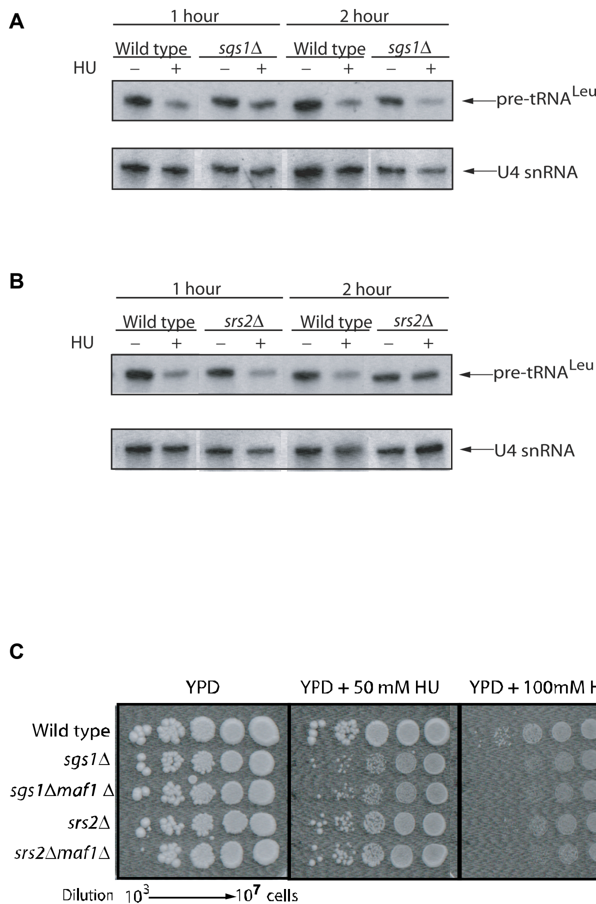
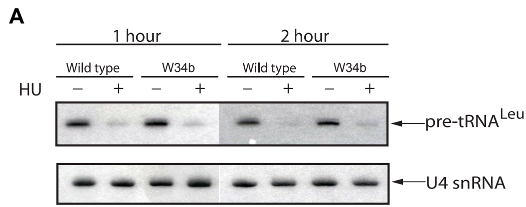
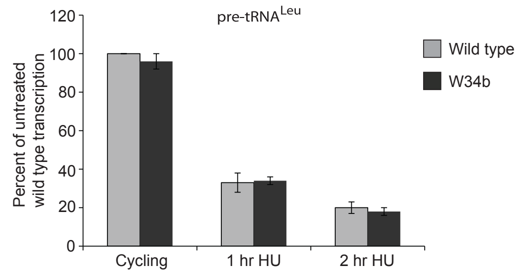


Figure 5-4. Sgs1 and Srs2 are not required for the repression of tRNA gene transcription during replication stress, and cell lacking Sgs1 or Srs2, in addition to Maf1, are not more sensitive to HU than cells lacking Sgs1 or Srs2 alone. A. Wild type and *sgs1Δ* cells were treated with HU or left untreated as previously described and total RNA extracted from cell samples was analyzed by Northern blotting. **B.** Wild type and *srs2Δ* cells were treated with HU or left untreated as previously described, followed by Northern blotting analysis of total RNA. Note that apparent failure to detect repression at 2 hour HU-treated *srs2Δ* reflects unequal sample loading as shown by the U4 signal. **C.** Ten-fold serial dilutions of cells grown in rich medium were spotted onto plates with no, 50 mM, or 100 mM HU. Plates cultured at 30 °C for 4 days.

A





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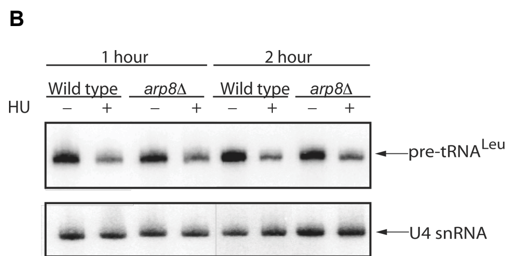

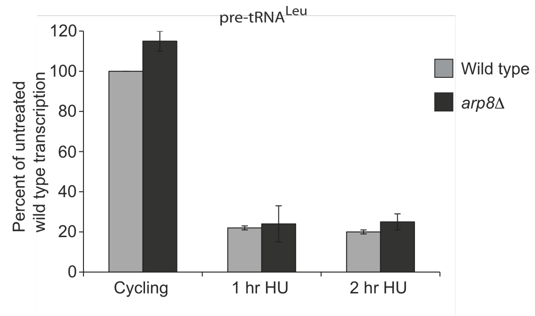

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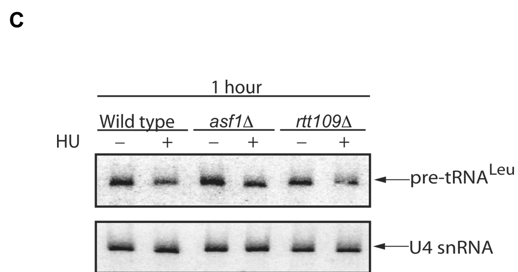


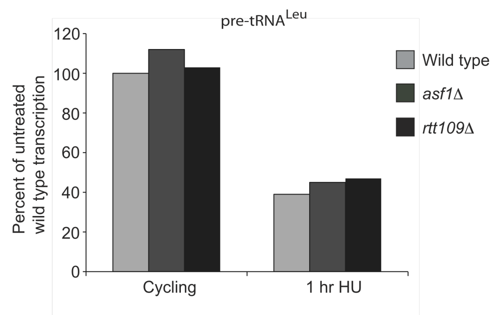
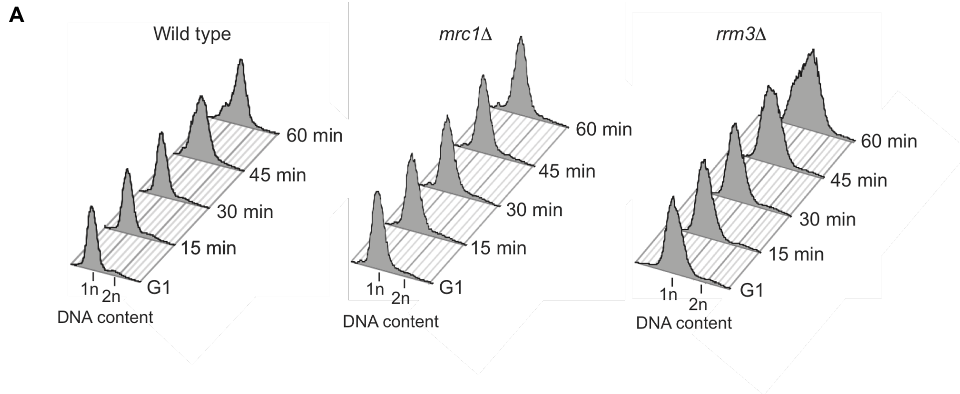


Figure 5-5. γ-H2A, INO80 chromatin remodeling, and H3K56 acetylation are not involved in repression of tRNA gene transcription during normal growth or replication stress. A. The γ-H2A chromatin mark is not required for repression of tRNA gene transcription during unchallenged and replication stress conditions. Wild type and W34b (*hta1*-S129A *hta2*-S129A) cells were grown to early log and were either treated with 0.2M HU or left untreated. Total RNA extracted from cell samples taken at one or two hours were analyzed by Northern blotting. Quantitation by phosphoimaging and ImageQuant software from three independent experiments is shown in bar graph. Relative transcription from cell samples is normalized to untreated wild type, which is set to 100 percent. Error bars represent +/- s.d. from the mean. **B.** Repression of tRNA gene transcription does not include the INO80 specific sub-unit Arp8. Wild type and *arp8Δ* cells were analyzed in their ability to repress transcription by Northern blotting of total RNA extracted from untreated cells or HU treated cells for one or two hours. Quantitation is as in A. **C.** H3K56 acetylation contributes little to the repression of tRNA gene transcription. Wild type, *asf1Δ*, and *rtt109Δ* strains were subjected to 0.2 M HU treatment for one hour. Northern blotting analysis was performed to monitor tRNA gene transcription. Quantitation of Northern blot is shown, n=1.

A



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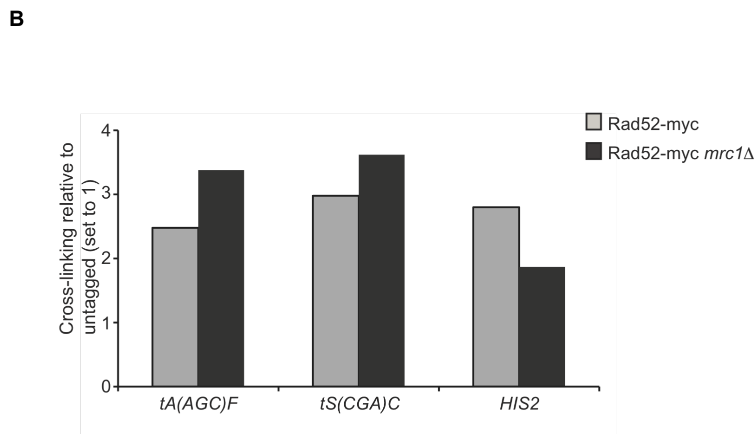

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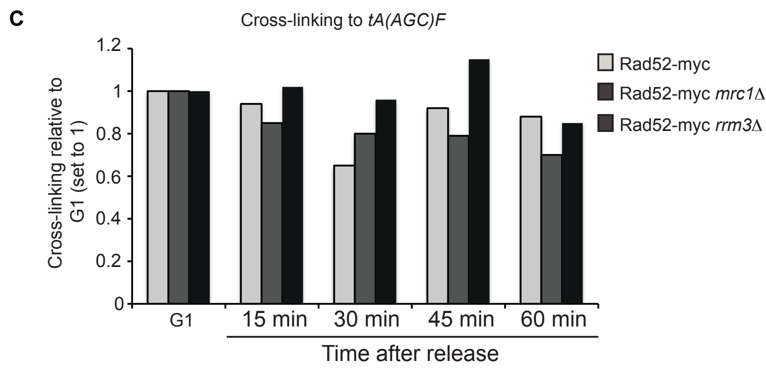

Figure 5-6. Rad52 is not detected at tRNA genes in cycling cells or in S-phase. A. Rad52-myc is not detected cross-linked to tRNA genes in cycling cells. Untagged wild type, Rad52-myc, and Rad52-myc *mrc1Δ* cells were treated with formaldehyde during exponential growth, harvested, and processed according to ChIP protocol. Primers that amplify up two tRNA genes, *tA(AGC)F* and *tS(CGA)C*, and a negative control region, *HIS2*, were used to probe purified immunoprecipitated DNA. Real-time PCR signal from untagged cells is used as reference and Rad52-myc signal is expressed as a fold-change from untagged signal. **B.** Wild type, *mrc1Δ*, and *rrm3Δ* strains all begin replication by 60 min following release from α-factor induced G1 arrest. DNA content is monitored by flow cytometry in cells synchronized by mating pheromone treatment and then released by removal of α-factor containing medium by vacuum filtration and re-suspension in pre-conditioned medium. *BAR1* deletion was used to increase sensitivity to α-factor. **C.** Rad52-myc is not found cross-linked to *tA(AGC)F* in S-phase of wild type, *mrc1Δ*, or *rrm3Δ* cells. Cells were treated with α-factor and released into S-phase as described in B. Samples of cells were taken in G1 and 15, 30, 45, and 60 minutes after α-factor removal. Processing of cells by ChIP procedure was followed by quantitation of immunoprecipitated DNA by real-time PCR using primers that amplify *tA(AGC)F* gene. Height of the bars represents PCR signal normalized to untagged background signal.

