tRNA GENES AFFECT MULTIPLE ASPECTS OF LOCAL CHROMOSOME BEHAVIOR

by

Matthew J Pratt-Hyatt

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
2008

Doctoral Committee:

Professor David R. Engelke, Chair
Professor Robert S. Fuller
Associate Professor Mats E. Ljungman
Associate Professor Thomas E. Wilson
Assistant Professor Daniel A. Bochar

.

To my loving family

Who've helped me so much

Acknowledgements

I would first like to thank my mentor, David Engelke. His help has been imperative to the development of my scientific reasoning, writing, and public speaking. He has been incredibly supportive at each phase of my graduate career here at the University of Michigan. Second, I would like to thank the past and present members of the Engelke lab. I would especially like to thank Paul Good, May Tsoi, Glenn Wozniak, Kevin Kapadia, and Becky Haeusler for all of their help.

For this dissertation I would like to thank the following people for their assistance. For Chapter II, I would like to thank Kevin Kapadia and Paul Good for technical assistance. I would also like to thank David Engelke and Tom Wilson for help with critical thinking and writing. For Chapter III, I would like to thank David Engelke and Rebecca Haeusler for their major writing contribution. I would also like to acknowledge that Rebecca Haeusler did the majority of the work that led to figures 1-3 in that chapter. For Chapter IV, I would like to thank Anita Hopper, David Engelke and Rebecca Haeusler for their writing contributions. I would also like to acknowledge Rebecca Haeusler's work that led to figure 4.2 in that chapter.

I would also like to thank my parents, Peggy and John Hyatt, for their encouragement and support. They instilled in me the importance of intellectual investigation.. Finally, I wish to thank my wife, Jennifer, and my daughter, Genevieve, who make my life fun and exciting. Jen is such a supportive person and I don't know where I would be without her. She is always there to help me see the big picture.

Table of Contents

List of Figures

Figure

List of Tables

Table

List of Abbreviations

pol II	RNA polymerase II
pol III	RNA polymerase III
mRNA	Messenger RNA
SINES	Short interspersed repetitive elements
tRNA	Transfer RNA
LTR	Long terminal repeats
SSA	Single strand annealing
tgm	tRNA gene mediated
SMC	Structural maintenance of chromosomes
DMAPP	Dimethylallyl diphosphate
ChIP	Chromatin immunoprecipitation

Abstract

In eukaryotic organisms the tRNA genes, which exist in multiple copies, are spread throughout the genome. Although it had been assumed that these genes are spread throughout the nucleus, it has recently shown that tRNA genes are actually clustered at the nucleolus. Since the tRNA genes seem to play a role in organizing the entire genome, their transcription may have dramatic influences. The three studies presented here were undergone to provide insight into these influences and their maintenance.

The first study provides evidence that tRNA gene transcription influences recombination in the genome. The study examined two different circumstances. The first situation was used to tested if the transcription of repetitive tRNA genes influenced their tendency to undergo homologous recombination and the second situation was used to test how a tRNA gene influences the homologous recombination of two nearby repetitive sequences. It was determined that two transcriptionally active tRNA genes had a five times greater rate of recombination than if one or both tRNA genes were inactivated, however tRNA gene transcription had no discernible influence over the recombination between nearby repetitive elements. This could have implications in the human genome, which contains over 1 million RNA polymerase III transcribed SINE elements.

The second study explores how tRNA gene transcription influences their clustering and localization at the nucleolus. This study demonstrated that the condensin complex associates with tRNA genes and physically interacts with RNA polymerase III transcription factors. Condensin's interactions with these two elements may lead to the clustering and localization of the tRNA genes, which could play an important role in the organization of the entire genome in eukaryotes.

The third study found a gene deletion, *mod5*Δ, that results in the alleviation of silencing near tRNA genes. The results of this study indicate that Mod5p's catalytic activity is necessary for tgm silencing. The protein was also shown to be present at

tRNA genes and seems to maintain the silencing in *cis*. These results indicate that Mod5p performs an activity necessary for the repression of pol II transcription and this may have implications for the regulation of many genes in yeast and higher eukaryotes.

Chapter I

Introduction

In most eukaryotic genomes the hundreds of transfer RNA (tRNA) genes are dispersed throughout the chromosomes through RNA-mediated transposition and are heavily transcribed (1,13,15,16). The minimal complex responsible for tRNA gene transcription is made up of three subcomplexes: TFIIIB, TFIIIC, and RNA polymerase III (pol III). The binding of the two transcription factors, TFIIIB and TFIIIC, are necessary for the recruitment of pol III to the transcription sites (1) (Figure 1.1). Recent studies in *Saccharomyces cerevisiae* (bakers' yeast) have shown that tRNA genes play many important roles in the cell. These roles include: genomic organization (7), being barriers to silencing at the mating loci (2), RNA pol II transcriptional repression (6), and being "hot spots" for recombination (17). The research presented in this dissertation was undertaken to better understand how tRNA genes play roles in recombination, genomic organization, and silencing near tRNA genes.

tRNA genes and Homologous Recombination

In yeast there are multiple types of repetitive elements spread throughout the genome. An example is the previously mentioned tRNA genes, which are present in multiple copies and have large complexes involved with their transcription. One other example is the retrotransposons, which contain long terminal repeats (LTR) at their ends. It has previously been shown that several types of retrotransposons, Ty1-Ty4, preferentially insert themselves at or near the transcription start site of tRNA genes (27, 180, 181). If tRNA genes are able to discourage deleterious recombination in their immediate vicinity, such a phenomenon would explain the selective advantage of Ty retroelements juxtaposing themselves next to tRNA genes.

However, other evidence exists that suggests that tRNA gene transcription actually increases recombination in the genome. One of the first studies that indicated this was done on replication fork pause sites (RFP)(18). These pauses are often caused when RNA polymerases and DNA polymerases collide (19, 20). Looking at previously discovered RFPs in *Saccharomyces cerevisiae,* a study concluded that a RFP site was caused by the transcription of a single *SUP53* tRNA gene (18). By eliminating the upstream sequence of the tRNA gene, and thus lower its transcription, they were able to dramatically reduce the amount of RFPs at this site. They further proved that transcription of the tRNA gene was necessary by using strain with a pol III (RPC160p) temperature sensitive mutation. The mutant strain possessed a 8-fold decrease of RFP activity at the nonpermissive temperature (18).

A later study demonstrated that repetitive elements, such as tRNA genes, are frequently found near DNA breakpoints. This study used PCR to determine translocation breakpoints in *Saccharomyces cerevisiae*. The data indicated that the breakpoints occurred at repetitive sigma sequences as well as tRNA genes (21). A third study looked into regions of chromosome instability using markers Can^R and *ADE* to assay chromosome stability and uncovered two regions that contained multiple tRNA genes that when deleted significantly reduced the frequency of unstable chromosomes (17).

In order to better elucidate if tRNA gene transcription suppresses or encourages recombination, I performed the studies detailed in Chapter II.

tRNA genes are localized to the nucleolus

DNA in living cells is not only compacted but also actively organized into specific areas for many different organisms. The mechanisms behind this organization has been shown to respond to many different forms of stimuli and controlled by many different factors (reviewed in 69,119,120,131)

Although tRNA genes in eukaryotes are linearly separated it has become evident that in at least some eukaryotes these genes are grouped together spatially. Some of the first evidence for this was a study that saw that new RNA transcripts concentrated in the nucleoli during the use of RNA polymerase II (pol II) inhibitors in

HeLa cells (23, 24). Later, more concrete evidence came with the visualization of intron containing tRNAs, which in *Saccharomyces cerevisiae* are found only in the nucleus (reviewed in 25). The study using fluorescent oligodeoxynucleotide probes, designed to anneal to the nuclear tRNA precursors, found clusters of these intron-containing pre-tRNAs at the nucleolus (2,12). A more recent study was able to visualize tRNA genes using fluorescent oligonucleotide gene family-specific probes in *Saccharomyces cerevisiae*. This study demonstrated that tRNA genes that are transcriptionally active are localized to the nucleolus, however if the tRNA gene was inactivated this localization was negated (26). These results indicated that spatial organization of the large number of tRNA genes plays an important part in the organization of nuclear information. This localization also contributes to a novel form of silencing discussed later in this dissertation.

Silencing of pol II transcription in *Saccharomyces cerevisiae*

There are many different forms of pol II transcriptional silencing in yeast, such as silencing near the mating loci, at the ribosomal RNA (rRNA) gene repeats, and near telomeres. These three areas make up as much as 10% of the silent chromatin in yeast (167). There are many different proteins that are necessary to maintain these types of silencing, and most of these do this through interactions with histones and nucleosomes. Some examples of these include the Silent Information Regulators (SIR genes), which are necessary for the maintenance of the silencing in the previously discussed regions (168-170). Another example includes Set1p, which is important for telomeric silencing by assisting in the tri-methylation modification to H3 Lys4 (171). Two other examples of deletions that affect these forms of silencing are the H2A variant Htz1p, and the protein necessary for its inclusion in histones Swr1p (172-173).

Silencing near tRNA genes

Silencing near tRNA genes is unaffected by deletions or conditionally defect mutations to any of the above genes or most genes that are necessary for other types of silencing (5). This phenomenon has been labeled tRNA gene-mediated (tgm)

silencing. The first studies on this novel form of silencing used several different constructs consisting of tRNA genes directly upstream of a pol II specific promoter (from *CUP1* or *GAL1-GAL10*) controlling the expression of reporter genes. The main assay used to test for tgm silencing utilizes the tRNA genes $tRNA^{Leu}$ variant, *SUP53*, and a $tRNA^{Tyr}$ variant, *SUP4* (6). The assay tested if the transcription of a *HIS3* reporter gene was affect by these tRNA genes. The assay tests for the production of histidine in cells. Cells containing an active tRNA gene exhibited no growth, however in strains where the construct contained no tRNA gene or an inactivated tRNA gene there was significant growth (6). The assay also demonstrated that the orientation of the tRNA gene was irrelevant.

An experiment using chromatin footprinting showed that pol II transcription factors were able to bind to the appropriate sites. This demonstrated that steric interference and read-through transcription by pol III do not play a role in the silencing (6)

A second test using the *lacZ* gene in a β−galactosidase assay examined how different distances between the tRNA gene and the *lacZ* pol II promoter would affect the silencing affect. The two distances tested were 240 bp and 400 bp, and they observed only slight loss of silencing at the greater distance (6). The distance that tRNA genes are able to enact their silencing affect has not been completely studied. However, the Boeke lab performed a study using the gene database to determine if tRNA genes influence the genomic landscape around them. This study determined that pol II genes are underrepresented around tRNA genes up to 1 kilobase and hypothesized this to be caused by transcriptional interference of tRNA genes (10).

Other studies have also been done to test if this phenomenon is present at native genomic locations. Two of these studies examined the previously mentioned juxtaposition of tRNA genes with pol II promoters at the retrotransposon Ty3 insertions sites. The first study utilized a temperature sensitive mutant in the largest subunit of pol III, RPC160p. After shifting to the nonpermissive temperature (37^{o}) for 18 hours, transcription of Ty3 elements was induced with α factor for 30 minutes. Primer extensions to detect the sigma LTR transcripts of Ty3 elements had a 60.4 fold increase in the mutant compared to the wildtype cells (6). A second study saw a

similar effect using Ty1 and inactivating the nearby tRNA gene by mutagenesis to its promoter (10).

Another study looking at native locations mutated a tRNA gene adjacent to a pol II gene (*PTR3*). This study replaced the functional tRNA gene with different mutant copies that displayed different levels of transcription using homologous recombination. Using quantitative RNA analysis, the mutant strains exhibited greater than a 2.5 fold increase in transcription compared to the wildtype (10). These observations suggest that tgm silencing actually influences genes in native situations.

In order to find proteins necessary for tgm silencing a mutational study was undertaken to find mutations that caused an alleviation of silencing of a *HIS3* reporter gene that was silenced by a proximal tRNA gene. The results of this screen were initially confusing, since not only were mutations mostly pleiotropic, but isolated suppressors did not have any obvious relationship to pol III transcription (6). The first definitive result from this screen was that an under-expressing allele of the gene *CBF5* abolished silencing. The Cbf5 protein is a pseudouridine synthetase for rRNA that is important in rRNA precursor processing (12). This mutation, as well as several others was later shown to cause the alleviation of silencing by causing mislocalization of the tRNA genes from their normal spatial organization at the nucleolus (5). This result demonstrates that three dimensional localization of the tRNA genes is necessary for tgm silencing. However, the results presented in this dissertation are evidence that localization to the nucleolus is not sufficient for silencing.