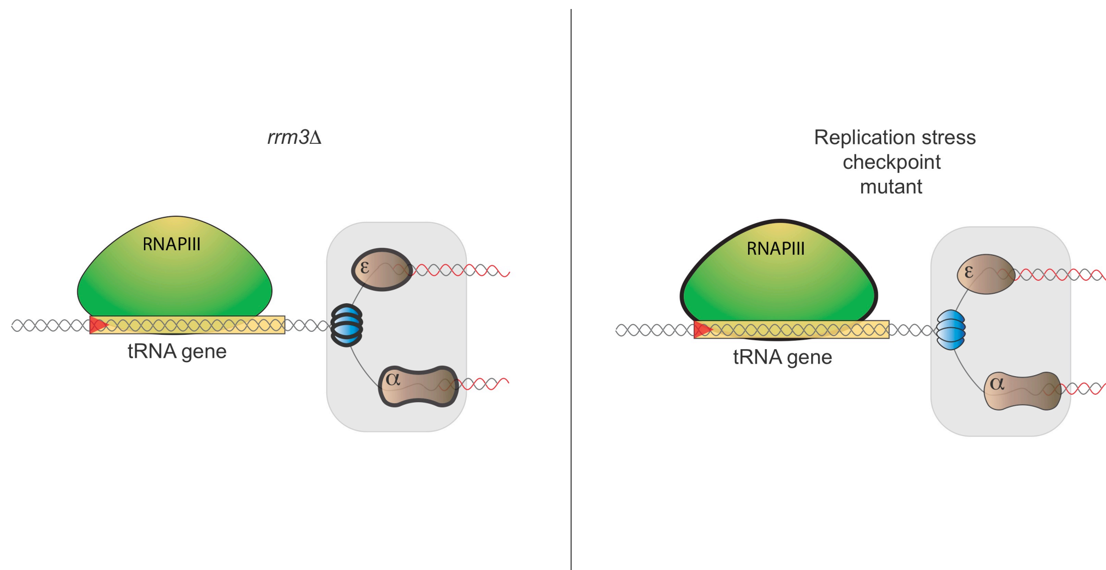

Figure 5-7. Mutation of replication stress checkpoint proteins results in increased occupancy of RNAPIII at tRNA genes, but not an increase in replication fork association. A. Deletion of *RRM3* results in increased association of replication fork proteins with tRNA genes (indicated by thick lines around fork proteins). **B.** Mutation of replication stress checkpoint proteins results in increased occupancy of RNAPIII at tRNA genes (thick line around RNAPIII), but not an increase in association of fork proteins.

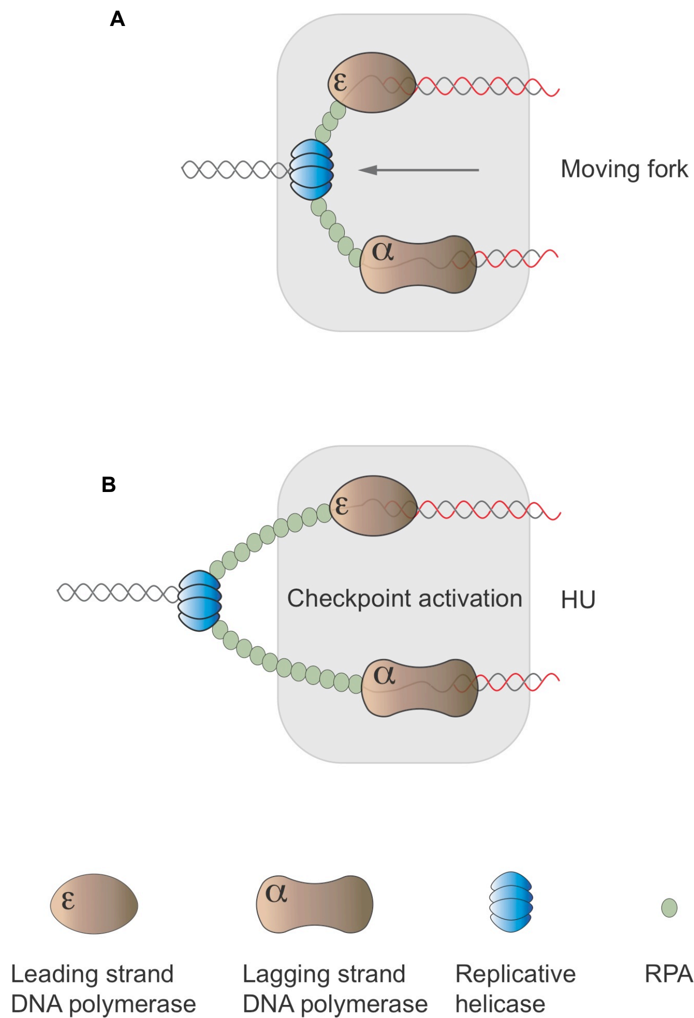

Figure 5-8. The amount of RPA-coated ssDNA at HU stalled forks determines the activation of the replication stress checkpoint. A. A normal progressing fork does not produce enough ssDNA to activate checkpoint signaling. **B.** By depleting nucleotide pools, HU treatment slows DNA polymerase progression without inhibiting helicase movement. This generates increased ssDNA coated with RPA, which is sufficient to activate the checkpoint when a sufficient number of forks are arrested. Leading strand DNA polymerase, lagging strand DNA polymerase, replicative helicase, and RPA are indicated by the shapes shown.

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Chapter 6

New links between tRNA gene transcription and cell cycle control

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Introduction

In order to proliferate, all cells execute a series of distinct phases of the cell cycle consisting of periods called G1, S, G2, and mitosis[1-3]. G1 is a noteworthy phase in the cell cycle because this period is where a choice is made between differing developmental pathways based on the interpretation of environmental and cellular cues. In G1, a yeast cell can either commit to go one round in the cell cycle, pause cell cycle progression, or enter a state of no proliferation when nutrients are limiting[1-3]. If the decision is made during G1 to divide, the rest of G1 is devoted to preparation for DNA replication[4]. This includes induction of transcriptional programs to up-regulate the expression of genes required for replication and the assembly of replication proteins on future origins. G2 and mitosis follow S-phase. Mitosis is the period where newly synthesized sister chromatids are pulled apart to prepare for cell division[5]. S-phase and mitosis are potentially hazardous periods in the life of a cell because events interfering with either DNA replication or sister chromatid separation could result in gross chromosomal changes in genomes inherited by progeny cells.

There is some evidence linking tRNA transcription to cell cycle control. First, it has been shown that inactivating the function of an essential yeast sub-unit of RNAPIII, Rpc53, results in a rapid and preferential arrest of cells in G1[6]. Six

conditional temperature sensitive alleles of *RPC53* were isolated, which arrest in G1. These cells maintain a normal level of protein synthesis and viability suggesting cell cycle arrest involved RNAPIII transcription, instead of an effect on translation. Similar results have been reported for a temperature sensitive allele of Rpc160[6], the catalytic sub-unit of RNAPIII. Rpc53 is significantly similar to the human protein BN51 suggesting that BN51 may be the human homologue of Rpc53[7]. BN51 also appears to be important for progression through the cell cycle since its cDNA was isolated by its ability to complement the cell cycle defect of a human mutant cell line, which arrests in G1 at elevated temperatures[7]. Lastly, Niu *et al.* reported that over-expression of *RPC82*, a gene that encodes another RNAPIII sub-unit, results in cells arresting in G2/M[8].

Building upon previous work from our lab, I attempted to test if repression of tRNA gene transcription by the replication stress checkpoint occurs only during S-phase. Replication checkpoint signaling is thought to be sensitive only to perturbation of replication forks and thus activation of this type of signaling should only occur during S-phase[9]. Consequently, restraint of tRNA transcription by the replication checkpoint should also be limited to S-phase (Fig. 6-1). In the data presented in this chapter, I attempted to test this hypothesis. However, in process of doing this, I discovered new and unexpected links between tRNA gene transcription and cell cycle progression.

Initially, I used the microtubule polymerization inhibitor nocodazole to arrest cells in G2/M[10], and then released them by removal of drug from the medium. Using Northern blotting, I assayed tRNA gene transcription as cells proceeded

synchronously through a round of the cell cycle following nocodazole arrest. Instead of transcription being repressed during S-phase as predicted, I found repression occurs before S-phase begins, possibly during late G2 or mitosis. Interestingly, this repression required the presence of the replication stress checkpoint protein Mrc1.

Treating yeast cells with the mating pheromone α-factor is an often used method to reversibly synchronize cultures in G1 to study cell cycle effects[11]. While trying to utilize α-factor to arrest cells in G1 and release them into S-phase, I discovered that cells repress tRNA gene transcription during treatment with α-factor alone. Releasing cells from pheromone induced G1 arrest illustrated that tRNA transcription is not repressed in a cell cycle correlated manner, suggesting that the repression of tRNA transcription during α-factor treatment is not due to all cells being in G1. Additionally, repression in response to exposure of pheromone is correlated with a decrease in the abundance of the RNAPIII sub-unit Rpc160.

Lastly, α-factor treated cells arrest in G1 due to inhibition of the yeast's main cyclin-dependant kinase, Cdc28[12,13]. Negative regulation of Cdc28 during α-factor treatment is brought about by the activation of a well-studied signaling cascade elicited by the pheromone binding to a cell-surface receptor. To determine if repression of tRNA gene transcription with α-factor treatment is related to the activity of Cdc28, I examined tRNA gene transcription in cells harbouring a *CDC28* mutant allele, *cdc28-13*[14]. I found that *cdc28-13* cells have five-fold more tRNA gene transcription than wild type cells.

This data illustrate some possible new connections between control of the progression of the cell cycle and RNAPIII transcription of tRNA genes.

Results

tRNA gene transcription is repressed following release from nocodazole arrest.

In order to test our hypothesis that replication stress checkpoint inhibition of tRNA gene transcription occurs during S-phase, I began by arresting cells in G2/M using the drug nocodazole under conditions known to cause microtubule disassembly[10]. Nocodazole arrest is reversible by removing the cells from drug containing medium, which I did by vacuum filtration, and re-suspension of the cells in pre-conditioned medium without drug. Cells then progress synchronously through mitosis and into the subsequent round of the cell cycle. Using flow cytometry, I monitored the cell cycle progression of wild type and *mrc1Δ* cells that had been treated with nocodazole for three hours and then released (Fig. 6-2A). From 30 to 60 minutes after removal of nocodazole both wild type and *mrc1Δ* cells were dividing and by 90 minutes the strains had completed cell division and had began to replicate their DNA. During the course of the same nocodazole arrest and release experiment, cell samples were taken to extract total RNA for Northern blotting analysis to monitor tRNA gene transcription. Wild type and *mrc1Δ* cells displayed slight repression of transcription of about 15% following 3 hours of nocodazole exposure (Fig. 6-2B - compare cycling versus G2/M lanes). Following removal the drug, wild type cells exhibited a significant degree of

repression that peaked around 60 minutes after release from nocodazole arrest when a majority of cells are in late G2 or mitosis (Fig. 6-2B and C). By 120 minutes after release tRNA gene transcription had increased back to levels near that associated with nocodazole arrest. In contrast to wild type, *mrc1Δ* cells did not display any repression of tRNA gene transcription following release from nocodazole arrest, even though they progressed though the division cell cycle with approximately the same kinetics as wild type (Fig. 6-2B and C).

Cells exhibit an Mrc1 independent repression of tRNA gene transcription when treated with α-factor.

To attempt to extend and confirm the previous nocodazole results, I used α-factor to block cells in G1 and released cells into S-phase by removing pheromone from medium[15]. Samples of wild type and *mrc1Δ* cells were taken at 20, 60, 90, and 120 minutes following release from G1 for both flow cytometry analysis and Northern blotting of total RNA (Fig. 6-3A, B, and C). Both wild type and *mrc1Δ* cells entered S-phase by 20 min post G1 release, and wild type cells had completed S-phase by 60 minutes, whereas *mrc1Δ* cells took until 120 minutes to complete the initial S-phase (Fig. 6-3A). Interestingly, exposure to α-factor resulted in a substantial decrease of tRNA gene transcription. Subsequent release from arrest revealed that transcription gradually returned to cycling levels by 120 minutes (Fig. 6-3B and C). Mrc1 is not required for restraint of transcription in the presence of α-factor because *mrc1Δ* cells repress transcription to similar levels as wild type when treated with α-factor (Fig. 6-3B

and C). This data suggests that tRNA gene transcription is repressed when cells are treated with mating pheromone.

Recovery from pheromone-dependent repression seems to be by a mechanism that is gradual and not specifically coupled to the progression through any particular phase of the division cycle. For example, at the 60- and 120-minute time points, wild type populations are similarly enriched for cells in G2/M. These populations however do not support the same amount of tRNA gene transcription (transcription is about 2.5-fold higher in the second G2/M). Collectively, these results suggest that inhibition is not likely due to cells being arrested in G1, but rather due to another effect of pheromone treatment.

Repression of tRNA gene transcription following pheromone treatment is associated with decrease in abundance of RNAPIII sub-unit Rpc160.

Immunoblotting was used to monitor the abundance of some of the subunits of RNAPIII to ascertain if repression is correlated with decrease in abundance of these subunits. HA, TAP, and c-myc epitope tagged sub-units of RNAPIII were used in these experiments. Rpc160-HA, Rpc82-HA, Rpc37-TAP, Rpc53-myc, and Rpc128-TAP cells were subjected to the same G1 arrest and release procedure as before. Total protein was extracted from cell samples taken at each time point and subjected to immunoblotting analysis. It was observed that Rpc160-HA, the catalytic sub-unit of RNAPIII, decreased in abundance upon α-factor treatment (Fig. 6-4A). Similar to transcription, the abundance of Rpc160-HA returned to cycling levels by 120 minutes after release. Other tested subunits

of RNAPIII do not decrease in their cellular levels during mating pheromone treatment, (Fig. 6-4B-E).

***RPC160* and *RPC82* message levels change very little in cells treated with α-factor.**

To determine whether the decrease in abundance of Rpc160-HA and Rpc82-HA was due to regulation at the level of mRNA, I subjected total RNA extracted from α-factor arrested and released cells to reverse transcription followed by real-time PCR to monitor *RPC160* and *RPC82* cDNA. Primers were designed to amplify *RPC160*, *RPC82*, and *POL1* cDNA and all real-time signals from these primers were normalized to *ACT1* levels. I found that message levels for *RCP160* and *RPC82* changed very little with α-factor G1 arrest and release (Fig. 6-5). *POL1* encodes for a replication protein whose mRNA level is known to be induced during S-phase[16]. I used *POL1* as a positive control here and indeed I found that S-phase cells (20 minutes after release) display a 2.5 fold increase in the abundance of this message.

SUMO-directed ubiquitination is not required for the decline in Rpc160 abundance observed during α-factor treatment.

The ubiquitin-like protein Smt3 of budding yeast (SUMO-1 in humans) is conjugated to substrate proteins typically on a lysine of the consensus site hKxE, where h is a hydrophobic amino acid[17,18]. Sumoylation can lead to diverse affects on the function of a target protein including altering subcellular localization,

modulating protein-DNA interactions, or regulating enzymatic activity[19,20]. Additionally, through the use of a SUMO-directed ubiquitin ligase, sumoylation can be the first step to target a protein for degradation by the proteasome[21]. Many sub-units of RNAPIII are known to be sumoylated in cycling cultures[22], however the purpose of this sumoylation remains unknown. I wondered if SUMO-targeted ubiquitination followed by proteasome degradation was involved in the decrease of abundance of Rpc160 seen upon α-factor treatment.

Slx5 and Slx8 are sub-units of the sumo-directed ubiquitin ligase in yeast and deletion of *SLX5* or *SLX8* results in viable cells that are unable to ubiquitinate sumoylated targets to direct them to degradation[23]. I treated Rpc160-HA *slx5Δ* and Rpc160-HA *slx8Δ* cells with the same G1 arrest and release procedure as before and monitored Rpc160-HA levels by immunoblotting (Fig. 6-6A-C). However, I found that cells lacking SUMO-targeted ubiquitin ligase abilities still displayed a decrease in abundance of Rpc160-HA upon α-factor treatment, indicating that this pathway was not involved in decline in Rpc160 levels.

***cdc28-13* cells have elevated tRNA gene transcription**

Progression through the various stages of the cell cycle is directed by cyclin dependant kinases (CDK)[2]. The main CDK catalytic sub-unit is Cdc28 in budding yeast (homologues include CDK1 in animals and cdc2 in *S. pombe*)[2,24]. Waves of phosphorylation of key Cdc28 substrates are what drive the events of the cell cycle. To accomplish these waves of activity, nine different regulatory proteins, called cyclins, associate with and direct Cdc28 to different substrates at different

cell cycle stages. The expression of several of these cyclins is concurrent with the cell cycle. This serves to limit their period of action to the proper time in the cell cycle[2].

During G1, Cdc28 associates with three G1 cyclins: Cln1, Cln2, and Cln3, all of which target Cdc28 to proper substrates for G1-S transition[2,25]. During late G1, the expression of *CLN1*, *CLN2*, and *CLN3* genes is highly induced leading to increased association of Cln-Cdc28 proteins and once a certain threshold of Cln-Cdc28 is met, the cell enters S-phase and commits to another complete round of cell division[25].

α-factor induced G1 arrest is made possible through inhibition of Cln-Cdc28 association[25]. Through a well-known mating signaling response that is activated upon α-factor binding to a receptor on the cell surface, the cell increases the expression of the Far1 protein, which associates with Cdc28 and inhibits Cln-Cdc28 association, thus blocking the cell in G1[25,26].

Knowing that α-factor treatment leads to repression of tRNA gene transcription, we hypothesized that Cdc28 may be involved in regulating RNAPIII. To preliminarily test this, we obtained a mutant allele of *CDC28*, *cdc28-13*[14], and assayed this mutant's level of tRNA gene transcription by Northern blotting.

This mutant strain can be used to synchronize cells in G1 by shifting exponentially growing *cdc28-13* cells to the restrictive temperature for this mutant, 37 °C, for 2.5 hours[14]. Elevated temperature causes these cells to arrest in G1 at the same G1 position as α-factor arrest. Rapid cooling to 25 °C allows the cells together to enter S-phase and continue cycling.

In order to ensure that the strain that I had obtained was defective in cell cycle progression at elevated temperatures, I grew these cells to early log phase at the permissive temperature, 25 °C, and shifted these cells to 37 °C for 2.5 hours. Using light microscopy I determined that a majority (>80%) of the cells were unbudded. Following arrest, I shifted these cultures rapidly to 25 °C on an ice bath and cultured them at this permissive temperature. I took cell samples every 10 minutes following the shift to lower temperature for DNA content analysis by flow cytometry (Fig. 6-7A). It was observed that following shift to elevated temperature for 2.5 hours the culture was enriched in G1 cells. Following release from cell cycle arrest the cells synchronously entered S-phase and progressed through the cell cycle, consistent with previous studies using this mutant allele.

Given these results, I was encouraged to perform Northern blotting to establish the rate of tRNA gene transcription of *cdc28-13* cells. I cultured wild type and *cdc28-13* cells to early log at 25 °C and shifted them to 37 °C for 2.5 hours. Cell samples were taken before and after cell cycle arrest for extraction of total RNA followed by Northern blotting. I found that *cdc28-13* cells had about 5 times higher tRNA gene transcription than wild type cells during growth at permissive temperature for this mutant (Fig. 6-7B and C). This increased transcription is not due to a cell cycle defect of *cdc28-13* at 25 °C because cell cycle profiles of wild type and *cdc28-13* are similar at this temperature (Fig. 6-7D). Following shift to non-permissive temperature the *cdc28-13* culture was enriched in G1 cells, as expected, and still showed elevated transcription, although it was dampened to about 2-3 fold over wild type. This raises the

exciting possibility that Cdc28 may be involved in a pathway that is restraining tRNA gene transcription, although at this point it is undetermined if a wild type version of *CDC28* added back to *cdc28-13* will complement the effect on transcription.

Treatment with MG132 is associated with decreased tRNA gene transcription

As mentioned before, Cdc28 is often targeted to appropriate substrates during the cell cycle by regulatory cyclin proteins and in many instances the proteasome degrades these cyclin proteins[2,3,25]. To extend these previous results, I investigated if the proteasome inhibitor MG132 affects tRNA gene transcription. We speculated that if Cdc28 is targeted by a cyclin protein to a pathway involved in repression of tRNA gene transcription, then treatment with MG132 would stabilize the proposed cyclin in non-synchronous cultures and lead to greater inhibition of tRNA gene transcription (Fig. 6-8). I used deletion of *PDR5* to increase MG132 concentration inside the cell as this gene encodes for a multi-drug pump, which removes intracellular MG132 by it efflux activity[27].

Early log *pdr5Δ* and *pdr5Δ mrc1Δ* cells were either treated with MG132 or drug carrier DMSO for 1.5 hours. Total RNA was extracted from these cells along with exponentially growing wild type cells and Northern blotting analysis was performed to monitor tRNA gene transcription (Fig. 6-9A and B). Deletion of *pdr5Δ* and treatment with DMSO had little effect on tRNA transcription. However, MG132 treated *pdr5Δ* cells have lower tRNA transcription than these cells treated

with DMSO alone (Fig. 6-9A and B). *MRC1* deletion has little effect on the decrease in transcription upon MG132 treatment because *pdr5Δ mrc1Δ* cells have significant repression of tRNA gene transcription, similar to wild type cells. This drug inhibition of transcription is not likely due to defects in cell cycle progression of MG132 treated cells because cell cycle profiles of MG132 and DMSO treated *pdr5Δ* or *pdr5Δ mrc1Δ* cells were similar (Fig. 6-9C and [27]).

Lastly, treatment of cells with MG132 is correlated with decrease in the abundance of the catalytic subunit of RNAPIII, Rpc160. I treated Rpc160-HA *pdr5Δ* cells either with DMSO or MG132 and using immunoblotting examined Rpc160-HA abundance (Fig. 6-9D). I discovered that cells treated with MG132 had a lower level of Rpc160-HA protein compared to untreated and DMSO treated levels.

Discussion

Here, basing our studies on the hypothesis that tRNA gene transcription is repressed during S-phase of the cell cycle, we uncovered new preliminary evidence that links regulation of tRNA gene transcription to the cellular machinery used to control cell cycle progression.

Firstly, I discovered that cells repress RNAPIII transcription of tRNA genes after release from nocodazole arrest. Interestingly, this repression requires the presence of the replication stress checkpoint adaptor protein Mrc1, indicating that checkpoint signaling is important in this repression[28,29].

During the course of experiments that were aimed at extending the nocodazole results, I found that there is restraint of tRNA transcription in cells treated with the mating pheromone α-factor. Following removal of pheromone, transcription increases back to untreated levels of transcription by two hours. This repression is associated with a decrease in the abundance of Rpc160, the catalytic sub-unit of RNAPIII. A proteome-wide approach has revealed that many of RNAPIII subunits are sumoylated in cycling cells[22], although the reason for this, and any involvement of sumoylayion in regulation of RNAPIII transcription, is unclear at this point. I tested if SUMO-directed ubiquitination followed by degradation by the proteasome was required for the decrease in abundance of Rpc160 upon pheromone treatment. I found that this is not likely the case. Using *slx5Δ* and *slx8Δ* strains, I found that levels of Rpc160-HA decreased during G1 arrest with α-factor in cells that are unable to ubiquitinate SUMO-conjugated targets, similar to wild type.

Lastly, we found a mutant allele of *CDC28* displays elevated tRNA gene transcription during normal growth conditions at the permissive temperature for this allele. In addition, I found that cells treated with proteasome inhibitor MG132 have less RNAPIII tRNA gene transcription than cells treated with drug carrier alone, consistent with the hypothesis that a proteasome degraded cyclin is directing Cdc28 to a pathway implicated in control of tRNA gene transcription.