Mod5p, a tRNA modifying enzyme

Mod5p will be shown in this dissertation to be necessary for tgm silencing. Previously, Mod5p's characterized activity was modifying the exocyclic amine on position 37 (the adenine adjacent to the anticodon of 3 yeast tRNAs) by the addition of an isopentenyl group from dimethylallyl pyrophosphate (DMAPP) (28-30) (Figure 1.2). Mod5p is present in two different isoforms resulting from the presence of two different AUG translational start sites separated by 11 amino acids. Using high copy plasmids expressing these proteins and indirect immunofluorescence it was

determined these two isoforms are present in different cellular compartments. The first isoform was shown to be located in the mitochondria and the cytoplasm (32). The second isoform was shown to be located in the nucleus even though it is believed that Mod5p only modifies tRNAs in the cytoplasm and in the mitochondria (32,33). Mutations to the gene *MAF1* were shown to affect Mod5p subcellular localization from the nucleus to the cytoplasm as well as a loss of tRNA isopentenylation (33, 34). Five residues were found in the C-terminus of the protein that if mutated could case a change of localization of the protein from cytoplasmic to nuclear (35). It is not clear to what extent these results are specific to yeast, since none of these residues are conserved in higher eukaryotes, even though Mod5p is highly conserved (unpublished observations).

Homologous recombination is increased between tRNA genes

Since tRNA genes are spread throughout the genome in multiple copies it was hypothesized that there may be mechanisms in place to prevent homologous recombination between these repeats. In Chapter II, it will be shown that the opposite case is true and that the transcription of two active tRNA genes actually increases the recombination between them. This chapter will also show that tRNA gene transcription has no discernible influence over the recombination between the sigma elements of nearby Ty3 retrotransposons.

Proteins necessary for tRNA gene clustering

Our lab is interested in the mechanisms that are involved with the localization of tRNA genes to the nucleolus. One finding that was of great interest to us came from the laboratory of Frank Uhlmann (Cancer UK, personal communication) that the condensin complex binds preferentially to tRNA genes. This suggested that the condensin protein complex might be an active participant in the clustering of tRNA genes, since condensin had previously been found to influence ribosomal DNA. In the data presented in chapter III it will be demonstrated through temperature-sensitive mutants that condensin is necessary for the clustering of the tRNA genes. It will also be shown that condensin is preferentially located at tRNA genes and physically

interacts with pol III transcription factors TFIIIB and TFIIIC in a DNA independent manner.

Mod5p is necessary for tgm silencing and is located at tRNA genes

Previously Mod5p's only known activity, in yeast, was the isopentenylation of three tRNAs. However, in a screen of potential alleviators we discovered that Mod5p is necessary for tgm silencing. Studies were done to determine: if Mod5p's catalytic activity is necessary for tgm silencing, if Mod5p is present at tRNA genes, and what other proteins physically interact with Mod5p. The data presented in Chapter IV is the first evidence that Mod5p has a secondary activity that is necessary for tgm silencing, is located at tRNA genes, and interacts with the pol III transcription machinery.

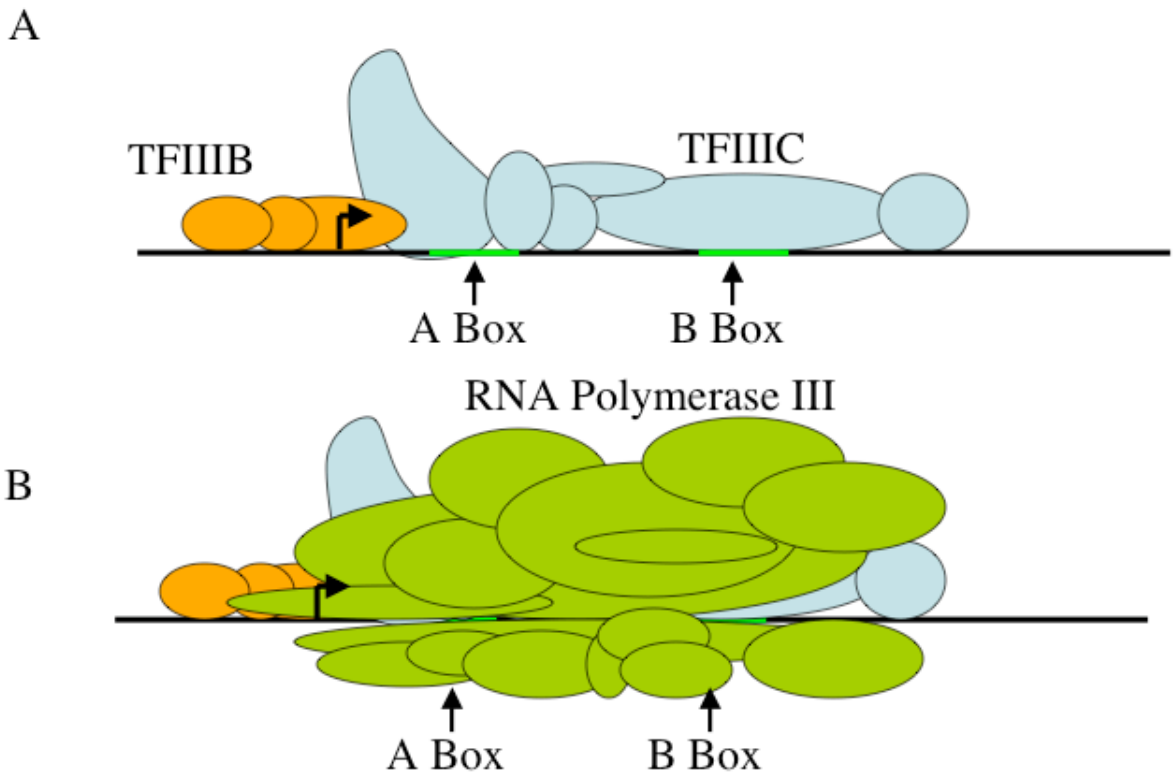


Figure 1.1. Three subcomplexes bind sequentially to induce tRNA gene transcription. **(A)** As reviewed in (1), TFIIIC, represented by six subunits in blue, is the first to the gene promoter. Two internal promoter elements, termed the A and the B boxes, are necessary for this binding. TFIIIC then recruits TFIIIB, three subunits in orange, which contains that TATA binding protein. **(B)** These two subcomplexes then recruit the sixteen member RNA polymerase III, depicted in green. These three subcomplexes then promote the transcription of the tRNA gene.

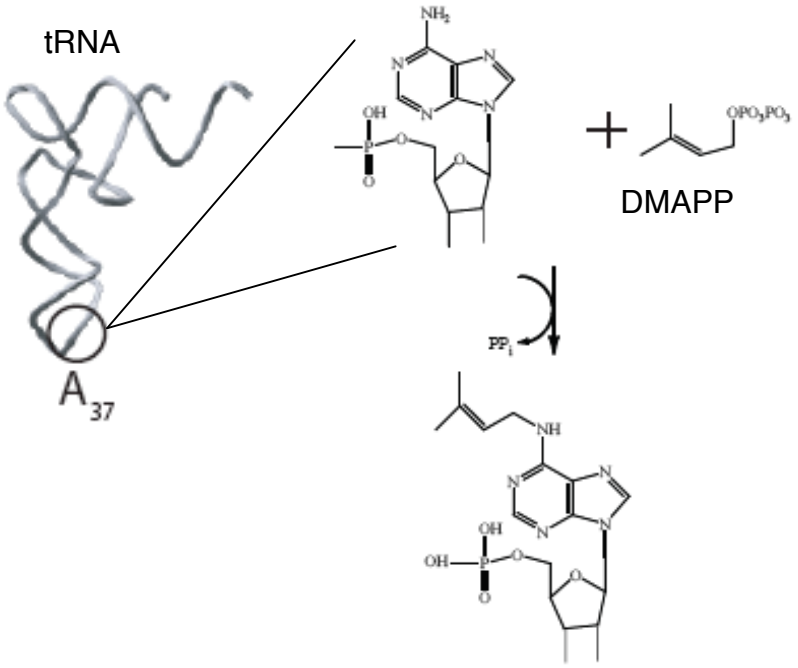


Figure 1.2 Mod5p catalyzes the addition of an isopentenyl group from dimethylallylpyrophosphate (DMAPP) onto position 37 of the tRNA. This modification alters the efficiency of tRNA-mediated nonsense suppression by the *SUP7* ochre suppressor tRNA (28), but is not essential for viability of most yeast strains in most media.

Chapter II

Increased recombination between tRNA genes

Abstract

Transfer RNA genes are distributed throughout eukaryotic genomes and are frequently found as multicopy families. In *Saccharomyces cerevisiae* tRNA gene transcription by RNA polymerase III suppresses nearby transcription by RNA polymerase II, partially because the tRNA genes are clustered near the nucleolus. We have tested whether active transcription of tRNA genes might also suppress recombination, since recombination between identical copies of the repetitive tRNA genes could delete intervening genes and be detrimental to survival. The opposite proved to be the case. Recombination between active tRNA genes was elevated, but only when both genes are transcribed. We also tested the effects of tRNA genes on recombination between the direct terminal repeats of a neighboring retrotransposon, since most Ty retrotransposons reside next to tRNA genes and the selective advantage of this arrangement is not known.

INTRODUCTION

Most tRNA genes exist in multiple copies distributed throughout the genome, and appear to have duplicated in dispersed locations through RNA-mediated transposition (15, 16). Little is known about which predicted tRNA genes in multicellular eukaryotes are transcribed, but there are indications from a limited number of studies that there might be considerable developmental regulation in the transcription of tRNA gene subclasses (47,67). In addition to tRNA genes, vertebrates have highly repetitive DNA elements, termed SINEs (short interspersed repetitive elements) that are derived from small RNA genes with tRNA-class promoters for RNA polymerase III (pol III) (46). The major SINE in humans, the Alu elements, are originally derived from the 7SL RNA found in signal recognition particles and are found in more than 500,000 copies per haploid genome (41). Although few of these elements appear transcriptionally active under normal conditions, extensive Alu expression can be observed during viral infection or in response to cellular stress (40, 49) and most cloned Alu repeats can be transcribed by pol III in vitro (38, 48).

The existence of such frequent, highly similar DNA sequences in the genome raises the question of whether there is some mechanism to protect against frequent deletion of chromosome segments between repeats by homologous recombination, since such deletions could lead to death of single cells or developmental abnormalities in complex organisms. In adults, such deletions can lead to unregulated growth, such as human mammary tumors with deletions between Alu elements at the BRCA1 loci (57, 62, 66).

Transcription of genes by RNA polymerase II (pol II) increases their susceptibility to recombination (65), but this has not been addressed for pol III transcription units, which are very short and almost entirely covered by the active transcription complex when present. Although transfer of genetic information between identical pol III genes is possible (e.g. tRNA) (53) recombination that deletes intervening material might be predicted to be suppressed as being counter to survival.

Here we use a yeast model system to address the effects of pol III transcriptional activity on recombination between and near tRNA genes in the yeast chromosome. We examined the rate of recombination between two nearly identical tRNA genes in the yeast *Saccharomyces cerevisiae* that were either transcriptionally active or inactive. The results were unexpected, in that recombination increased when both tRNA genes were active.

In addition to testing the effect of pol III transcription on recombination between tRNA genes, there was reason to suspect that homologous recombination within a few hundred base pairs of active tRNA genes might also be suppressed. Most of the Ty retrotransposons in yeast (Ty1 - Ty4) have evolved mechanisms for preferential insertion and retention near tRNA genes (6, 10, 12, 27). A rare fifth class, Ty5, prefers transcriptionally silent regions such as telomeres and silent mating type loci (8). Proximity to a tRNA gene might have the selective advantage of conferring conditional pol II transcriptional silencing (6), but reduced recombination between the direct, long terminal repeats (LTRs) of the Ty retrotransposons might also have provided the selective advantage for the Ty elements to have developed this insertion preference.

Materials and Methods

Yeast Strains

Strains were derived from Saccharomyces cerevisiae W3031A (MAT□ *leu2-3, 112 his3-11, 15 ade2-1 trp1-1 ura3-1 can1-100*) and BY4741 (MATa *his3*Δ*1, leu2*Δ*0,met15*Δ*0, ura3*Δ*0).* Growth was performed in YPD (64) except where noted. For testing recombination between identical $tRNA^{Leu}$ genes, constructs were made in W3031A, the coding sequence of *LEU2* gene, on chromosome III, adjacent to the SUP53 $tRNA^{Leu}$ gene was precisely replaced with the coding region of *URA3*. A second copy of the *SUP53* gene was introduced 200 bp downstream from the 3' end of the *URA3* coding region, 1.3 kb downstream from the first tRNA gene and in the same orientation. The insertion contained *SUP53* sequence from 59 bp upstream to 29 bp downstream of the tRNA coding sequence. The strains contained either a wild type $tRNA^{SUP53}$ promoter at each site or a transcriptionally inactive promoter

containing previously characterized point mutations both the A box and B box (6,55). The mutated gene is designated *sup53*ΔAB (Figure 2.1A).