Repression of tRNA gene transcription in wild type cells seen following release from nocodazole arrest peaks about 60 minutes after removal of drug. Flow cytometry analysis illustrates that cells are still in G2/M at 60 minutes. Mrc1 is

needed for this repression. Therefore, one particularly surprising interpretation of these data is that replication stress checkpoint signaling represses tRNA transcription during G2/M, not during S-phase as expected.

These results could indicate that replication stress checkpoint control of RNAPIII transcription may be involved in replication termination at tRNA genes. Recently, work that has mapped regions where replication termination takes place genome-wide has been reported[30]. This evidence suggests that replication termination often occurs at loci of replication fork pausing, such as tRNA genes. This raises the possibility that the replication stress checkpoint dependant repression that I observe may be important for the controlled completion of replication. It could be that repression of tRNA gene transcription supports the organized fusion of converging replication forks and be important to help maintain orderly replication termination.

Previous to these results, repression of tRNA gene transcription during mitosis has never been reported in budding yeast, although mitotic repression has been reported for higher eukaryotes[31-33]. In HeLa cells, this repression is conferred by phosphorylation of a component of TFIIIB. Using an *in vitro* transcription assay, it was shown that extracts produced from mitotic cells displayed dampened RNAPIII transcription[32]. TFIIIB sub-units (BRF1 or BRF1 and BDP1) purified from cycling cells added to the mitotic extracts reconstituted transcription, indicating that TFIIIB is a likely target for mitotic repression. Further, it is likely that TFIIIB sub-unit BDP1 is phosphorylated by casein kinase II (CKII) during mitosis,

leading to the repression of RNAPIII transcription[32]. Regulatory functions of CKII on RNAPIII transcription have also been reported in yeast[34].

On the other hand, inhibition of tRNA transcription following release from nocodazole arrest could be associated with re-establishing nucleolar localization of tRNA genes. As previously mentioned, tRNA gene are clustered together and localized to the nucleolus throughout the cell cycle[35-37]. Additionally, microtubules have been shown to be important in maintaining nucleolar localization since treatment of cells with nocodazole, a microtubule poison, has been shown to disrupt this localization, divorcing tRNA genes from the nucleolus[36]. In order to return to their typical location following release from nocodazole arrest, the tRNA genes presumably would re-establish connections with newly polymerized microtubules and localize back to the nucleolus. It may be that the process of developing connections with the microtubules or movement of tRNA genes back to the nucleolus is associated with the repression of tRNA gene transcription that I observe. However, how the replication stress checkpoint would be involved in this scenario is unclear.

I also discovered that cells respond to treatment with α-factor by down-regulating tRNA transcription. Possibly, this repression is important for cells to prepare for mating. In normal mating conditions, mating pheromone causes the arrest of cells in G1 to prepare two haploid cells for cell fusion during the mating process. Our newly discovered repression of tRNA transcription during α-factor exposure may be significant in preparation for the combining of the two haploid

genomes to form a diploid during mating, especially since tRNA genes are significant loci in determining overall genome architecture[35,37].

α-factor repression of tRNA gene transcription is in harmony with a recent report that suggests that treatment with mating pheromone not only arrests cell division, but also restricts cellular growth[38]. For example, these authours found that arresting cells in G1 using *CDC28* mutants led to cells continuing to grow by increasing cell volume to about 10-fold of cycling cells. α-factor treatment, on the other hand, while arresting cells at the same point in the cell cycle as the *cdc28* cells, did not increase cell volume. Further, treatment of *cdc28-arrested* cells with pheromone resulted in restriction of cell growth of these cells. Therefore, it seems likely that mating pheromone contributes to the repression of bio-mass growth of cells. As part of the mechanism for controlling protein synthesis, repressing tRNA gene transcription during pheromone treatment would be consistent with restriction of growth.

Also, the result that tRNA gene transcription is repressed during α-factor treatment has implications for the interpretation of some experiments reported in the literature. Repeatedly, experiments have used α-factor arrested and released cells to synchronize cultures to monitor cell cycle effects connected to tRNA genes, including replication and condensin loading at tRNA genes[39-42] ENREF 33. For the proper analysis of these studies, it is important to note that there is repression of tRNA gene transcription during α-factor mediated G1 arrest.

α-factor associated repression of tRNA genes is associated with a decrease in abundance of the catalytic subunit of RNAPIII, Rpc160. We find that this

decrease in abundance is not likely due to a regulation of *RPC160* message level or SUMO-directed RNAPIII ubiquitination followed by targeting to the proteasome for degradation. Whether or not this decrease in the abundance of this protein is affecting transcription during these conditions is unclear at this point and requires further experimentation. There is also a possibility that during these conditions there is degradation or cleavage of the HA epitope tag and not the actual Rpc160 protein itself leading to the decreased signal from these fusion proteins during immunoblotting of extracts of α-factor treated cells.

To finish, mutation of *CDC28* is linked with increased tRNA gene transcription during unchallenged, normal growth conditions. I also find evidence that proteasome inhibition results in limitation of tRNA transcription. This data is consistent with our hypothesis that a cyclin may be directing Cdc28 to phosphorylate a substrate protein(s) that is somehow involved in signaling to tRNA genes. Repression upon MG132 treatment seems not to require replication stress checkpoint signaling because there was still significant repression in MG132 treated cells lacking Mrc1. All of this data taken together has led us to propose the possibility that Cdc28 may be in a previously unappreciated pathway that is signaling to tRNA genes. Although at this point, until further experimentation is performed, we cannot rule out the possibility that MG132 is leading to repression of tRNA gene transcription that is independent from Cdc28.

Previous links between tRNA gene transcription and cell cycle progression have been established in the literature[6-8]. However, our results shown here indicate that there are possible new avenues of research that can be fruitful in

elucidating more about exact reasons and mechanisms of tRNA gene transcription regulation by machinery involved in cell cycle control.

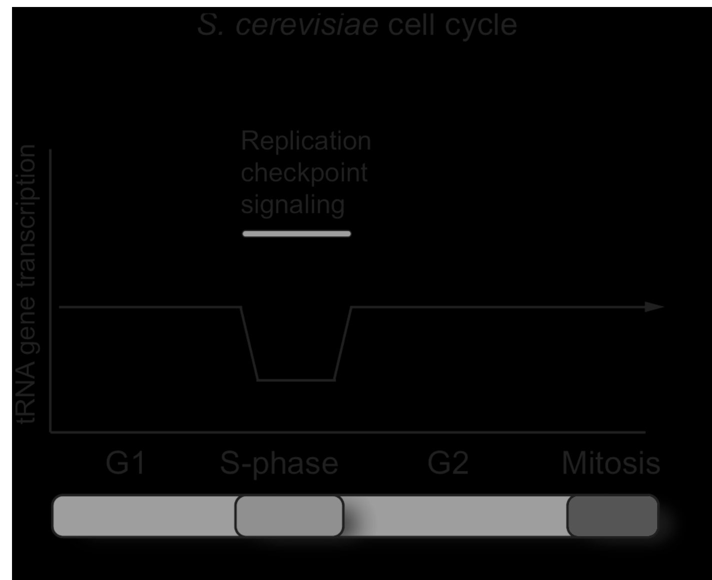
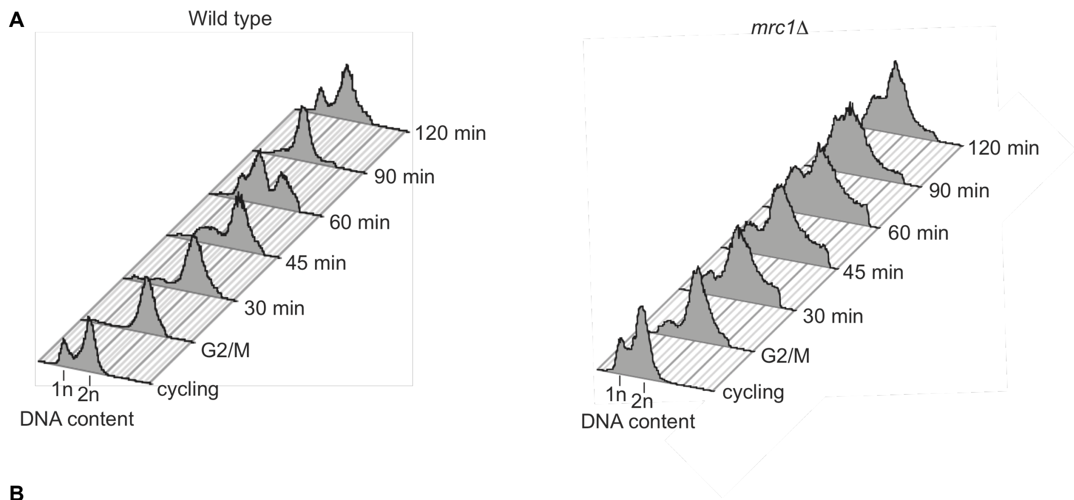
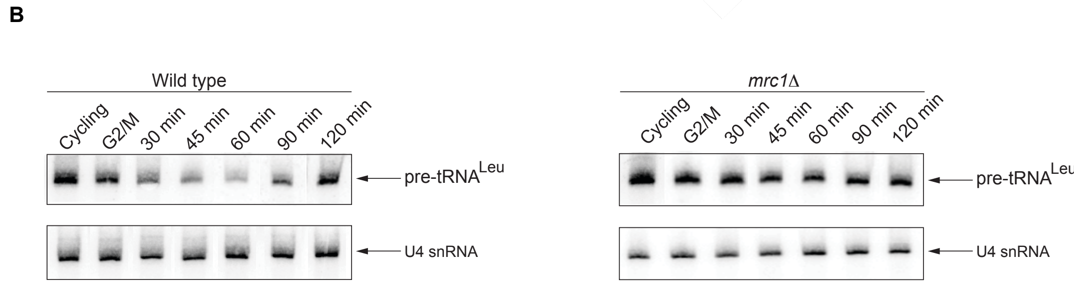


Figure 6-1. Hypothesis: Replication stress checkpoint signaling should only limit tRNA gene transcription during S-phase. Because replication stress checkpoint signaling is thought only to be active during S-phase, control of tRNA gene transcription is hypothesized to only occur during S-phase during unchallenged growth. The graph plotting tRNA gene transcription against cell cycle progression, shown here, illustrates this proposition.