To test recombination rates near a tRNA gene, *URA3* was inserted at the Ty3-1 locus on chromosome VII in strain BY4741, (YGRWTy3-1) replacing all but 100 bp of each end of the central epsilon region. A second strain was created by then mutating the neighboring $tRNA^{Cys}$ gene promoter (triple B box mutation G52A, T53A, C55G) by homologous recombination of a PCR product with integration selected by G418 resistance and confirmed by sequencing genomic PCR fragments from the strains. The Kan gene was inserted 152 bp downstream of the tRNA gene in the opposite orientation in the strain with and without mutations to the neighboring $tRNA^{Cys}$.

PCR fragments for deletions of *RAD51* and *RAD52* were produced by replacement of their coding regions with the *KAN* cassette from the plasmid pFA6a-KanMX6 (50).

Recombination assay

For each experiment three cultures were inoculated from individual colonies and were grown in SDC-ura (64) medium to ensure retention of the *URA3* genes. These cultures were used to inoculate 200 ml of YPD cultures to a starting OD600 of 0.2 and grown for seven hours to allow loss of *URA3*. Cells were collected and resuspended in 10ml of 10 mM Tris-HCl pH 7.5, 1 mM EDTA. Cells are plated on SDC+5-fluorootic acid (5-FOA) medium, to select against *URA3*. The number of 5-FOA resistant colonies is expressed relative to the number of cells plated**.**

To test whether 5-FOA resistance arose from homologous recombination to delete *URA3,* 24 to 32 5-FOA resistant colonies were tested in 2 or more separate experiments by PCR of genomic DNA from flanking primers**.**

Results

Recombination between identical tRNA genes

Since many tRNA genes in yeast are duplicated up to fifteen times throughout the genome, suppression of recombination between duplicates could have survival

value. The question addressed here is whether transcription of tRNA genes by RNA polymerase III affects the recombination rate between identical tRNA genes. We modified the *LEU2* locus on chromosome III as shown in Figure 2.1A. The $tRNA^{SUP53}$ gene is normally located upstream of the *LEU2* gene and its transcription has been extensively characterized (43,55). The coding sequence of *LEU2* was precisely replaced with that of *URA3,* along with inserting a second copy of $tRNA^{SUP53}$ gene (*SUP53*) 200 base pairs downstream from *URA3*. Strains were produced containing either active tRNA genes or tRNA genes inactivated by point mutations in the internal promoters ($G_{19}C$, $C_{56}G$; *sup53*ΔAB) that prevent formation of any part of the RNA polymerase III complex (43,55). The four tested constructs had either both tRNA genes active (*SUP53/SUP53*), both inactive (*sup53*ΔAB/*sup53*ΔAB), or only one at a time active.

Loss of *URA3* function was selected on media containing the 5-FOA, and the normalized rate of number of 5-FOA resistant colonies is shown in Figure 2.1B. These data are averages from five different experiments, each done in duplicate.

To determine if 5-FOA resistance arose through deletion between the tRNA genes, PCR was done on genomic DNA from independent isolates using primers flanking the tRNA genes. The starting strains produce a 1.8 kb product spanning both tRNA genes and the *URA3* gene, whereas precise homologous recombination between the tRNA repeats produces a 175 bp product. Sixteen 5-FOA resistant colonies from two separate experiments on each strain all showed a 175 bp product, consistent with a precise deletion between sequences in the tRNA genes.

In contrast to our original hypothesis that recombination would be repressed between transcribed tRNA genes, the highest homologous recombination rate was found to be the one where both $tRNA^{SUP53}$ genes were active. This strain had a recombination rate averaging five times greater than the other three strains in four separate experiments. It is particularly interesting that inactivating either tRNA gene gave the same recombination as inactivating both. The failure of only a single active tRNA transcription unit to stimulate recombination implies that the increase in recombination between active tRNA genes is the result of some direct or indirect communication between components of active pol III complexes on both genes.

Recombination Path

Single strand annealing (SSA) is one of the common mechanisms leading to deletions between direct repeats. When a break occurs between the two repeats the 5' ends are resected. This allows the 3' strands to anneal within the repeated elements, followed by removal of the nonhomologous 3' tails corresponding to the sequence between the repeats, gap filling and ligation (48,59,66). Deletions caused by recombination between direct repeats of untranscribed regions have been shown to be reduced 10- to 100- fold in *rad52* mutants consistent with the role of Rad52p in SSA (45,58). In contrast, *rad52* mutation did not affect deletion rates of direct repeats in rDNA and high-copy *CUP1* tandem arrays (56). Another participant in homologous recombination, Rad51p, acts by promoting pairing and strand exchange with an intact homologous duplex. Rad51p is required for most recombination but dispensable for SSA (56). To examine whether recombination between the tRNA genes was proceeding through a SSA path, *RAD52* and *RAD51* were individually deleted from our strains with either two active or two inactive tRNA genes flanking the *URA3* gene.

The *rad52*Δstrains consistently showed reduced 5-FOA resistant colonies. In the *SUP53/SUP53* strain there was a 20-fold decrease and in the *sup53*ΔAB/*sup53*ΔAB strain there was a 33-fold decrease in homologous recombination (Table 2.1). This sensitivity to *rad52* deletion distinguishes recombination between these direct repeats transcribed by pol III from the rDNA and *CUP1* repeats transcribed by RNA polymerases I and II. In contrast, *rad51*Δ caused little change in the number of 5-FOA resistant colonies in the *SUP53/SUP53* strain and caused a slight increase in 5-FOA resistance in *sup53*ΔAB/*sup53*ΔAB strains (Figure 2.1B). This is consistent with previous demonstrations that recombination between direct repeats can occur in the absence of strand exchange (44,52,61). These results suggest that recombination between tRNA genes is occurring in the absence of strand exchange regardless of their transcriptional activity, with Rad52p facilitating SSA.

To test whether the residual 5-FOA resistant colonies arose through recombination between the tRNA genes or some other defect, we again tested genomic numerous 5-FOA isolates by PCR. This analysis showed that only 39% of the *SUP53/SUP53* and 5% of the *sup53*ΔAB/*sup53*ΔAB 5FOA resistant colonies underwent deletion by homologous recombination in the Δ*rad52* strain, compared to 100% in *RAD52* strains. In the *SUP53/SUP53*-Δ*rad52* strain 22% of the 5FOA resistant colonies came through a probable *URA3* mutation, since deletions were not detected, and 39% appear to have a large chromosomal deletion in the region. In the *sup53*ΔAB/*sup53*ΔAB-Δ*rad52* strain most (95%) of the 5-FOA resistant colonies represented a probable small mutation in *URA3* (Table 2.1).

Recombination between retrotransposon LTR elements

We also tested the effect of a nearby tRNA gene on the recombination between LTR elements of a well-characterized yeast retrotransposon locus Ty3-1, in its native chromosomal position on chromosome VII (11). We replaced the interior epsilon region of the retrotransposon Ty3-1 with *URA3*. We then either left the neighboring $tRNA^{Cys}$ gene intact or mutated three base pairs in the B box internal promoter (figure 2.2), which dramatically decreases pol III transcription (9, 36,37).

The strains with active vs. inactive tRNA genes were tested for their relative frequency of *URA3* loss. The averages from four separate experiments demonstrated that the frequencies for the active tRNA gene and inactive gene were essentially identical ($9.5x10^{-6}$ and $9.0x10^{-6}$ respectively) within error for these experiments. Recombination between the LTR direct repeats therefore appears to be unaffected by the presence of a nearby active tRNA gene.

Discussion

Contrary to our original hypothesis, the work presented here showed that the occurrences of recombination between two identical tRNA genes is higher when both tRNA genes are being actively transcribed. In this respect pol III transcription appears to behave similarly to pol II transcription in that transcription stimulates recombination (36). However, there are also distinct differences. It is unclear why both tRNA genes needed to be actively transcribed in order for the increase in

homologous recombination to occur. One possibility is having two active tRNA genes causes an increase in breakage, consistent with tRNA genes causing replication fork pause (18), which have been shown to cause increased homologous recombination (11,36). If this is the case, it is not clear why a single active tRNA gene might not give an intermediate level of recombination increase. A second possibility is that having two active tRNA genes changes the type of repair that is used. If this is the case perhaps the spatial organization of these tRNA genes is playing a part in their repair. However, the *rad52*Δ data suggest that the path of this increased recombination is still through SSA.

Recent findings showing spatial clustering of the linearly dispersed tRNA genes suggest a possible explanation for our results. Yeast tRNA genes are largely localized to the nucleolus when actively transcribed, but not when inactivated by promoter point mutations (7). The mechanism of this co-localization is not currently known, but it is conceivable that tRNA transcription complexes associate with some sort of framework that brings them into proximity, increasing the likelihood of physical interactions. This would be consistent with evidence that the frequency of disease-specific chromosomal translocations are non-random, and have been correlated to the spatial proximity of the sites involved (51,54,63).

The increased recombination between identical tRNA genes when they are transcriptionally active could help explain why families of tRNA genes in yeast are not found tandemly repeated, or even in close proximity. The closest pair of identical tRNA genes occurs on chromosome IX where two tRNA genes Asp are about 12 kb apart from each other. Studies using plasmid constructs and non-tRNA repeats have shown that increasing the distance between repeats decreases the efficiency of SSA in competition with gene conversion (39,42,60).

The results presented here might be pertinent in considering the occurrence of deletions, inversions, and duplications at Alu elements throughout the human genome. Alu elements contain tRNA-class internal promoters from the 7SL RNA genes (41). While this pol III promoter in cloned Alu repeats is generally found to be transcriptionally competent in vitro, most of the hundreds of thousands of Alu repeats in human cells are inactive in cells and little is known as to which of the elements are

activated in response to cellular insults (41,40,49). An example of recombination between Alu repeats has been studied is the human tumor suppressor gene, BRCA1. BRCA1 genomic sequence is composed of 41.5% Alu sequence, corresponding to an Alu element every 650 bp average (65). Alu recombination seems to be the main source of genomic rearrangements in patients with a hereditary predisposition to breast and ovarian cancers (57,60,62). The data in this report suggests that the transcriptional activation of these Alu repeats, possibly through stress response or viral infection, might increase their ability to recombine.

Acknowledgements

This chapter is based on a published article "Increased recombination between active tRNA genes" (Pratt-Hyatt, M., Kapadia, K.M., Wilson, T.E., and Engelke, D.R. (2006) DNA and Cell Biol 25, 359-364). I would like to thank Kevin Kapadia for his technical assistance and I would like to thank Drs. Engelke and Wilson for their help with planning experiments, interpretation of results, and writing. This work was supported by the National Institutes of Health (GM063142 to DRE and CA102563 to TEW). MJP-H was supported by the Cellular and Molecular Biology Training Grant at the University of Michigan (T32 GM007315).

.

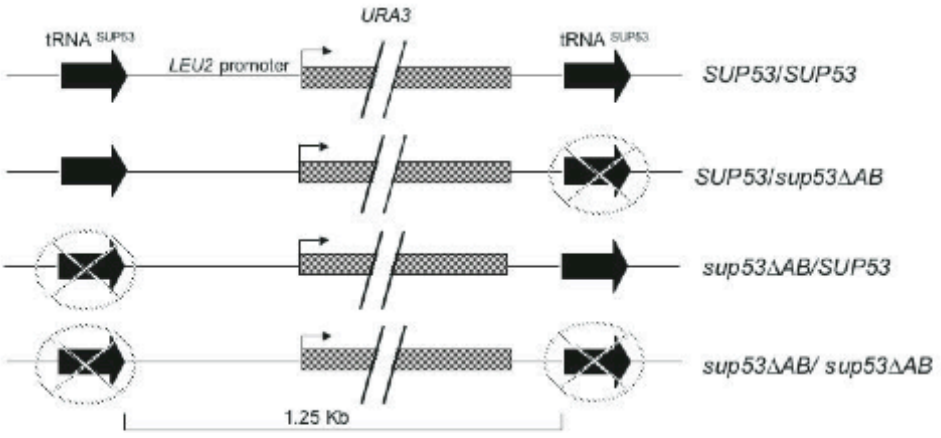
Table 2.1. Rates of homologous recombination

Strain	Rate of 5-FOA Resistance ($x10^{-10}$)	% Undergone homologous recombination
SUP53/*SUP53*	9.47 +/- 0.71	100
*sup53*ΔAB/*sup53*ΔAB	1.76 +/- 0.75	100
SUP53/*SUP53* *rad52Δ*	1.23 +/- 0.28	39
*sup53*ΔAB/*sup53*ΔAB *rad52Δ*	1.04 +/- 0.19	5
SUP53/*SUP53* *rad51Δ*	10.17 +/- 2.61	ND
*sup53*ΔAB/*sup53*ΔAB *rad51Δ*	5.68 +/- 1.23	ND

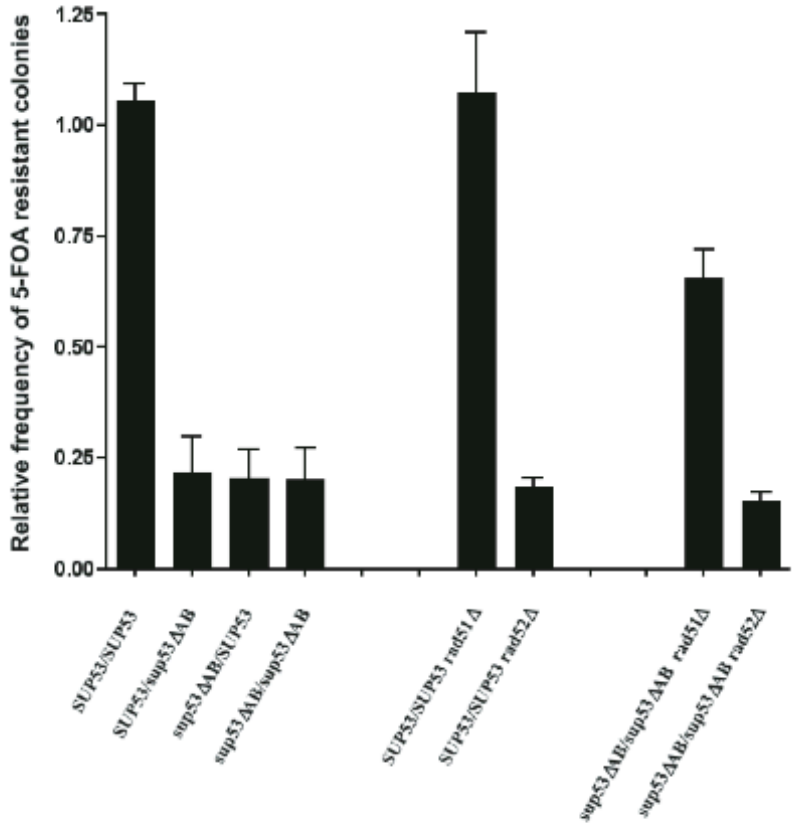
1. ND = not determined

Figure 2.1 Recombination between tRNA genes. (A) Four strains were modified by replacing *LEU2* coding sequence with that of *URA3* in strains containing either an active or transcriptionally inactive tRNA gene. An additional active or inactive $tRNA^{SUP53}$ gene was then placed 200 bp downstream of *URA3* in each strain. Deletion events between the two tRNA genes was selected by growth on plates containing 5-FOA media and deletions were confirmed by PCR. (B) The first four bars are average values of five independent experiments. The following four values are an average from three experiments. The number of viable colonies on the 5-FOA plates was divided by the number of cells plated and normalized to recombination in the *SUP53/SUP53* strain. The frequency of 5-FOA resistance in the *SUP53/SUP53* strain was ($9.47 +/- 0.71 x10^{-10}$). *RAD51* and *RAD52* were deleted in the strain constructs (Figure 2.1) containing either two active tRNA genes (*SUP53/SUP53)* or two inactive tRNA genes (*sup53*ΔAB/*sup53*ΔAB). Deletion of *RAD51* causes a significant increase in 5-FOA resistant colonies between inactive tRNA genes, but only a modest, if any, increase between active tRNA genes. Deletion of *RAD52* caused about a ten-fold decrease in the amount of 5-FOA resistant colonies in the *SUP53*/*SUP53* strain, but only a modest decrease in the *sup53*ΔAB/*sup53*ΔAB strain.

A


URA3
tRNA SUP53
LEU2 promoter
tRNA SUP53
SUP53/SUP53
SUP53/sup53ΔAB
sup53ΔAB/SUP53
sup53ΔAB/ sup53ΔAB
1.25 Kb

B


Relative frequency of 5-FOA resistant colonies
1.25
1.00
0.75
0.50
0.25
0.00
SUP53/SUP53
SUP53/sup53ΔAB
sup53ΔAB/SUP53
sup53ΔAB/sup53ΔAB
SUP53/SUP53 rad51Δ
SUP53/SUP53 rad52Δ
sup53ΔAB/sup53ΔAB rad51Δ
sup53ΔAB/sup53ΔAB rad52Δ

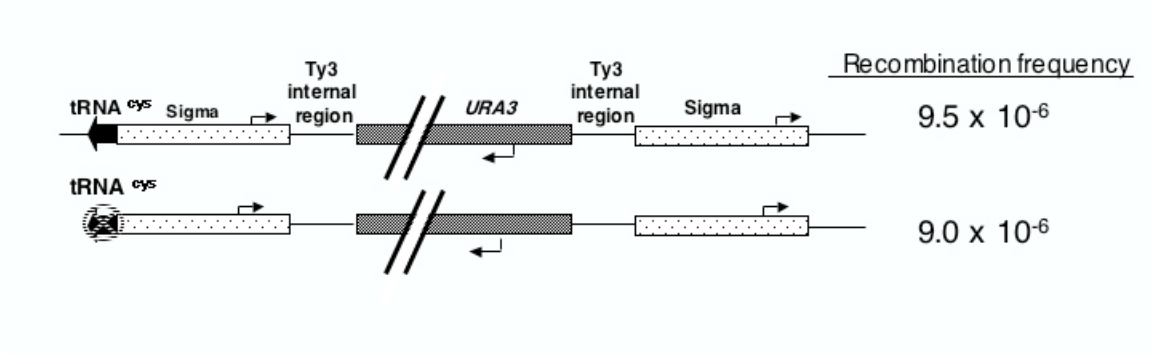


Figure 2.2 Recombination between Ty3 sigma direct repeats. The *URA3* gene was inserted at the Ty3-1 locus, replacing all but 100 bp of either end of the epsilon region. Multiple B box promoter mutations were then created in the neighboring $tRNA^{Cys}$ gene in a second strain, inactivating the transcription promoter. Recombination between the two sigma elements was identified by section against the *URA3* gene through growth on 5-FOA media and screening for precise recombination between sigmas by PCR from genomic DNA. Recombination frequency is shown as number of recombinants divided by the total number of cells plated.

Chapter III

Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complex

Abstract

The 274 tRNA genes in *Saccharomyces cerevisiae* are scattered throughout the linear maps of the sixteen chromosomes, but the genes are clustered at the nucleolus when compacted in the nucleus. This clustering is dependent on intact nucleolar organization and contributes to tRNA gene-mediated (tgm) silencing of RNA polymerase II-transcription near tRNA genes. After examination of the localization mechanism, we find that the chromosome-condensing complex, condensin, is involved in the clustering of tRNA genes. Conditionally defective mutations in all five subunits of condensin, which we confirm is bound to active tRNA genes in the yeast genome, lead to loss of both pol II transcriptional silencing near tRNA genes and nucleolar clustering of the genes. Furthermore, we show that condensin physically associates with a subcomplex of RNA polymerase III transcription factors on the tRNA genes. Clustering of tRNA genes by condensin appears to be a separate mechanism from their nucleolar localization, as microtubule disruption releases tRNA gene clusters from the nucleolus, but does not disperse the clusters. These observations suggest a widespread role for condensin in gene organization and packaging of the interphase yeast nucleus.

INTRODUCTION

Numerous levels of preferential DNA positioning within nuclei have now been described (69,78,81,92,98,115,119,120,130,131). Examples include global effects like chromosome territories (78,81) and transcription factories(79), and specific effects such as telomeres and centromeres at the yeast nuclear periphery (90, 91), the large ribosomal DNA repeats at the nucleoli of all organisms (125), and the 5S rDNA at or near the nucleoli of many organisms (13). Multiple genes alter their position when transcriptionally activated (72, 73,75,76,89,102,132), and some genes are brought together with linearly distant enhancer elements (106,107,127,129). In some cases genes that are co-regulated but not linearly connected are brought together in three dimensions, including β-globin genes that are found clustered when active (71), and the nucleolar clustering of most of the 274 tRNA genes in *S. cerevisiae*, which are transcribed by RNA polymerase III (pol III) (5,26). Although the mechanisms of spatial genome organization are not well defined, a few elements have been determined for specific cases. The long-range movement of at least one inducible gene requires actin/myosin (77). The transcription-dependent tethering of some genes at the nuclear pore depends on interactions between the gene promoters and the basket of the nuclear pore complex (124). Centromere clustering at the spindle pole body requires microtubules (74), as does homologous recombination, which requires spatial proximity of the two loci (22).

The nucleolar localization of most tRNA genes in *Saccharomyces cerevisiae* is a particularly pervasive example of genome-wide gene positioning. The spatial clustering was originally discovered because proximity to tRNA genes leads to transcriptional silencing of nearby RNA polymerase II (pol II) promoters, termed tRNA gene mediated (tgm) silencing (6,9,12). The co-localization of the tRNA genes may also contribute to the elevated rate of recombination between active tRNA genes in yeast (122).

To identify requirements for nucleolar clustering of tRNA genes, we first examined the influence of the cell cycle and the involvement of microtubules on tRNA gene positions. We observed that tRNA genes associate with the nucleolus throughout division, and that microtubule depolymerization releases intact clusters of tRNA genes away from the nucleolus, suggesting that some separate mechanism was responsible for bringing the tRNA genes together.

To investigate the mechanism of tRNA gene clustering, the involvement of the protein complex condensin was examined. This highly conserved complex (93,105) is involved in the compaction of chromosomes, and the mechanism of compaction is generally thought to involve direct condensin-condensin interactions among condensin complexes bound to dispersed sites in the genome (110, 117,131). Condensin complexes have five essential subunits: two core 'structural maintenance of chromosomes' (SMC) subunits (Smc2 and Smc4), which are thought to be targeted to chromosomes by the three regulatory non-SMC subunits (Ycs4, Ycg1, and Brn1 in *S. cerevisiae*). Prior studies of yeast condensin have emphasized its role in the dramatic condensation of the nucleolar rDNA locus (82,86 103,104,165), and using chromatin immunoprecipitation with PCR and standard microarray chips, the complex has previously been found to bind to multiple preferential sites along every yeast chromosome (133). However, binding to a short DNA elements such as tRNA genes might not be detected in these earlier microarray approaches, and results presented here and in an accompanying paper (166) show that condensin is preferentially bound at tRNA genes.

The role of condensin in structural organization of nucleolar DNA, and its specific binding to tRNA genes prompted the examination of mutations in all five condensin subunits for effects on tRNA gene organization: first using tgm silencing as an indirect, functional evaluation for proper localization, and then directly by DNA *in situ* fluorescence microscopy. The results show that the condensin complex is required for clustering of dispersed tRNA genes in yeast. We go on to show that condensin is stably associated with a complex of bound transcription factors on the

tRNA genes, accounting for the specificity of the condensin interactions at tRNA gene sites and pol III transcription factor sites in the yeast genome.

RESULTS

tRNA genes remain clustered at the nucleolus throughout the cell cycle.

Clustering of most of the 274 yeast tRNA genes near the nucleolus affects the organization of much of the interphase genome, but previously little was known about the mechanism. As a first step, we asked whether the localization was dynamic during the cell cycle, or whether tRNA gene clusters continually associated with the nucleolus, which remains intact throughout mitosis in *S. cerevisiae* (108). Staining of nucleolar proteins and ribosomal DNA (rDNA) throughout mitosis has indicated that this region is one of the last in the genome to divide (83,112). To examine the nucleolar association of tRNA genes during mitosis, extensive imaging was first undertaken in unsynchronized cell populations, using *in situ* hybridization to directly visualize a representative $tRNA^{Leu}$(CAA) gene family, one of several tRNA gene families originally tested (26). Cells captured throughout mitosis, as determined by bud size in the DIC image, were imaged and analyzed. Representative cells undergoing nuclear division are shown in Figure 3.1A. In these cells and in cells from each visually distinct phase, we observed nearly complete overlap of tRNA gene signal with a nucleolar marker. Similar results were obtained when cells were synchronized in G1 with the mating pheromone, α factor, or synchronized with α factor and then released for varying time periods, or synchronized in S phase with hydroxyurea (not shown). Although it is possible that individual tRNA genes become transiently dissociated, since signal from individual genes is not detectable, the observation that tRNA genes continue to associate with a dividing nucleolus argues that the bulk of the tRNA genes remain clustered with the nucleolus during its partitioning late in nuclear division.

Nucleolar localization of tRNA gene clusters requires microtubules.