A
Wild type
120 min
90 min
60 min
45 min
30 min
G2/M
cycling
1n
2n
DNA content
mrc1Δ
120 min
90 min
60 min
45 min
30 min
G2/M
cycling
1n
2n
DNA content


B
Wild type
Cycling
G2/M
30 min
45 min
60 min
90 min
120 min
pre-tRNALeu
U4 snRNA
mrc1Δ
Cycling
G2/M
30 min
45 min
60 min
90 min
120 min
pre-tRNALeu
U4 snRNA

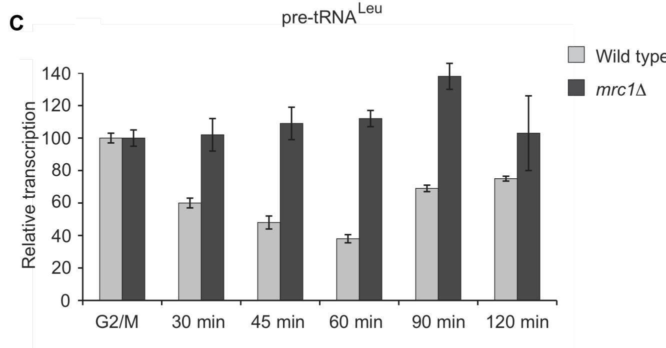
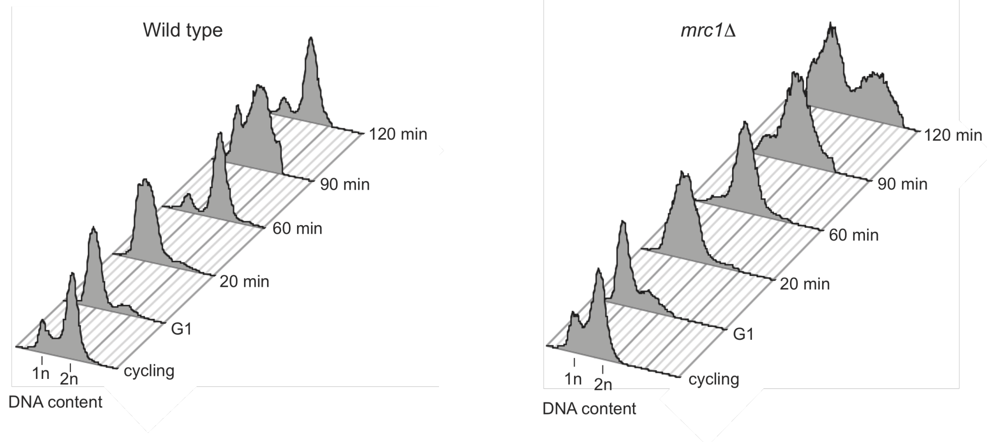
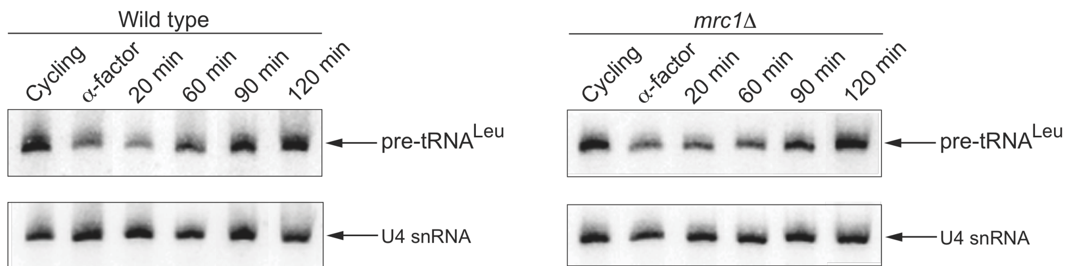

C
pre-tRNALeu
Wild type
mrc1Δ
Relative transcription
140
120
100
80
60
40
20
0
G2/M
30 min
45 min
60 min
90 min
120 min

Figure 6-2. Cells repress tRNA gene transcription following release from nocodazole G2/M arrest. A. Flow cytometry illustrates progression of wild type and *mrc1Δ* cells progress through the cell cycle after release from nocodazole arrest. Wild type and *mrc1Δ* cultures were grown to early log and treated with 15 μg/mL of nocodazole for 3 hours to arrest cell cycle progression in G2/M (cells were checked by microscopy to ensure >80% of cells were in the large-budded state). Cells were released from G2/M arrest by removal from nocodazole containing medium by rapid vacuum filtration and re-suspension in pre-conditioned medium. Samples of cultures were taken from cycling cells, nocodazole arrested cells, and 30, 45, 60, 90, and 120 minutes following release. DNA was stained by propridium iodide and DNA content was analyzed by flow cytometry. **B.** tRNA gene transcription is repressed following release from nocodazole release. Wild type and *mrc1Δ* cultures were subjected to the exact nocodazole treatment and release protocol as in A. Total RNA was extracted and probed by Northern blotting. Repression peaks around 60 minutes following release and requires Mrc1 **C.** Quantitation of Northern blot shown in B performed in biological triplicate using phosphoimaging and ImageQuant software. Leucine pre-tRNA abundance was normalized to U4 snRNA. Error bars represent +/- s.d. from the mean.

A



B



C

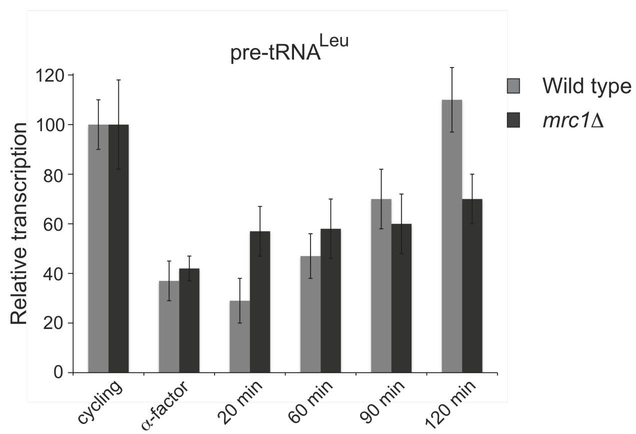


Figure 6-3. Treatment with α-factor is associated with repression of tRNA gene transcription that does not require Mrc1. A. Flow cytometry analysis shows that wild type and *mrc1Δ* cells arrest in G1 upon pheromone treatment and synchronously enter S-phase upon removal of α-factor from medium. Wild type and *mrc1Δ* cells were grown to early log and were treated with 10 μg/mL α-factor for 3 hours to synchronize cells in G1 (>90% of cells were determined unbudded by light microscopy). Cells were released from arrest by filtration of cells from pheromone containing medium by vacuum filtration and resuspention into pre-conditioned medium. Samples of cells were taken from cycling cells, G1 arrested, and 20, 60, 90, and 120 minutes following release. Flow cytometry was used to analyze DNA content. **B.** Cells treated with α-factor repress tRNA gene transcription. Wild type and *mrc1Δ* cultures were G1 arrested and released as in A and total RNA from cell samples was extracted. Northern blotting was used to probe total RNA. **C.** Quatitation of Northern blotting in B. Pre-tRNA levels were normalized to U4 RNA and plotted on this bar graph. Error bars are +/- s.d., n=3.

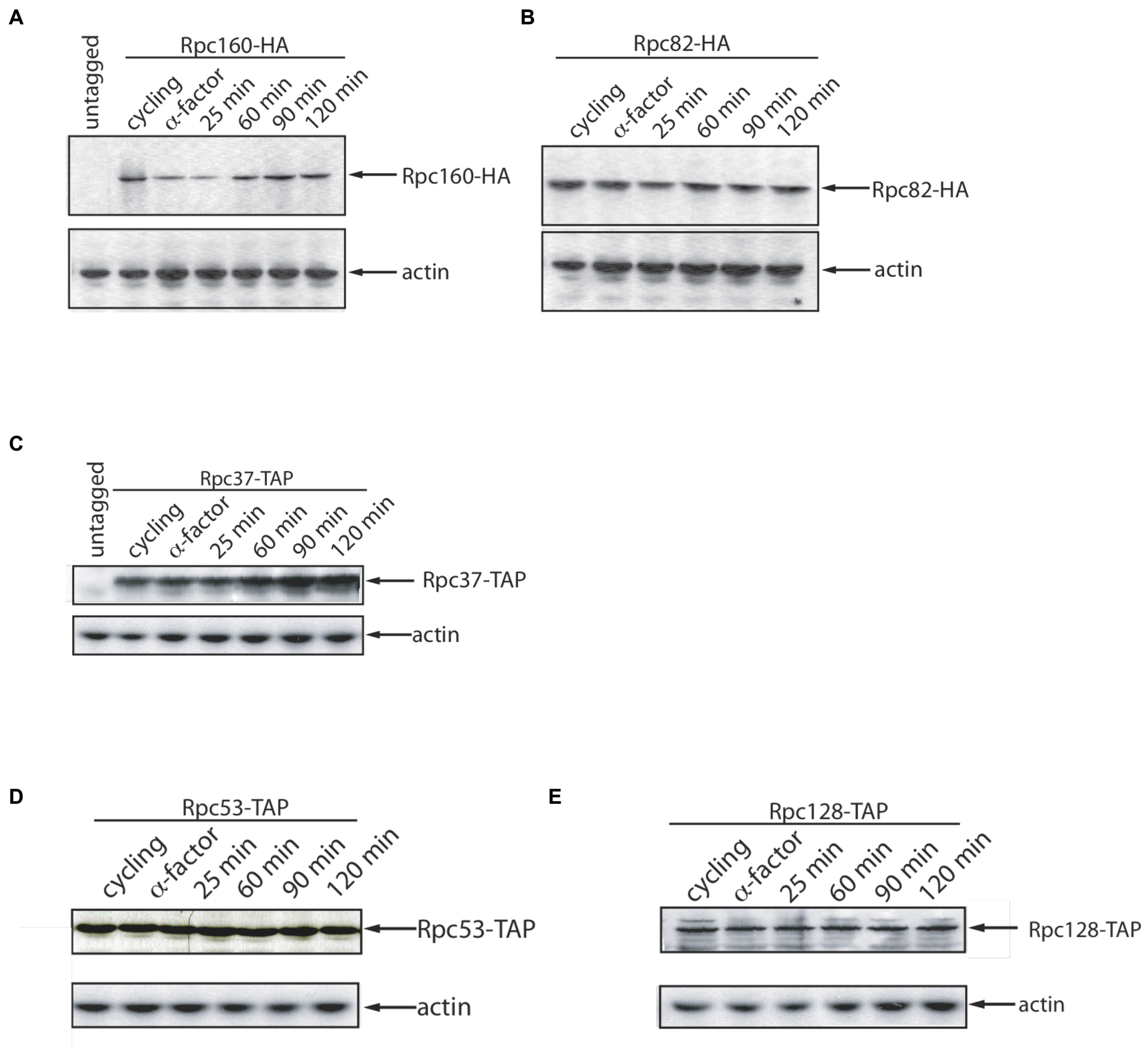


Figure 6-4. Repression of tRNA gene transcription by α-factor is correlated with a decrease in abundance of Rpc160. A-E. Rpc160-HA, Rpc82-HA, Rpc37-TAP, Rpc53-myc, and Rpc128-TAP cells were treated with α-factor to synchronize cells in G1 and released as before. Protein extracts from samples of these cells was subjected to immunoblotting using primary antibodies against epitopes. Actin abundance is shown here as a loading control.

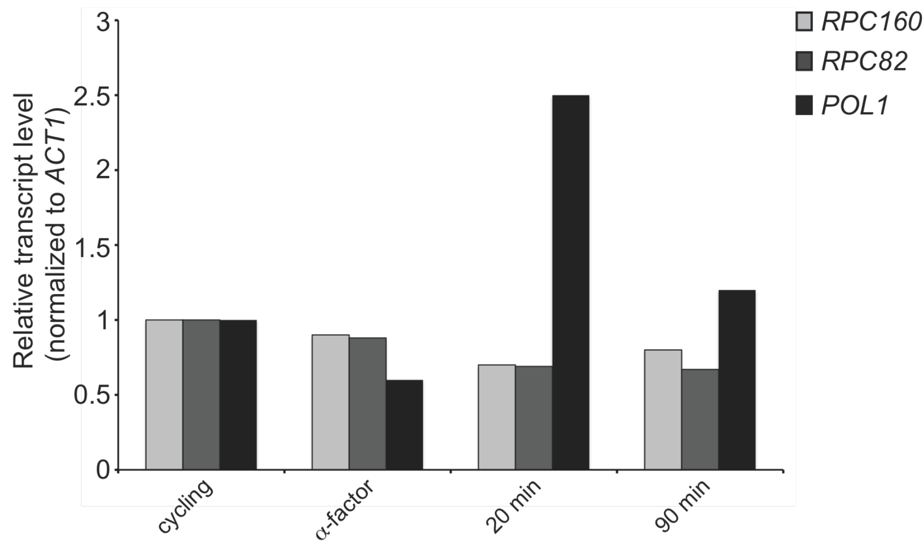


Figure 6-5. *RPC160* and *RPC82* message abundance changes little in response to α-factor arrest and release. cDNA from reverse transcribed total RNA from α-factor treated and released cells was probed by real-time PCR using primers for *RPC160, RPC82,* and *POL1. POL1* encodes a replication protein whose message level is induced during S-phase and *POL1* is used here as a positive control. Height of the bars represents real-time PCR signal normalized to PCR signal from primers designed to amplify *ACT1* cDNA. Real-time PCR signal using *RPC160* primers of cDNA from cycling cells is set to one and all other PCR signals are compared to this.