One hypothesis for the mechanism of nucleolar tRNA gene clustering was that a structural element, such as microtubules, might be involved. Deletions and mutations of various microtubule motor and actin microfilament components did not affect the silencing of pol II transcription near tRNA genes (data not shown), which requires nucleolar clustering of tRNA genes (5), but many of these genes are redundant or essential, so deletions that are viable might not be informative. To directly assess the involvement of microtubules in tRNA gene positioning, nocodazole was used at conditions known to cause microtubule disassembly (99), and tRNA genes were subsequently visualized as described (26). The nocodazole treatment of these cells did not strongly inhibit tRNA expression or release tgm silencing (data not shown), but did result in a shift in the subnuclear positioning of the tRNA gene clusters. As shown in Figure 3.1B, cells arrested with nocodazole in late G2 phase of the cell cycle show tRNA genes that are gathered in one or two clusters, but divorced from the nucleolus. The proportion of cells with a single nucleolar cluster of tRNA genes was greatly reduced from 76% in wild type, unsynchronized cells to 30% in nocodazole-arrested cells. A similar pattern was observed for cells treated with nocodazole for only one hour (not shown). This phenotype is distinct from one in which tRNA genes become dispersed throughout the nucleoplasm, as seen in some nucleolar mutants that release tgm silencing (5). To ascertain whether non-nucleolar tRNA gene clusters were due to nocodazole disruption of nuclear structure, and not a result of arrest in G2/M, untreated cells in a large-budded state (similar to nocodazole-arrested cells) were evaluated. The proportion of cells with a single cluster of tRNA genes, where the cluster was nucleolar, was similar to the overall unsynchronized cell population (83%), indicating that cell cycle stage was not the cause of the divorce of tRNA gene clusters from the nucleolus. Furthermore, nocodazole depolymerization of microtubules in G1-arrested cells also loses nucleolar localization of tRNA gene clusters, whereas cells arrested in G1 with α factor alone show tRNA genes at the nucleolus (Fig. 3.1C). Thus, maintenance of tRNA gene positioning is dependent throughout the cell cycle on intact microtubules,

although this requirement could be either direct or indirect. In either case, the persistent tRNA gene clustering in the absence of microtubules suggests tRNA gene clustering is caused by a separate clustering mechanism that independent of overall nuclear organization.

Clustering of tRNA genes is condensin-dependent.

To identify potential genetic components of tRNA gene clustering, release of tRNA gene mediated (tgm) silencing of pol II transcription was used as an initial screen. tgm silencing requires nucleolar clustering of tRNA genes (5). The condensin complex was chosen as one target in this screen because of its known involvement in chromosome organization, particularly at the nucleolar rDNA repeats, and the recent discovery of condensin occupancy at yeast tRNA genes (166). Because the five condensin subunits are all essential, temperature sensitive mutants in all five subunits were tested: *smc2-8* (82), *smc4-1* (82), *ycg1-2* (103), *ycs4-1* (165), and *brn1-9* (103). Loss of tgm silencing from a reporter plasmid in mutant strains allows growth on media lacking histidine (Fig. 3.2A) (5,6,12). Plates were grown at 23°C, the permissible temperature for each strain (165,82; 103), and 30°C, where the strains can still grow but the mutant proteins may be partially defective. Growth of these mutants is arrested at 37°C.

Growth at 30°C is shown in Figure 3.2B. In contrast to wild type yeast, each of the condensin mutants shows some growth in the absence of histidine, consistent with release of tgm silencing. As with all mutations previously shown to release tgm silencing (5,12, 116), a minority of cells are able to grow on selective media, and the extent varies between strains. The reasons for this are unknown, but it persists when cells are re-plated to selective media. Surprisingly, each mutant also demonstrates release of silencing at 23°C, where the condensin defects are not sufficient to cause severe growth defects (data not shown). The variability in colony size seen in some mutants is of unknown origin but is reproducible at both growth temperatures, and restreaking various sized colonies continues to give mixed colony sizes. The release

of tgm silencing in condensin mutants suggests its involvement in the behavior of tRNA genes, potentially their clustering and/or localization mechanisms.

The subnuclear distribution of tRNA genes was analyzed by *in situ* hybridization in wild-type cells and in each mutant strain, where yeast were grown at 30°C prior to fixation and slide preparation. As previously noted, the permissive growth temperature for all five mutant strains is 23°C, and growth stops at 37°C. We used 30°C as an intermediate condition where the complex might be partially defective. In wild-type cells, tRNA genes are clustered at the nucleolus (Fig. 3.3) (26; 5). However in all five mutant strains, tRNA gene clustering is either absent or defective. In *smc2-8*, *smc4-1*, and *ycs4-1*, the tRNA gene (red) signal is no longer detectable above the background, as previously observed for nucleolar disruptions that disperse the tRNA genes (5). In *ycg1-2* and *brn1-9*, tRNA genes appear to still be at least partially clustered, but divorced from the nucleolus. In *ycg1-2*, only 36% of all nuclei showed a single tRNA gene cluster (compared to 83% in wild type), and of those single-cluster nuclei, only 23% showed nucleolar localization (compared to 76% in wild type). Many more *ycg1-2* nuclei showed two or more clusters (51%), and the clusters were non-nucleolar in 94% of these nuclei. A small number of *ycg1-2* nuclei showed no clustering (13%). In *brn1-9*, 49% of nuclei showed a single tRNA gene cluster, where only 27% of these were nucleolar. 38% of *brn1-9* nuclei showed two or more clusters, and clusters were non-nucleolar in 88% of these nuclei. 13% of *brn1-9* cells showed no clusters. The different phenotypes observed may be due either to differences in mutation severity or to the different functions of the subunits. It can be concluded that defective mutations in any of the five condensin subunits can cause severe defects in tRNA gene positioning.

As confirmation of mislocalized pre-tRNA synthesis in strains where the genes are too dispersed to give focal signals, the locations of pre-tRNA^{Leu}(CAA) primary transcripts were also probed (3,5). Because there are hundreds of copies of this precursor RNA per nucleus, the fluorescent signal is detectable even when the corresponding tRNA genes are dispersed in the nucleoplasm (5). In wild-type cells,

pre-tRNAs are concentrated at the nucleolus (Fig. 3.3), as expected (3,5). In each condensin mutant, pre-tRNAs are aberrantly localized. Instead of majority overlap with the nucleolar marker, pre-tRNAs in the mutants are found either more dispersed throughout the nucleoplasm or localized mostly to the nuclear periphery. Representative cells are shown in Figure 3.3. The more diffuse nucleoplasmic staining seen in *smc2-8*, *smc4-1*, and *ycg1-2* is similar to that seen in several RNA polymerase I mutants and rRNA gene transcription factor mutants that also release tgm silencing (5). The pre-tRNAs in *ycs4-1* and *brn1-9* appear concentrated at the nuclear periphery, a phenotype that has not previously been seen. The reason for this is unclear, but it is possible that residual function of these subunits at 30º leads to partial, but aberrant, pre-tRNA localization.

Condensin is bound preferentially to tRNA genes.

The involvement of condensin at tRNA gene loci suggests a possible direct interaction of the complex with tRNA genes, with subsequent clustering due to interactions between condensin complexes bound at different loci, as has been proposed for other forms of condensin-mediated chromosome condensation. Recent ChIP analysis with high resolution oligonucleotide arrays shows a sharp peak of condensin bound to each tRNA gene in the yeast genome (166). To independently verify the binding of condensin to tRNA genes, we tested condensin occupancy of two distinct tRNA genes relative to chromosomal locations devoid of tRNA genes. Chromatin associated with affinity tagged Smc2p or Smc4p was used for semi-quantitative PCR amplification of a $tRNA^{Lys}$ on chromosome VII or a $tRNA^{Phe}$ on chromosome XVI (Fig. 3.4). Two separate internal amplification controls regions were from pol II-transcribed genes (coding regions) with no nearby tRNA genes or TFIIIC binding sites. Both Smc2p and Smc4p show specific binding to each of the representative tRNA genes, with enrichment similar to that of affinity tagged Brf1p, a subunit of the pol III transcription factor TFIIIB, which continuously occupies activated tRNA genes.

The preferential binding of condensin to tRNA genes strongly implies that condensin is interacting specifically with some component(s) of the pol III transcription complex on the genes, since the only conserved sequences of all tRNA genes are the internal promoters within the coding sequences (121). tRNA genes are recognized by initial binding of the multisubunit TFIIIC to these internal promoters, followed by binding of TFIIIB upstream of the gene but in contact with TFIIIC, followed by pol III (68,43,103). We tested for direct association of pol III components with condensin using co-immunoprecipitation. TAP-tagged proteins from pol III (Rpc82), TFIIIB (Brf1), or TFIIIC (Tfc1) were isolated and blotted for a 13xMyc tagged condensin subunit (Smc2 or Smc4, depending on viability of dual-tagged strains). We observed a low-level association with all three pol III components in cell lysates (Fig. 3.5); suggesting condensin was stably associated with the assembled tRNA gene complexes released by the lysis procedure, but not preferentially with any single soluble component, since these components do not form stable complexes away from the DNA. To test if these interactions were direct *versus* DNA-mediated, we performed the same immunoprecipitation in parallel after treating lysates with enough DNase I to completely digest any exposed DNA. This DNase treatment consistently removed the association of pol III with condensin, but left the condensin signal in TFIIIB and TFIIIC pulldowns essentially unchanged (Figure 3.5). This strongly suggests that there is a direct interaction between condensin and a stable TFIIIB/TFIIIC complex, and that maintenance of this complex does not require the presence of RNA polymerase III.

DISCUSSION

The spatial organization of genes within a eukaryotic nucleus is extraordinarily complex. Examples of this in yeast are the preference of telomeres (90), centromeres (91), and silenced chromatin (80,113) for the nuclear periphery, the localization of various highly expressed and inducible genes to nuclear pores (70,75,76,124), and the co-localization of ribosomal DNA and a majority of tRNA genes at the nucleolus. To accommodate each of these known levels of organization, there are likely to be multiple collaborating mechanisms. Two factors are shown here to affect the positions of tRNA genes: condensin-dependent clustering and separable

association of clusters with the nucleolus. Because the tRNA genes are distributed throughout the linear maps of the sixteen chromosomes, this phenomenon affects the condensation and spatial organization of much of the yeast genome.

The involvement of microtubules in nuclear genome organization has previously been observed in a variety of other studies, although the mechanistic details for these effects are not entirely clear. Microtubules were found to be responsible for the constraint on chromosome motion in the yeast nucleus (114), and they are also required for centromere clustering at the spindle pole body (74). Our observation that intact microtubules are required for localizing tRNA genes is consistent with microtubule-dependent structures being required for the general positioning of chromosomes. It is possible that, in addition to passive foundational support, microtubules might be involved in the dynamic positioning of specific DNA regions. For example, homologous recombination, which requires two DNA loci to be spatially adjacent, is facilitated by microtubules in *S. cerevisiae* (22). While preliminary investigations of microtubules interacting directly with pol III transcription units in yeast have proven inconclusive (unpublished observations), in human cell culture β-tubulin is one of the few non-subunit proteins associated with highly purified RNA polymerase III (95, 96), and it remains possible that microtubules have a direct effect on tRNA gene positioning.

The association of the condensin complex with tRNA gene complexes appears to be direct. Prior to this work, only a small number of interphase condensin functions were explored in various organisms (87), including gene regulation and centromere organization. Of particular interest is the condensin-like dosage compensation complex in *Caenorhabditis elegans*, which is responsible for the sex-determining X chromosome repression (87). In *S. cerevisiae*, condensin's role in the architecture of the rDNA locus during division has been documented (82,103,104,165), but the complex might have other non-mitotic functions that are distinct from its role in chromosome segregation. For example, condensin has been implicated in the *SIR2*-mediated silencing at the rDNA and telomeres (111), and

certain condensin mutations can also affect silencing at the mating type loci (165), which is also *SIR2*-mediated. While silencing near tRNA genes (and their nucleolar localization) are mechanistically distinct from the other types of silencing and independent of *SIR2* (5), it has previously been suggested that condensin may influence interphase chromosome arrangements in ways that impact gene regulation (87). Our combined evidence that condensin is required for tRNA gene clustering and for tgm silencing suggests that the complex plays a very important role in the nuclear architecture and that mutations could have broad, non-specific effects on nuclear functions.

As yet, the process by which condensin condenses chromosome structure is unknown, although several models have been suggested (87,93,147,128). Because the Smc2/4 complex is large (capable of spanning up to 150 base pairs of DNA (93), it is theoretically possible that each condensin molecule is associated with two or more regions of double stranded DNA. It is also possible that condensin molecules oligomerize, bringing together the chromatin regions to which they are bound. The condensin-dependent clustering of tRNA genes from multiple chromosomes argues for a model in which multiple DNA strands are brought together by multiple condensin molecules.

In order to bind tRNA genes, condensin appears to target one of the many proteins in the transcription factors TFIIIC or TFIIIB. The TFIIIC transcription factor, which initially recognizes the genes, has been identified as a likely binding partner (166), since genome sites bound by TFIIIC but not other members of the transcription complex also bind condensin. Most models for SMC complex interactions with bacterial and eukaryotic chromosomes assume direct contacts with DNA, and yeast condensin SMC complexes are known to bind naked DNA avidly in a non-sequence dependent fashion (128). Our observation of a DNase-resistant TFIIIB/TFIIIC/condensin complex indicates that condensin association with the complex is stable once the exposed DNA is removed, although it does not address whether condensin is also in contact with the DNA.

The interaction of condensin at sites that bind TFIIIC, but do not stably interact with the other pol III components (166) supports a model whereby condensin is brought to the tRNA gene through TFIIIC's sequence-specific binding to the internal promoters. It remains possible that additional contacts might also be made with the RNA polymerase, since Smc4 has also been found to co-isolate with Rpc40 (94). It is potentially interesting that Rpc40 is a subunit of both pol I and pol III, but not pol II. Thus condensin might be targeted to both tRNA genes and rDNA as a part of nucleolar organization through this interaction. However, the present data do not provide support for a stable interaction between polymerase subunits and condensin.

The condensin-dependent clustering of linearly scattered tRNA genes affects the spatial organization of most of the yeast genome, but the extent to which this phenomenon affects larger eukaryotic genomes has not yet been explored. Vertebrate genomes can be two orders of magnitude larger than yeast, yet the number of active tRNA genes is estimated to be only a few fold higher (85) and their positions in nuclei have not been determined. It would be surprising, though not impossible, for the metazoan transcribed tRNA genes to also be localized to nucleoli during interphase. It is perhaps also worth considering the extent to which the many non-transcribed, tRNA-like promoter elements are organized. Vertebrate genomes tend to have over a million copies of short interspersed nuclear elements (SINEs) (118,134) that were originally derived from retrotransposition of either tRNA genes or 7SL RNA genes, which have tRNA-class pol III promoters. Although these elements are normally expressed only at very low levels, if at all, most have consensus TFIIIC binding sites and cloned copies can be transcribed by RNA polymerase III in vitro. It is not currently known to what extent TFIIIC and other pol III components are bound to the elements *in vivo*, but imaging of SINE (human Alu element) positions in HeLa cells shows a punctate pattern (100) that is consistent with local clustering. If pol III components and condensin associate with some subset of the SINEs, it is interesting to speculate that they might serve as symbiotic, rather than selfish DNA elements, in that they provide condensation signals for the relatively large metazoan genomes.

MATERIALS AND METHODS

Yeast strains

For tgm silencing assays and *in situ* hybridizations, wild type parent strains included BY4741 (MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL4 GAL80*) and W3031A (MAT**a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*). Mutant strains included 1bAS330 (MAT**a** *smc2-8 ade2 leu2 lys2 his3 trp1 ura3*) and 1bAS342 (MAT**α** *smc4-1 ade2 leu2 lys2 his3 ura3*) (82), BLY07 (MAT**a** *ycg1-2:KAN ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2-801 ade2-101*) (103), NBY481 (MAT**a** *ycs4-1 ura3-1:: PGAL-MPS1::TRP1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 lys2Δ hmlΔ::LEU2*) (165), and BHY78 (MAT**a** *brn1-9:TRP1 ura3-52 leu2Δ1 trp1Δ63 his3::natMX4*) which was created by replacing *HIS3* with the natMX4 nourseothricin resistance cassette in the strain CH2524 (103).

For ChIP assays, strains were derived from YPH500α (MATα *ade2-101 his3-Δ200 leu2Δ1 lys2-801 trp1-Δ63 ura3-52*) (126) (gift of Olivier Lefebvre). Smc2p and Smc4p were C-terminally tagged with 13xMyc epitope by methods previously described (50). Brf1p is C-terminally tagged with 3xHA in strain MW4034 (88) (derivative of YPH500α, gift of Olivier Lefebvre). For protein-protein interaction studies, the C-terminally 13xMyc epitope tagged Smc2p was also incorporated into BRF1-TAP and RPC82-TAP strains. Smc4p was C-terminally tagged with 13xmyc in a Tcf1-TAP strain, since tagging Smc2p in this strain did not give healthy isolates. Tandem affinity strains were obtained from Open Biosystems (84).

Nocodazole treatment and cell cycle synchronization

BY4741 cultures were grown in SDC media to an OD_{600} of 0.2-0.4 prior to addition of drug or pheromone. Nocodazole (Sigma-Aldrich) was added to a final concentration of 15 μg/ml, and 30°C incubation continued for either one hour or until >80% of cells appeared by microscopy to be arrested in a large-budded state, 3.5-4.5 hours. Cells were subsequently fixed, and immunofluorescence with an anti-α-tubulin antibody confirmed the absence of microtubules in treated cells (not shown).

For α factor synchronization, α factor was added to 10 μg/ml and 30°C incubation continued until cells arrested in a G1 "shmoo" state, 2-2.5 hours. Cells were fixed at this point, or for subsequent treatment, yeast were harvested, washed in prewarmed SDC, and resuspended in prewarmed SDC containing 15 μg/ml nocodazole for one hour additional incubation at 30°C prior to fixation.

tgm silencing growth assay

To test for release of tgm silencing, cells were transformed with p*SUP4(-77)o* (5,6,12) and plated in serial dilutions on synthetic media containing galactose and raffinose (SGR) or dextrose (SD): SGR-ura, SGR-ura-his, SD-ura-his. There was no growth on SD-ura-his. Mutant cells were able to grow on SGR-ura-his at both 23°C and 30°C although the percentage of cells that grew was variable between repeat experiments. This phenotype is typical for mutations that relieve tgm silencing (5,12). Large and small colonies from mutant strains were restreaked onto SGR-ura-his and grown at 23°C, which resulted in growth of mixed sized colonies, regardless of original colony size.

In situ hybridization and microscopy

Yeast were grown in synthetic dextrose complete (SDC) media and fixed for 30 minutes by adding 3.6% paraformaldehyde directly to the growth media, to minimally disturb cellular and nuclear architecture (3, 26., 2003; 5). After fixation, cells were treated as previously described (3, 109), except that spheroplasting was performed by treating with 0.38 μg/μl Zymolyase 20T (Seikagaku, Tokyo, Japan) for 45 minutes at 37°C. Oligonucleotide probes, labeled with Oregon Green 488 (Molecular Probes) or Cy3 (Amersham), to U14 snoRNA, $tRNA^{Leu}$(CAA) and pre-$tRNA^{Leu}$(CAA) were previously described and *in situ* hybridization, microscopy, and deconvolution were performed as published (3,5,26,). After high-resolution imaging, cells that retained tRNA gene signal were scored: 216 untreated and 89 nocodazole-arrested cells, as well as 61 *ycg1-2* and 45 *brn1-9* mutant cells.

Chromatin immunoprecipitation

ChIP procedures were adapted from (123), with these exceptions: Fixation was performed with 1% paraformaldehyde for 30 min at room temperature and stopped with 400mM glycine for 10 min. Cell pellets were resuspended in lysis buffer (50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 1mM EDTA, 1% Triton, 0.1% deoxycholate, complete protease inhibitors (Roche)), and incubated with 0.3 μg/μl Zymolyase for 30 min at 30°C. Samples were lysed with glass beads in a Bead Beater (Biospec Products), sonicated, and spun out. Samples were incubated overnight with antibody against either Myc (9E10) or HA (Y11) (Santa Cruz Biotechnology). Myc tagged samples were also incubated with 1μl of a rabbit anti-mouse IgG (Jackson ImmunoResearch) for compatibility with Protein A beads. Antibody-bound samples were added to prewashed, immobilized Protein A beads (RepliGen) and incubated for 3 hours. Remaining procedures as described (123).

PCR and quantitation

Precipitated DNA was used as template in PCR reactions cycled at (94˚C for 30s, 63˚C for 30s, and 72˚C for 60s) x 25, 27, 29, or 31 cycles. Primers used: For $tRNA^{Lys}$(CUU) on chromosome VII: 5'AGTATATTCCAAACATGTG TTAATC and 5'CCAGAGCA CTTTTATGTGGGAC. For $tRNA^{Phe}$(GAA) on chromosome XVI: 5'GACGCTTG GACCATTTATAAAGCAC and 5'CCATAAGAGAAGGAGC AGTCAAGTTCA. For *UBR1*: 5'GCATCCATATTTGGAGAAC ATCCCATA and 5'CGTAGCTTGATTT CCTTAGATTGGGTA. For *ATG22*: 5'CAAAGTTTCGGTG GACTTCTAGTCAAA and 5'GCTTTAAACCGAACGCA TTGAAGAAAA. *ATG22* and *UBR1* genomic controls were selected because there was no polymerase III transcribed region within 5000 bp. EtBr-stained PCR products were imaged on a Syngene Bioimaging System with GeneSnap and quantitated with GeneTools software (Syngene).

Condensin-TFIIIC/TFIIIB/Pol III interaction study

Cells were grown in YPD to an OD600 of 0.6-0.8, and pellets were stored at –80ºC. Cells were thawed, lysed in buffer (175 mM NaCl, 20 mM Tris-HCl pH 7.9, 0.5% NP-40, complete protease inhibitors (Roche)), subjected to mechanical bead disruption, and spun at 14,000 rpm for 20 minutes. Of each supernatant, half was DNase I-treated with 50 units DNase I (Cooper Biomedical) per 3 ml extract. Elimination of DNA from extracts was verified by extracting and precipitating samples, then performing PCR using primers flanking the $tRNA^{Lys}$ locus. DNased samples produced no product after 40 cycles, whereas non-DNased samples produced product in less than 30 cycles.

Strains used in the pulldown were: SMC2-myc (no TAP tag); SMC4-myc (no TAP tag); BRF1-TAP + SMC2-myc; RPC82-TAP + SMC2-Myc; TFC1-TAP + SMC4-myc. Extracts were incubated with IgG Sepharose (GE healthcare) for 2.5 hours then washed four times with lysis buffer, and once with TEV cleavage buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT). Samples were eluted overnight with TEV protease, precipitated with trichloroacetic acid, electrophoresed, and blots probed with a Myc monoclonal antibody (9E10) (Santa Cruz).

ACKNOWLEDGEMENTS

This work is based on a submitted manuscript “Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complex.” (Haeusler RA, Pratt-Hyatt M, Good PD, Gipson TA, and Engelke DR) I would like to credit to both Rebecca Haeusler and David Engelke for writing large portions of this text. Rebecca Haeusler did the majority of the work that constitutes figures 3.1, 3.2, and 3.3 in this chapter. Figures 3.4 and 3.5 were done by myself as well as the accumulation of data that went into their production. We thank Alexander Strunnikov, Brigitte Lavoie, Andrew Murray, and Olivier Lefebvre for yeast strains, Claudio D’Ambrosio and Frank Uhlmann for communicating results prior to publication, and Engelke lab members for helpful discussion. This work was funded by NIH grant GM061342 to DRE and predoctoral fellowships to RAH from the

University of Michigan Rackham Graduate School and NIH Genetics Predoctoral Training Grant (T32 GM007544), and to M.P-H. from the NIH Cellular and Molecular Biology Training Grant (T32 GM007315).