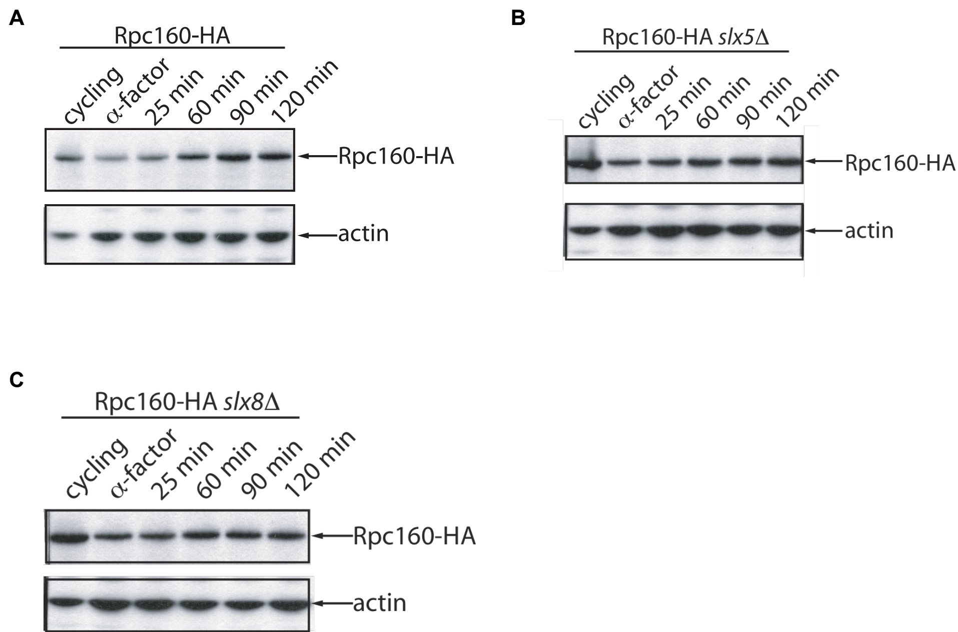


Figure 6-6. SUMO directed ubiquitination is not required for decrease in abundance of Rpc160 during α-factor treatment. A-C. Rpc160-HA, Rpc160 *slx5Δ,* and Rpc160-HA *slx8Δ* strains were G1 arrested and released as before. Total protein was extracted from cell samples and Rpc160-HA abundance was monitored by immunoblotting.

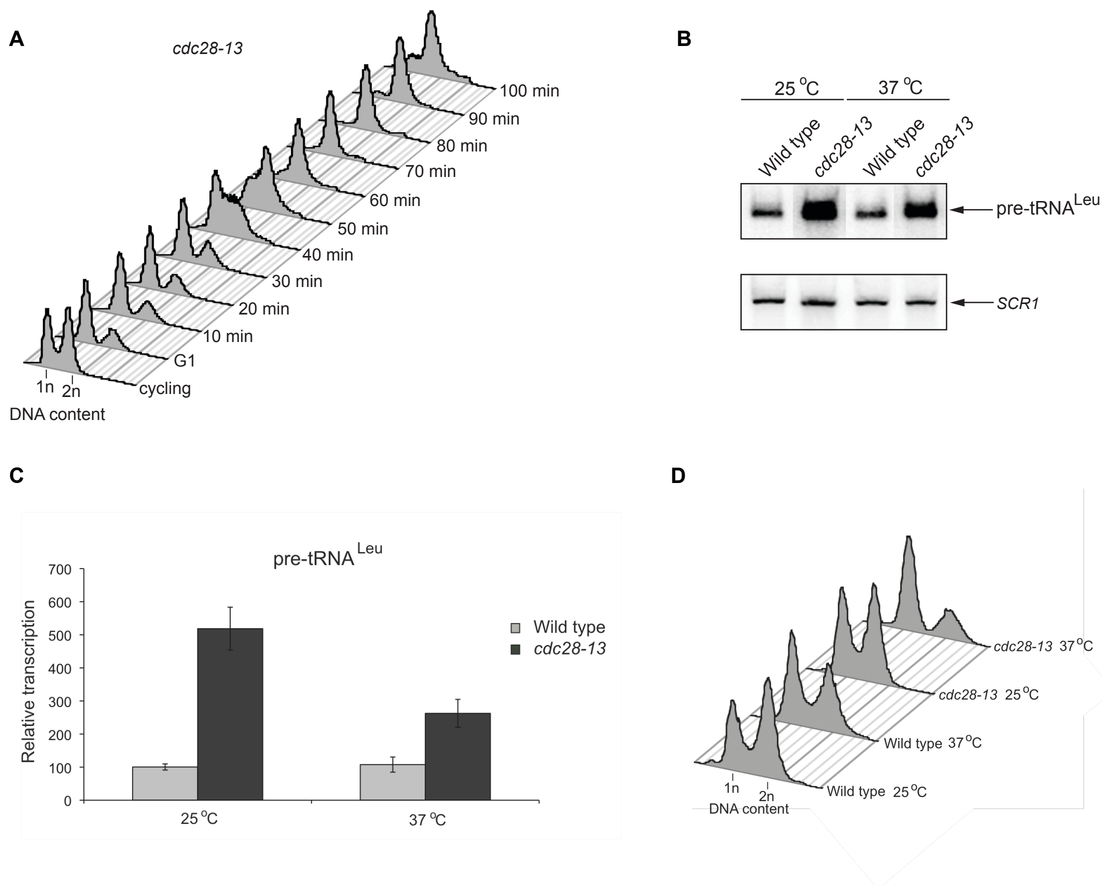


Figure 6-7. Cdc28 may be required for repression of tRNA gene transcription in cycling cells. A. *cdc28-13* cells arrest in G1 when shifted to 37 °C for 2.5 hours and synchronously begin cycling when shifted to 25 °C following arrest. Following arrest at elevated temperature, the culture was rapidly cooled in an ice-bath and cultured at 25 °C to release cells from G1 arrest. Cell samples from cycling cells, G1 arrested cells, and cells from the indicated times following release were analyzed for DNA content using flow cytometry. **B.** Total RNA from wild type and *cdc28-13* cells from B was probed by Northern blotting. *SCR1* is loading control. **C.** Quantitation of Northern blot shown in C performed in biological triplicate. Leucine pre-tRNA levels were normalized to *SCR1* levels. **D.** Cell cycle profiles of wild type and *cdc28-13* cells are similar in cells grown at 25 °C. Exponentially growing Wild type and *cdc28-13* cells culture at 25 °C were shifted to 37 °C for 2.5 hours. Flow cytometry analysis was used to determine DNA content from pre- and post-temperature shifted cells.

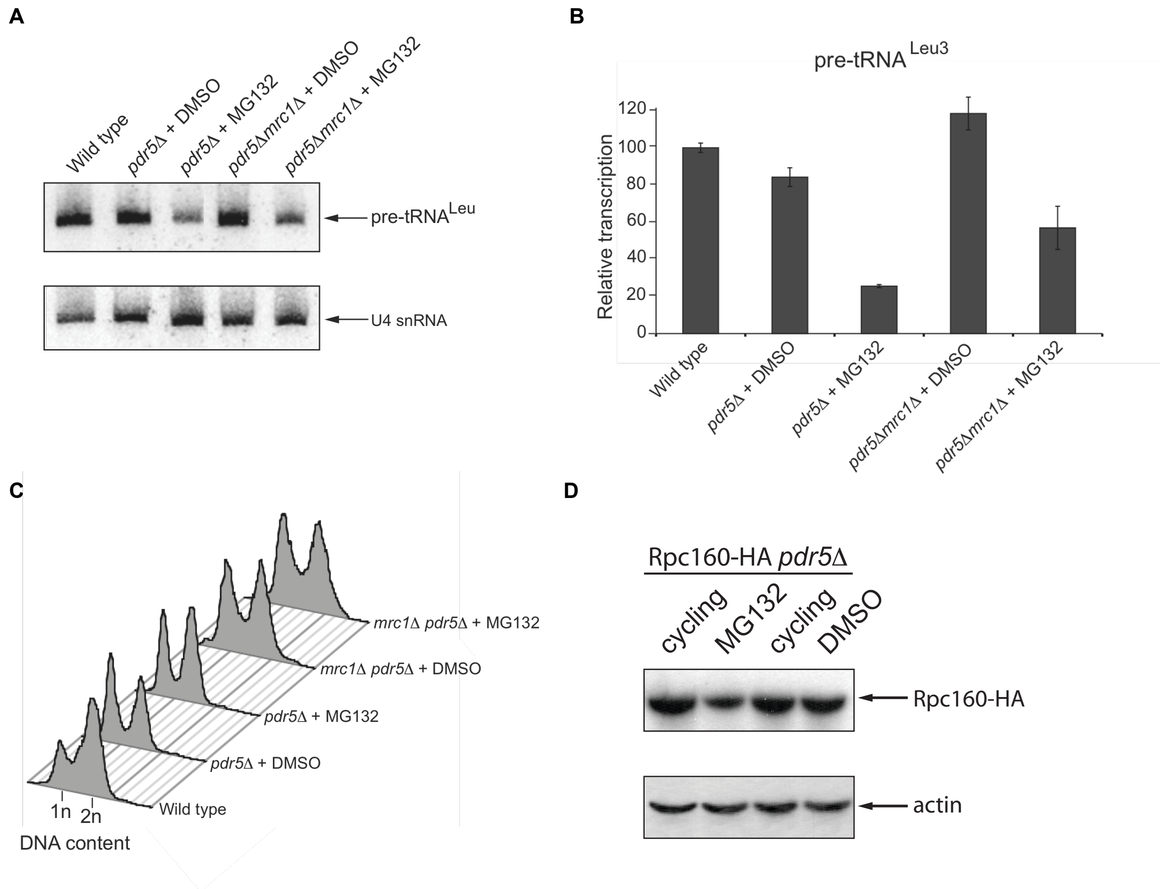


Figure 6-8. Treatment with proteasome inhibitor MG132 is correlated with a decrease in tRNA gene transcription. A. Total RNA from wild type cycling cells and *pdr5Δ* or *pdr5Δ mrc1Δ* cells either treated with 1% DMSO carrier or 70 μM MG132 for 1.5 hours was probed by Northern blotting. Deletion of *PDR5* was used to increase intracellular concentration of MG132. **B.** Three independent replicates of the experiment in A were performed and quantitation by phosphoimaging is shown in bar graph. **C.** Treatment with MG132 does not result in arrest of cell cycle progression. Samples of cells from A were subjected to flow cytometry analysis. **D.** Treatment with MG132 is correlated with decrease of Rpc160-HA abundance. Rpc160-HA cells were grown to early log and treated either with 1% DMSO carrier or 70 μM MG132 for 1.5 hours. Rpc160-HA abundance monitored by immunoblotting.

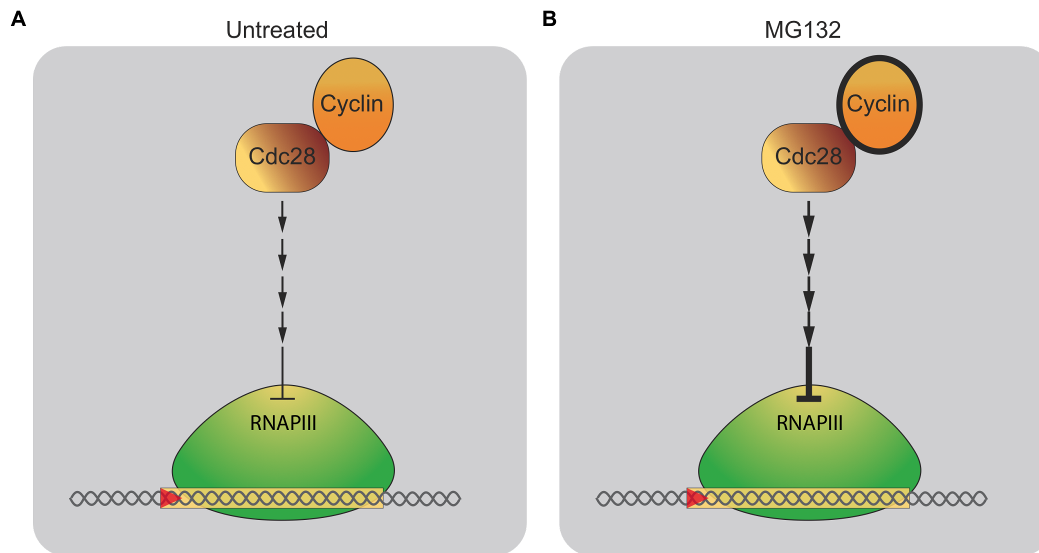


Figure 6-9. Rationale for experiment using proteasome inhibitor MG132. A. During normal growth conditions, it is proposed that Cdc28 is directed by a regulatory cycling to pathway correlated to repression of RNAPIII transcription of tRNA genes. **B.** Exposing a cycling culture of cells to MG132 would stabilize the predicted cyclin (thick line around cyclin), which would lead to greater targeting of Cdc28 to repressing pathway and lead to greater inhibition of RNAPIII transcription of these genes (thick repression lines).