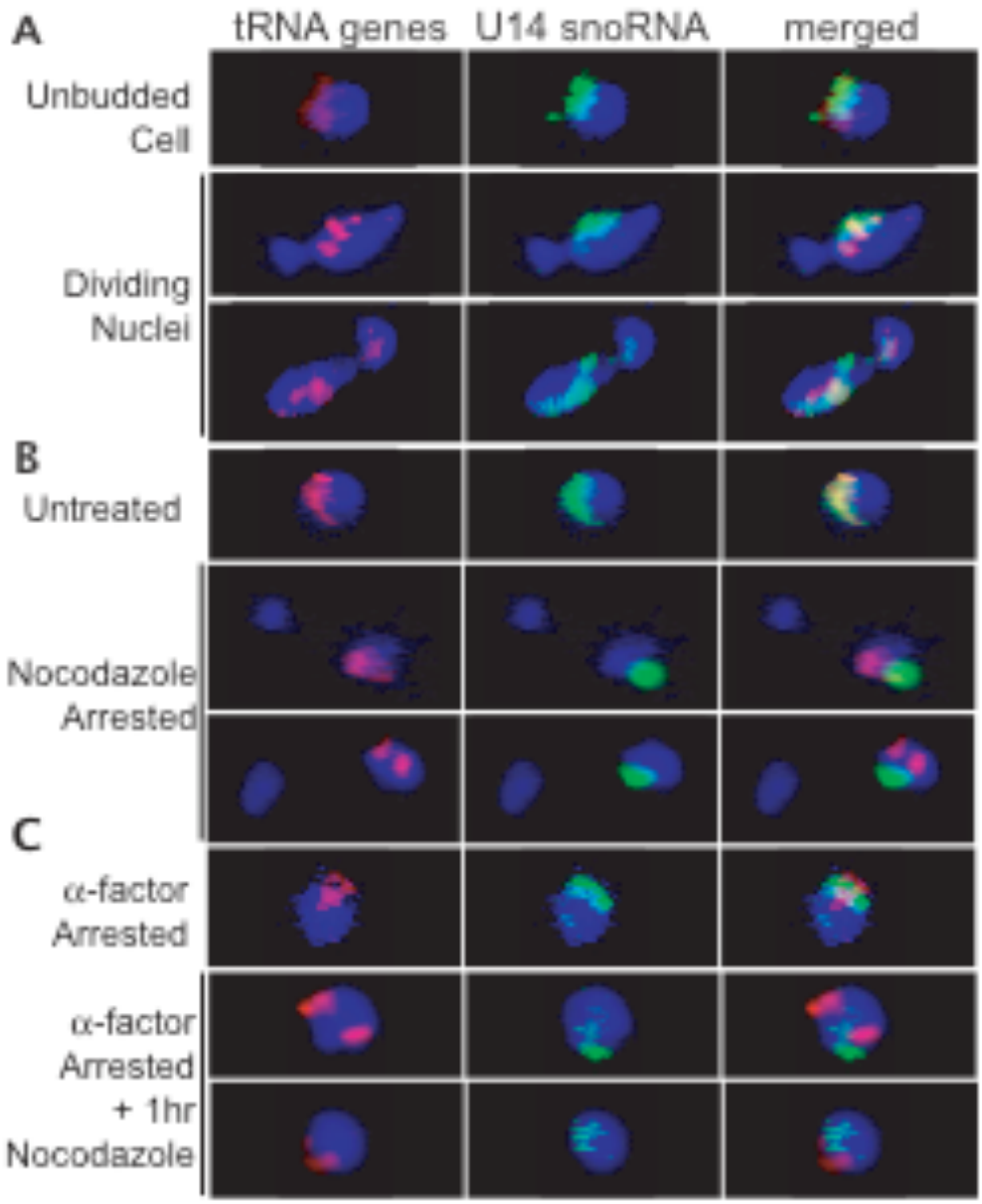


Figure 3.1. ***In situ*** **hybridization of unsynchronized and nocodazole treated cells.** In each panel, fluorescent oligonucleotide probes complementary to the U14 snoRNA (green) or 10 $tRNA^{Leu}$(CAA) genes (red) were used for hybridization. Blue represents DAPI staining of nucleoplasmic DNA. Nuclei from unsynchronized cells (A) show that tRNA gene signal (red) consistently overlaps the nucleolar signal (green) prior to and throughout division of the nucleus. Depolymerization of microtubules by arrest in nocodazole (B) causes partially divided nuclei in which tRNA genes remain clustered, but clusters are divorced from the nucleolus. This effect is not due to nocodazole blockage prior to nuclear division, as demonstrated by the release of tRNA gene clusters from the nucleolus by depolymerization of microtubules in cells arrested in G1 by α factor (C).

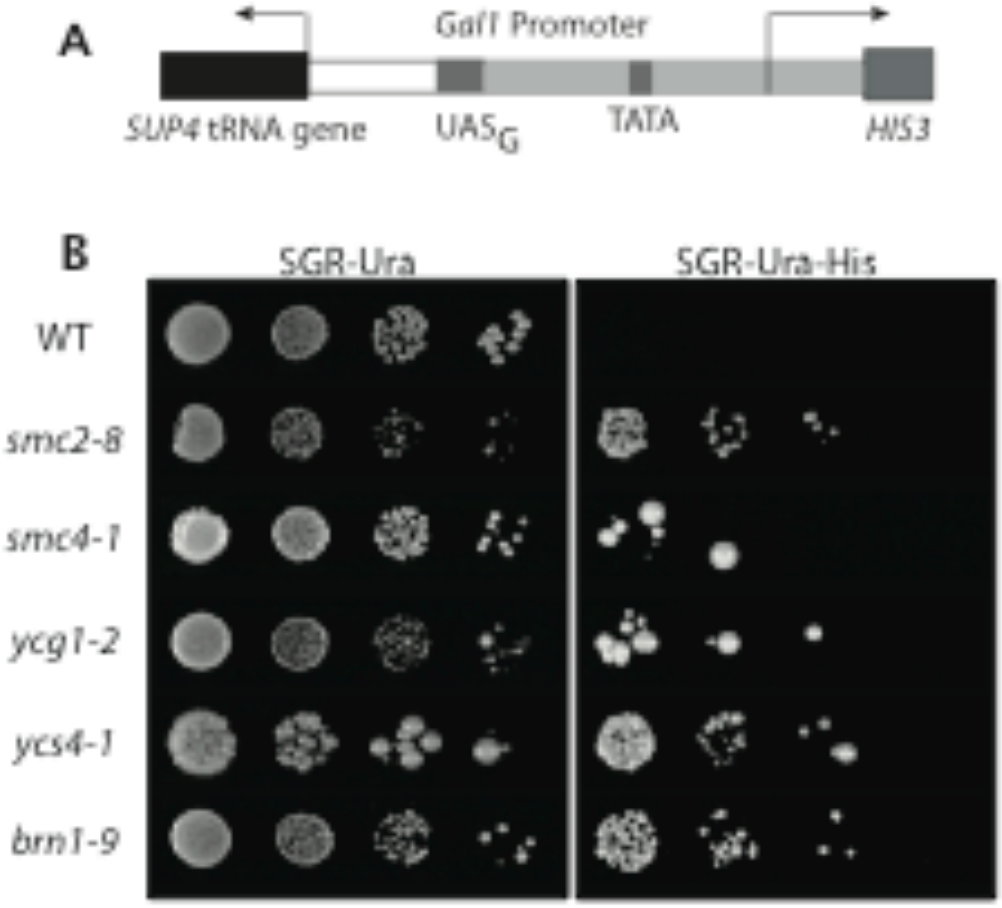


Figure 3.2. Condensin mutants release tgm silencing. Strains containing mutations in each condensin subunit were transformed with a plasmid containing a tgm silencing test construct (shown in A) (5,6,12), where the *SUP4* tRNA gene silences *HIS3* expression in wild type cells. Cells with defective tgm silencing are able to grow on media lacking histidine (SGR-ura-his) (B). Growth at semi-permissive temperature for the ts mutants (30°C) is shown, although growth at 23°C gave similar results (not shown). All five condensin mutants release tgm silencing to varying extents.

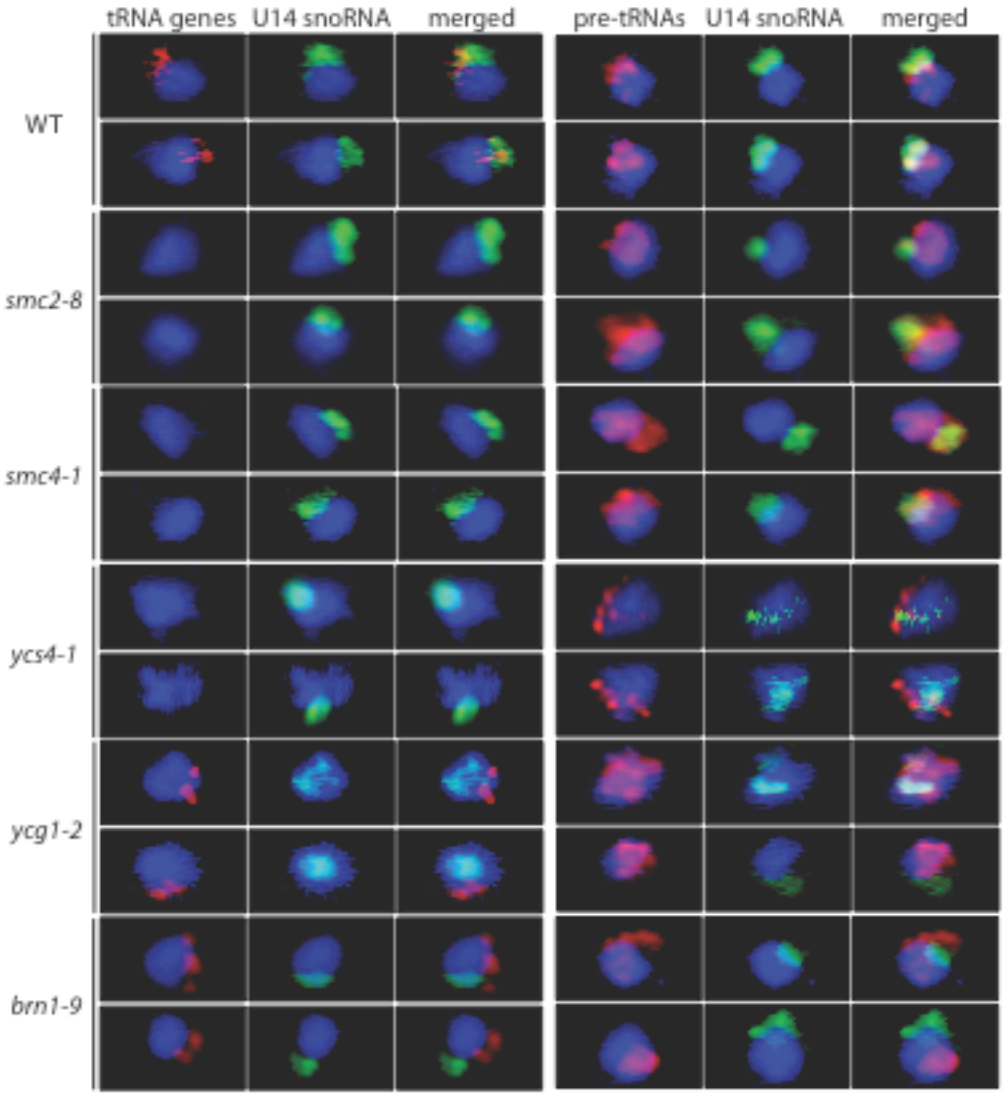


Figure 3.3. Condensin mutants show loss of tRNA gene and pre-tRNA nucleolar localization. Fluorescence *in situ* hybridization to the $tRNA^{Leu}$ genes and the early precursor tRNAs (pre-$tRNA^{Leu}$) in wild type cells shows the genes and their transcripts clustered at the nucleolus, as described previously (3,5,12,26). However, mutations in condensin subunits change the patterns of tRNA gene clusters. In some cases they disperse (*smc2-8*, *smc4-1*, *ycs4-1*) such that the fluorescent signal is no longer detectable above the background, and in other cases they mislocalize outside of the nucleolus (*ycg1-2*, *brn1-9*). Pre-tRNA positions are also altered, in some cases localizing near the nuclear periphery (*ycs4-1*, *brn1-9*) and in others diffusely in the nucleoplasm (*smc2-8*, *smc4-1*, *ycg1-2*).