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Chapter 7

General discussion and future directions

Chapter 7

General discussion and future directions

Building on the initial discovery that the replication stress checkpoint restrains RNAPIII transcription of tRNA genes[1,2], the work presented here reveals the steps of tRNA transcription that are targeted by checkpoint signaling and provides insight into how this phenomenon affects replication. In this concluding chapter, I will discuss the major findings presented in this thesis and describe our preferred speculative model of the spatial range of replication checkpoint signaling to tRNA genes[1]. I will then propose future work aimed at furthering our understanding of tRNA gene control by the replication stress checkpoint pathway and elucidating further the connections between tRNA gene transcription and cell cycle progression. In conclusion, I will briefly discuss the possible implications of our results for higher organisms, including potential associations with human cancers.

Steps of tRNA gene transcription targeted by the replication stress checkpoint during HU exposure.

I found that during HU-induced replication stress, tRNA gene transcription is repressed by the canonical Maf1-mediated mechanism[3-5]. That is, RNAPIII and TFIIIB dissociate from tRNA genes and TFIIIC increases association with these sites. Also, Maf1, which is known to be essential for restraint of RNAPIII transcription during HU treatment[2],

increases in its association with tRNA genes. From these data, we suggest that the level at which RNAPIII transcription is inhibited by Maf1 during replication stress is PIC assembly on the template DNA[6,7].

Further, genetic experiments showed that Maf1 functions in the same linear pathway as the checkpoint protein Rad53 during replication stress. Our lab has previously reported that Maf1 dephosphorylation requires Rad53 during replication stress and Rad53 is normally activated in HU treated *maf1Δ* cells[2]. All of this taken together strongly suggests that during replication stress, active checkpoint signaling impinges on Maf1 to signal to tRNA genes. Lastly, I found that under conditions of highly elevated tRNA transcription *maf1Δ* cells are sensitive to growth on HU-containing media. All of this work further characterizes this newly discovered method of RNAPIII regulation.

The effects of inactivating replication checkpoint signaling on the proteins present at tRNA genes during normal growth conditions.

I demonstrated that increased tRNA gene transcription in *mrc1Δ* cells is correlated with increased cross-linking of RNAPIII with template DNA. Interestingly, on the other hand, condensin's association was found to decrease in cells deficient in the replication stress checkpoint compared to wild type cells. Condensin is found enriched at tRNA genes and is an important complex in control of the overall architecture of a cell's genome[8-

[10]. Because of this, we propose that defects in replication stress checkpoint signaling likely has effects on genome organization.

Cis- and trans-repression of tRNA gene transcription by checkpoint signals: a speculative model.

The existence of a cis-acting signaling mechanism for checkpoint inhibition of tRNA genes is likely. In the model we suggest, a replication fork paused at a tRNA gene leads to a local activation of the replication stress checkpoint proteins resulting in removal of the fork blocking RNAPIII PIC. However, other evidence also indicates that trans-repression of tRNA genes by the replication stress checkpoint signaling pathway may exist.

First, there is evidence that replication stress checkpoint signals can affect regions of the genome where no replication abnormality exists. To explain, replication origin firing during S-phase is not simultaneous. Some origins fire first during S-phase initiation and are known as early firing origins, then other origins fire later in S-phase. Early replication origins fire during HU treatment, which leads to replication fork pausing due to dNTP starvation. These paused forks, which initiated at early firing origins, cause the activation of the replication stress checkpoint that is known to repress late origins. This prevents further accumulation of unstable stalled replication forks[11-13]. In other words, checkpoint signals can reach origins located at sites which have no perturbation of replication.

Second, because the tRNA genes are clustered in the nucleolus[9,14], the spatial barrier to checkpoint signaling between tRNA genes is decreased. Therefore, a paused replication fork at a tRNA gene is in close proximity to other tRNA genes and activated checkpoint signaling molecules do not need to travel far to signal in trans.

Putting together what we know so far leads to the following speculative model of the signal initiation and spatial range of checkpoint signaling (Fig. 7-1). We suggest that normal replication pausing at tRNA genes results in molecular transactions that produce an abnormal DNA structure that is sensed by the replication stress checkpoint. It may be that excess amounts of ssDNA produced by these interactions is sensed as 'damage' and is the first step in activation of the replication stress checkpoint[15,16]. A fork blocking tRNA gene that causes checkpoint activation is repressed by that signal, and other tRNA genes are weakly inhibited in trans (Fig. 7-1A). This trans signaling could be expected to reduce the chance that replication forks pause tRNA genes that have not yet been copied. Strong repression of a single tRNA gene during HU treatment could be due to replication stress checkpoint signals that came from forks that have stalled a sizeable distance away from the gene (Fig. 7-1B(i)). The localized signal in cis at a naturally paused fork in a cell treated with HU could be additive to the effect of genotoxin on that fork (Fig. 7-1B(ii)). Additional fork perturbation by HU might strengthen the local signal such that it results in full activation of the repressive pathway so that Maf1 is now involved. Together, these

replication interference incidents would strengthen repression and further decrease replication fork obstruction at tRNA genes.

Future Studies aimed at understanding replication fork interference by tRNA genes.

As mentioned in chapter 5, a number of discrepancies exist between replication fork pausing studied by ChIP-on-chip cross-linking studies and this phenomenon examined by 2D electrophoresis. Genome-wide analysis of Pol2 cross-linking revealed replication fork pausing at a sub-set of tRNA genes that were not detected to be fork blockers by 2D electrophoresis (i.e. co-directionally transcribed tRNA genes)[17]. The reverse was also found to be the case as well; some known and well-studied fork blocking tRNA genes, as determined by 2D electrophoresis, were not revealed to be fork blockers by Pol2 cross-linking[17]. Further, cross-linking of the GINS complex genome-wide suggested that all tRNA genes are barriers to replication[18]. Until such discrepancies are understood it will be challenging to determine how precisely tRNA genes interfere with the replication process. Therefore, future studies in this area could include a comprehensive examination of tRNA gene replication interference by ChIP and 2D electrophoresis experiments in parallel. Such experiments would monitor replication at both co-directionally and head-on transcribed tRNA genes and replication fork pausing should be monitored by these two methods in wild type and replication stress checkpoint mutants. This set of experiments will deepen

our understanding of the nature of replication fork pausing at tRNA genes by studying replication pausing by both methods in cells from the same cultures.

Next, according to our proposed model, tRNA gene repression during normal growth conditions is a downstream event that in elicited in response to replication difficulties. Future studies could focus on determining the nature of replication stress checkpoint signal initiation in normally cycling cells. Although we have ruled out the requirement of γ-H2A, H3K56 acetylation, Rrm3, and Tof1, there are other replication proteins that could be found to mediate the repression of tRNA genes in unchallenged cells. I anticipate the identification of replication protein mutants that display a lack of inhibition of tRNA gene transcription during exponential growth as measured by Northern blotting. Cells with inactivating mutations of certain replication proteins are known to be defective in the activation of the replication stress checkpoint. These mutants would be tested first. As previously mentioned, Rpa1 is of particular interest since it is generally accepted that excess ssDNA bound to RPA formed during various challenges to replication is the abnormal structure recognized by the cell to initiate checkpoint activation. Mutants of *RPA1* have been shown to have stable stalled replication forks, but have a defect in the ability to activate Mec1 in response to those stalled forks[15,16,19]. The GINS complex is another candidate that should be tested because it is already established that GINS sub-unit cross-linking is enriched at tRNA genes genome-wide[18]. Once

replication proteins are implicated in the regulation of tRNA genes in this manner, further experimentation could be directed at determining if and how identified replication proteins are involved in activation of checkpoint signaling at tRNA genes. These experiments will reveal important aspects of replication checkpoint signal initiation at tRNA genes and may offer clues into the nature of the replication pause at these endogenous replication pause sites.

Finally, future work in this area would be aimed at elucidating further the biological function(s) of repression of tRNA gene transcription by the replication stress checkpoint. For example, the effect of *MRC1* deletion on condensin association at tRNA genes is an interesting finding that should be examined further. Specifically, because condensin is a known requisite for the mitotic compaction of DNA[10,20-22], analysis of checkpoint mutants should be undertaken to determine if replication stress checkpoint mutants are defective in condensation. Further, condensin association at tRNA genes has been shown to be essential for the clustering of tRNA genes together throughout the cell cycle[9]. The implication here is that checkpoint mutants may have defects in tRNA gene clustering because of the decreased condensin loading at these sites. This could be tested by fluorescence microscopy.

Future studies aimed at understanding links between tRNA gene transcription and cell cycle progression.

In the course of studying tRNA gene transcription during cell cycle progression, we acquired preliminary evidence to suggest that the main CDK in yeast, Cdc28, is involved in a previously unknown regulation of RNAPIII transcription. However, further study is required to verify this. Specifically, it remains to be seen if a wild type version of *CDC28* will complement the increase in tRNA transcription seen in the *cdc28-13* cells. Presuming that the effect of *CDC28* mutation on RNAPIII transcription is the result of impaired kinase activity, a number of exciting possibilities can be envisaged. Initially, we proposed that a cyclin protein might be directing Cdc28 to substrate proteins that are involved in signaling to tRNA genes, an idea that is supported by evidence showing tRNA gene transcription is repressed during proteasome inhibition (see Chapter 6). To further our understanding, tRNA transcription could be measured by Northern blotting in strains harbouring mutations of putative cell cycle regulators. For example, the cyclins Cln1, Cln2, Cln3, Clb5, and Clb6 all regulate Cdc28 function at various stages of the cell division cycle and mutations of genes encoding these proteins should be examined for effects on RNAPIII transcription.

Another candidate gene that could be tested is *FAR1*. As discussed in Chapter 6, Far1 functions during pheromone exposure to arrest the cell cycle in G1 by inhibiting Cdc28-G1 cyclin association. Far1 expression is

up-regulated by active mating signaling induced by pheromone binding to its receptor[24,25]. Far1 could be targeting Cdc28 to a pathway leading to repression of tRNA transcription during α-factor treatment. Even though expression of Far1 is increased during mating pheromone treatment, a moderate amount of Far1 is produced during normal growth conditions, most of which is quickly degraded by targeting to the proteasome[23]. Therefore, two treatments (MG132 and α-factor), which stabilize Far1 expression, also cause inhibition of tRNA gene transcription, suggesting that Far1 may be involved in this repression.

Additionally, during repression of tRNA genes during both α-factor and MG132 treatment the abundance of Rpc160 decreases. This is particularly surprising because changes of Rpc160 abundance during repressing conditions has never been detected before and is noteworthy because this (Rpc160) is an essential sub-unit of a larger RNAPIII complex. We have ruled out that the decrease in abundance is due to SUMO-directed ubiquitination or changes in the message level during pheromone treatment. Future examination could be directed at determining the mechanism of the decrease in the abundance of this protein. One possibility is that the protein is targeted to proteasomal degradation by ubiquitination, as is the case with many unstable proteins[26]. One of the first steps to investigate this is to ascertain if Rpc160 is ubiquitinated during α-factor or MG132 treatment.

Phosphoryaltion of Cdc28 on tyrosine 19 is also an important method of regulation Cdc28 kinase activity in yeast[27]. To determine if phosphorylation of Cdc28 is involved in regulation of tRNA transcription, mutants of the Cdc28 kinase, Swe1, and phosphatase, Mih1, should be tested for the effect of mutation on tRNA gene transcription.

Lastly, I propose performing a synthetic genetic array (SGA) analysis of *cdc28-13* at the permissive temperature for this strain (25 ºC). In SGA studies, robotics are employed to mate a query strain (*cdc28-13* in our case) with the entire viable single gene deletion library to generate about 4800 different double mutants. The viability or growth defects of each double mutant can be determined to identify new possible genes that interact with *cdc28-13*. This experiment takes advantage of the fact that the *cdc28-13* cells at the permissive temperature (25 ºC) exhibit ~ 5-fold greater tRNA gene transcription than wild type, yet fully support proper cell cycle progression (Fig. 6-7D). Therefore, genes identified in SGA would likely exclude those interacting with *CDC28* in cell cycle regulation. The identification of genetic interactions using high-throughput SGA analysis provides an excellent starting point to identify and understand how a group of proteins might function in the cells, especially when a new function of a protein is discovered (for example, Cdc28 being involved in the regulation of tRNA gene transcription).

Significance of tRNA gene transcription and replication stress in yeast: links to oncogenesis.

Both replication stress and elevated tRNA gene transcription have been separately implicated in oncogenesis. To illustrate, the high rate of proliferation phenotype of some cancer cell types is partly achieved by the induction of tRNA transcription[28], possibly to sustain the high level of translation required by these cells. In addition, many tumour suppressors and proto-oncogenes directly inhibit and activate (respectively) tRNA gene transcription in order to increase the cell's capacity for tRNA synthesis[29].

There is also evidence that interference with DNA replication is important for oncogenesis. In particular, there are mounting data that suggests the development of replication stress as an early causal event in the chromosomal instability seen in the early stages of some sporadic tumours[30,31]. The most commonly accepted model presented in the literature is that the frequency of DNA double-strand breaks in precancerous cells is elevated as a result of oncogene-induced collapse of replication forks. Therefore, chromosomal instability of some types of tumour cells is thought to be caused by the genetic changes caused by early double-strand breaks formed by such challenges to replication.

Recently, more direct evidence linking replication stress and early oncogenesis has been reported. A paper published by the Kerem lab revealed that the activation of an important signaling pathway in regulating cell proliferation, the Rb-E2F pathway, results in shortages of nucleotides

for replication[32]. This nucleotide deficiency resulted in replication stress and genomic instability in cells. What is more, the addition of exogenous nucleotides, or up-regulating nucleotide biosynthesis, released the replication stress and also prevented the replication-associated DNA damage. For these reasons, the authours propose that aberrant activation of proteins responsible for cell proliferation in the early evolution of cancer cells can lead to insufficient nucleotides to support normal replication and maintain genome stability[32].

Working under the presumption that active tRNA genes interfere with replication in humans, we propose that elevated tRNA gene transcription may be a contributing factor to oncogene-induced replication fork collapse seen in pre-cancerous cells (Fig. 7-2). Several activated oncogenes, which are known to cause replication stress, also turn out to induce tRNA gene transcription, including Ras and c-myc[28,29]. This supports the notion that there may be causal links between RNAPIII transcription and replication-associated DNA damage.

Final comments.

Studies from the past two decades have increased our understanding of replication at regions of the genome that are difficult to copy. In particular, it has been demonstrated that tRNA genes are sites at which replication forks pause during normal S-phase in yeast[33,34]. Nevertheless, much still remains unknown about the precise way in which active tRNA genes interfere with

progressing replication forks. Work from our lab has demonstrated a novel mechanism that cells use to overcome the interference to replication that active tRNA genes present. A known DNA-structure checkpoint pathway that responds to replication difficulties has also been found to repress RNAPIII transcription of tRNA genes.

The work presented in this thesis builds and expands upon this initial discovery in two major ways. First, ChIP experiments provide an understanding of the Maf1-mediated mechanism of repression of tRNA gene transcription by the replication stress checkpoint. These results add 'replication stress' to the already lengthy list of stress conditions that impinge on Maf1 to repress RNAPIII transcription.

Second, preliminary data suggest possible new and exciting links between tRNA gene transcription and the cell cycle progression. For example, it is now plausible that Cdc28 is involved in regulation of RNAPIII. These results open up a variety of research avenues that can now be pursued. Specifically, if Cdc28 is confirmed to be part of a signaling pathway that controls tRNA gene transcription, what are the other signaling components to this pathway? Is protein degradation by the proteasome an important part of the repression that requires Cdc28? Given the robustness of these preliminary data, we predict that research in the next five years will elucidate novel cell cycle-linked pathway(s) that control tRNA gene transcription, and possibly lead to a more comprehensive appreciation of the specific reasons why tRNA genes are regulated by cell cycle machinery.

Research presented in this thesis is consistent with the overall hypothesis that tRNA genes are sites of the genome that are commonly associated with genome change[35]. Replication interference at tRNA genes needs to be managed by cells in order to minimize negative effects of such perturbation. It is becoming apparent is that tRNA gene transcription is associated with cellular phenomena that are not directly related to either tRNA production or translation. Interestingly, it is clear that tRNA genes are associated with a remarkable number of protein complexes that are involved in a number of signaling pathways[35]. Exciting and productive research focused on tRNA gene biology is envisioned in the near future, as the precise involvement of these complexes in tRNA gene biology is determined.

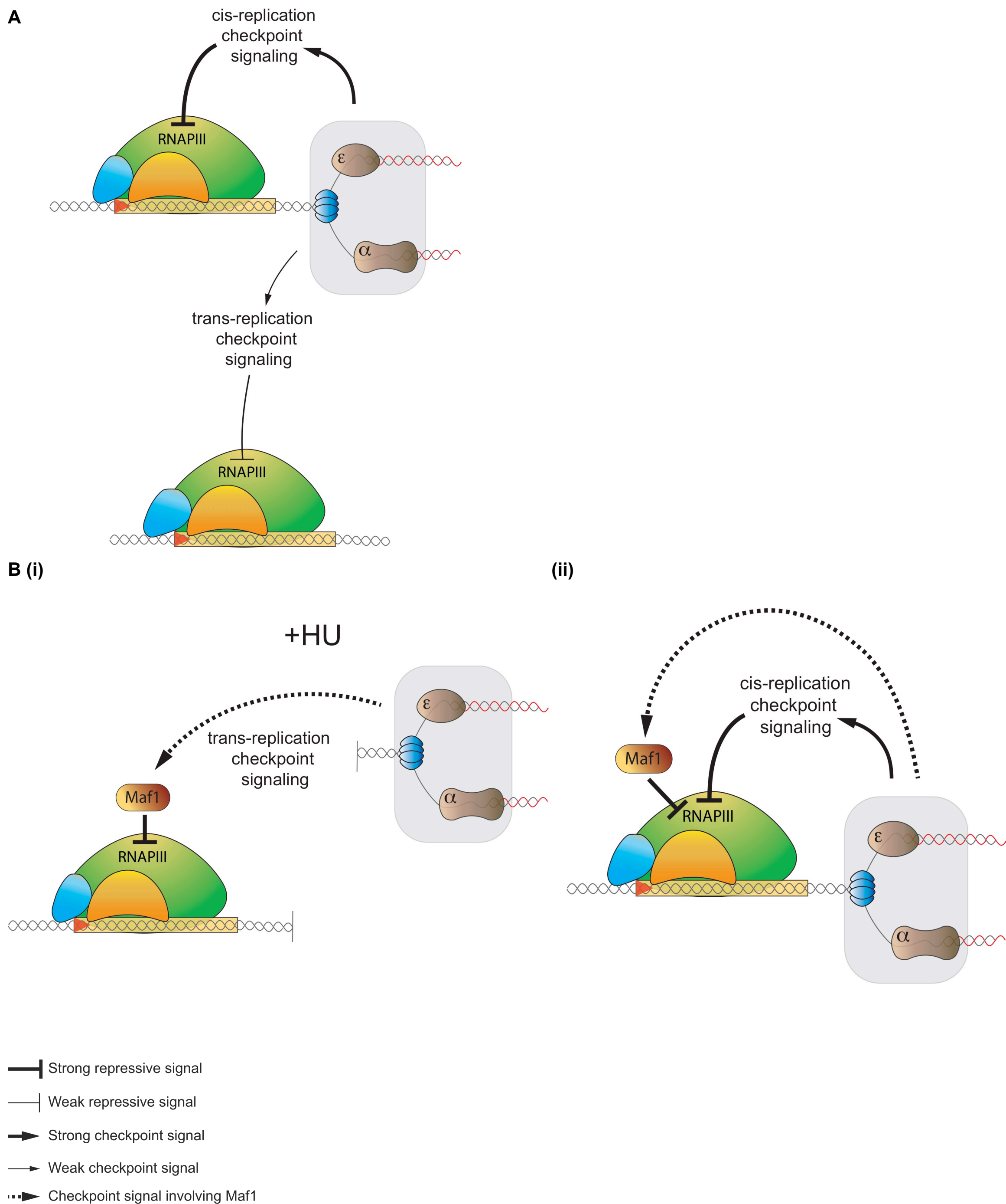

Figure 7-1. Speculative model of initiation and spatial range of checkpoint signals to tRNA genes. A. Model depicts events during normal cycling conditions. **B.** Events during replication stress are illustrative of replication fork stalling away from a tRNA gene (i) and in proximity to a tRNA gene (ii). Scenarios involve cis-signaling (A and B(ii)) and trans-signaling (A and B(i)).

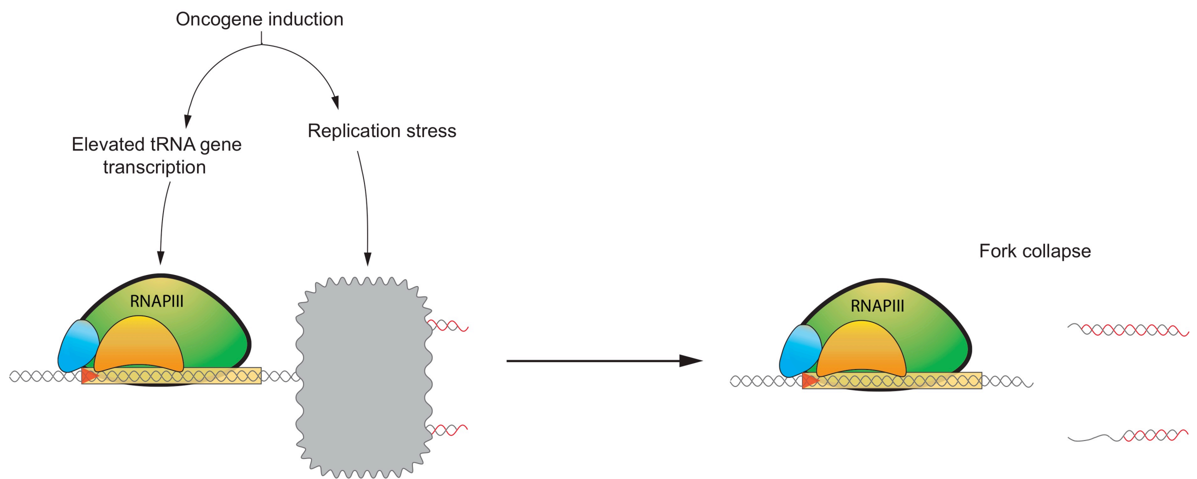

Figure 7-2. Elevated tRNA transcription may contribute to genomic instability seen in the early development of human tumours. Elevated RNAPIII transcription and replication stress are both observed in human cells with some activated oncogenes. Replication fork encounters with RNAPIII during this situation could contribute to increased fork collapse and result in DNA lesions.

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