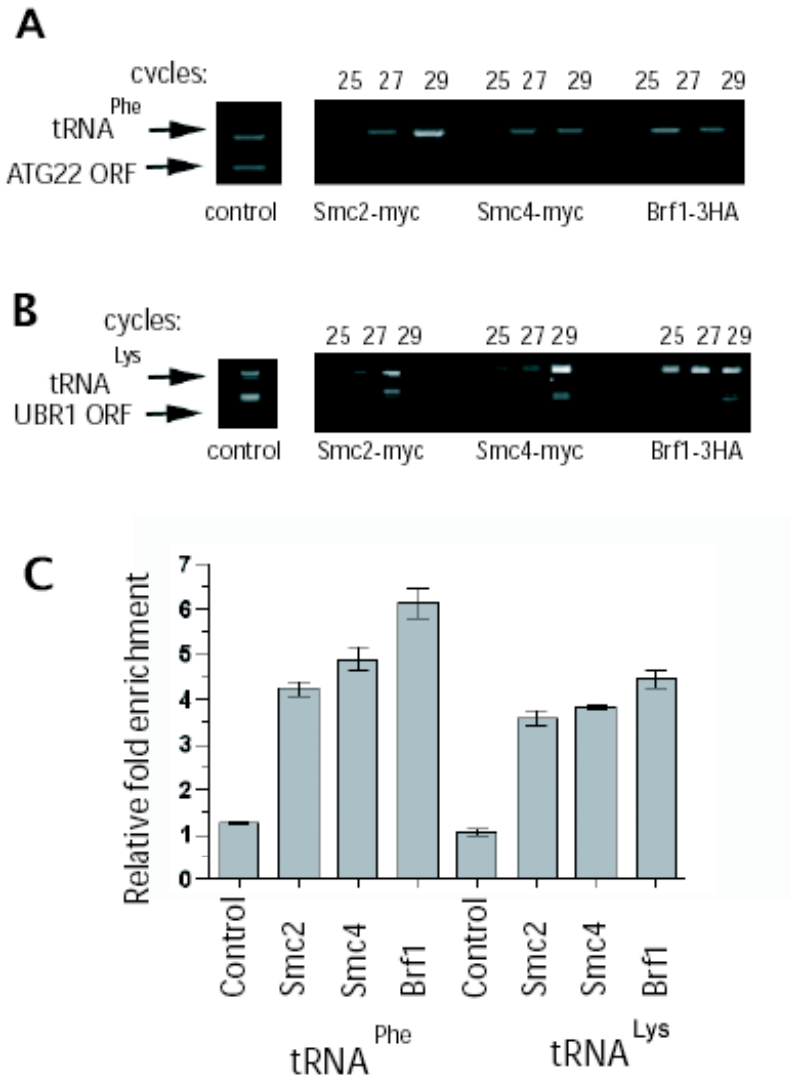


Figure 3.4. Condensin subunits Smc2 and Smc4 interact with tRNA genes. (A-B) ChIPs were performed using epitope tagged versions of Smc2, Smc4, and Brf1. 25, 27, or 29 rounds of PCR are shown with primers that simultaneously amplify a $tRNA^{Phe}$ gene and the *ATG22* ORF segment (panel A) or a $tRNA^{Lys}$ gene and a *UBR1* ORF segment (panel B). No antibody controls at high PCR cycles are also shown. (C) Ratios of relative signal intensity were quantified for each tRNA/ORF pair at subsaturating points (29 cycles in the examples shown). Error bars represent the standard deviation for four amplifications in two experiments.

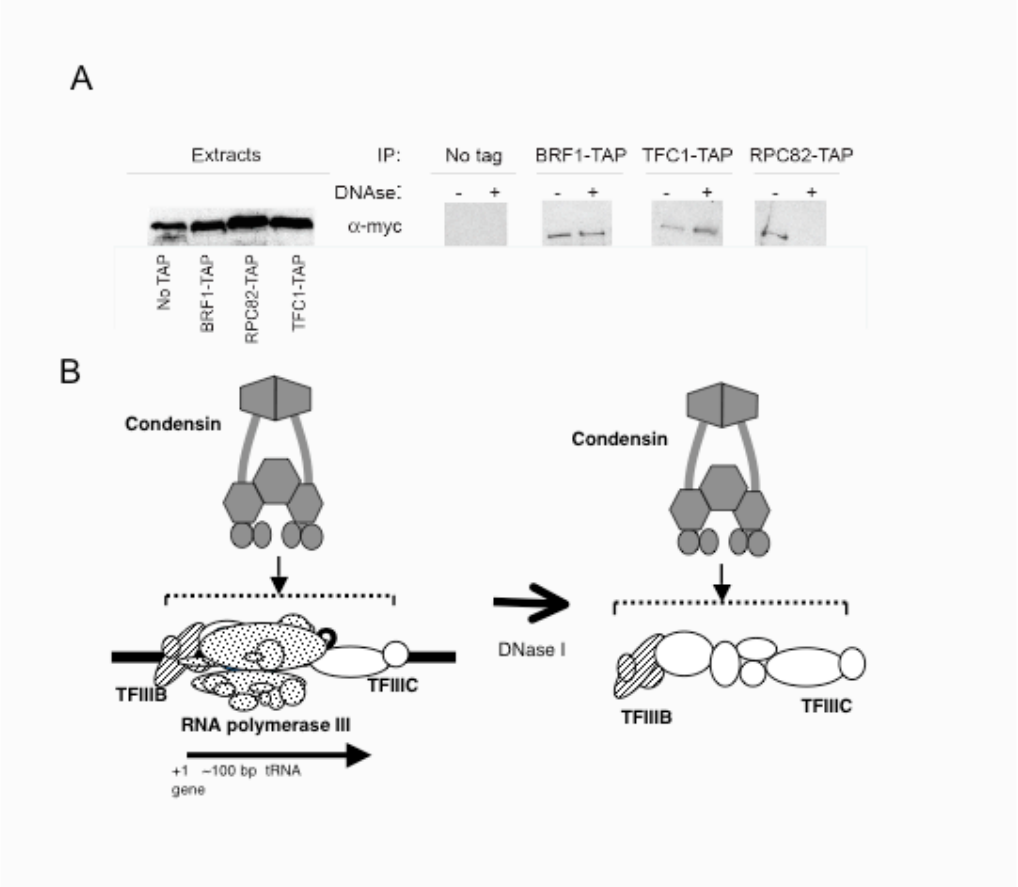


Figure 3.5. Condensin subunits Smc2 and Smc4 interact with subunits of RNA polymerase III and its transcription factors. (A) Strains containing a Myc-tagged Smc subunit and a TAP tagged subunit of RNA pol III, TFIIIB, or TFIIIC were lysed, TAP-containing complexes were affinity isolated and probed for Myc-tagged condensin. A portion of the condensin found in the extracts co-isolated with each of the pol III complexes. DNase I treatment removed pol III, as well as DNA, from the complexes while leaving condensin associated with TFIIIB and TFIIIC.
(B) A model for the association of condensin with the known complex of pol III transcription factors TFIIIC and TFIIIB. Although the DNase resistance of the complex suggests a direct and stable protein-protein association, it does not rule out contact of condensin with the DNA as well.

Chapter IV

Mod5p, a tRNA modifying enzyme, binds to tRNA gene complexes and affects local transcriptional silencing

Abstract

Transfer RNA (tRNA) genes are distributed throughout the linear chromosomes in eukaryotes, but recent studies show that most yeast tRNA genes are gathered near the nucleolus throughout the cell cycle. This nucleolar localization has been shown to be required for tRNA gene-mediated (tgm) silencing of nearby RNA polymerase II promoters, but tgm silencing also requires an additional mechanism besides nucleolar localization. Here we identify two genes required for tgm silencing, even though the nucleolar tRNA gene clustering is maintained when the genes are deleted. One of the gene products, Maf1p, was previously shown to bind to tRNA gene complexes and is required for repression of RNA polymerase III transcription, as well as nearby tgm silencing. The other gene product, Mod5p is a known tRNA modifying enzyme and suspected tumor suppressor in humans. Mod5p's previously characterized function is to add N6-isopentenyl adenosine at position 37 on a small number of tRNAs. Here we show that Mod5p associates with tRNA genes in vivo and contributes to local tgm silencing whether or not the target genes are producing substrates for Mod5p. Finally, we have evidence that Mod5p associates with a complex that contains RNA polymerase III transcription factors and condensin, which has recently been found to be involved in nucleolar tRNA gene clustering.

Introduction

The most widespread silencing influence in the yeast genome occurs near the 274 tRNA genes, which are distributed throughout the linear chromosomes (6,14). The tendency of tRNA genes to suppress transcription from nearby RNA polymerase II (pol II) promoters is mechanistically entirely from other forms of silencing in yeast, since gene mutations and deletions that affect other silencing mechanisms failed to affect tgm silencing (5). This tRNA gene mediated (tgm) silencing was previously shown to depend on the subnuclear localization of the tRNA loci to the nucleolus and to be relieved when nucleolar architecture is compromised (5,26). This raised the question of whether nucleolar localization alone was sufficient to suppress nearby pol II transcription. The existence of additional mechanisms required for the silencing was suggested by the requirement for Maf1p, which was originally shown to be a repressor of RNA polymerase III (pol III) transcription that acts through interactions with the upstream transcription factor, TFIIIB, although the precise mechanism of inhibition is not currently known (116,138,152,154).

Here we show that another protein, Mod5p, is also required for tgm silencing. Mod5p was originally tested for tgm silencing effects due to its previously discovered genetic interaction with Maf1p (33,34). Even so, it was not entirely expected that Mod5p would affect tgm silencing, since its only known activity involved the modification of only a few tRNA substrates, specifically isopentenylation of the A37 exocyclic amine in $tRNA^{Tyr}$, $tRNA^{Phe}$, and $tRNA^{Ser}$ in yeast (28,30,31,140,141). Here we demonstrate that Mod5p binds to tRNA genes in vivo and that its catalytic center is required for nearby tgm silencing even if the tRNA gene does not encode an appropriate substrate for Mod5p. Possible explanations for the role of Mod5p are discussed.

Materials and Methods

Yeast Strains

Strains were derived from *Saccharomyces cerevisiae* strains, W3031a (MATa *leu2-3*, 112 *his3-11*, 15 *ade2-1 trp1-1 ura3-1 can1*-100), MT-8 (MATą *SUP7, ura3-1, his5-2, leu2-3,112, ade2-1, trp1, lys1-1, lys2-1, can1-100, mod5:TRP1*) BY4741 (MATa hisΔ1, leu2Δ, met15Δ, ura3Δ) and YPH500α (MATα ade2-101, his3Δ200, leu2Δ1, lys2-801,trp1Δ63,ura3-52). Strains with TAP-tagged proteins were purchased from Open Biosystems (84). Strains were created by tagging chromosomal ORFs with 13xmyc-epitope by direct recombination at the C-termini as described in (50). Descriptions of all of the strains and plasmids used are listed in table 1.

Cloning of *MOD5*

MOD5 was PCR amplified from BY4741 from 500 base pairs upstream of the AUG start codon and 142 base pairs after the stop codon with BamHI sites added unto the ends. This product was ligated into the BamHI site of plasmid pRS316 (126) creating plasmid pMPH1. The TAP tag was PCR amplified from the MOD5-TAP strain from Open Biosystems using primers going 100 base pairs into the *MOD5* ORF and 50 base pairs from the pRS316 plasmid. The amplified TAP tag was added to the pMPH1 clone by gap repair by transforming the amplified product and pMPH1 digested with XhoI and HindIII into a *mod5*Δ strain, creating pMPH3.

In situ hybridization and microscopy

Yeast were grown in synthetic dextrose complete (SDC) media and fixed for 30 minutes by adding 3.6% paraformaldehyde directly to the growth media, to minimally disturb cellular and nuclear architecture before fixation (3,5,7). After fixation, cells were harvested and treated as previously described (3,109), except that spheroplasting was performed by treating with 0.38 mg/ml Zymolyase 20T (Seikagaku, Tokyo, Japan) for 45 minutes at 37°C. Oligonucleotide probes, labeled with Oregon Green 488 (Molecular Probes) or Cy3 (GE Healthcare), to U14

snoRNA, and $tRNA^{Leu}$(CAA) were previously described, as were *in situ* hybridization and deconvolution fluorescence microscopy (3,5,7).

Rapamycin treatment

Cells grown to an optical density of 600 nm 0.6-0.8 were treated with a final concentration of 0.2 µg/ml of rapamycin (LKT laboratories) for 40 minutes as described in (159). Cell pellets were harvested and stored at -80°C.

Atorvastatin treatment

Solid media of SDC-trp, SDC-ade, SGR-ura, or SGR-ura-his contained varied concentrations of Atorvastatin (Toronto Research Chemicals): 5, 10, 20, 50, 100, and 150 µg/ml.

Testing for silencing

Testing for silencing was done as described (5). Silencing was tested using plasmids p*SUP4*^{0}, p*SUP53*^{0}, pMPH2 (6).

Alignment of Mod5p homologs

Sequence alignments were obtained with ClustalX and Bioedit (144,158).

Testing for i6A modification of tRNAs

In order to test for i6A modification of tRNAs the strain MT-8 (141) was used to monitor modification-dependent translational ochre suppression. Plasmids were transformed into the MT-8 strain and then plated onto media lacking adenine. The presence of the modified tRNA was indicated by growth.

Site-directed mutagenesis of *MOD5*

Plasmid pMPH3 was used to produce two PCR products that have mutations (shown in Figure 4.5) in the overlapping region. Plasmid pMPH3 was digested with Age I and Kas I. The two PCR products were then transformed into BY4741 + *mod5*Δ with the digested plasmid. Gap repair of the plasmid was selected for by growth on SDC-ura and then confirmed the modifications through sequencing.

Random mutagenesis of *MOD5*

Mutations were inserted into *MOD5* by UV irradiation of the plasmid pMPH3. The plasmid pMPH3 (5µg) was subjected to 6 minutes of UV (254 nm) light by pipetting the plasmid directly onto the transilluminator (Fotodyne) covered with Saran wrap. The plasmid was then transformed into the strain BY4741 *mod5*Δ + pMPH2 to test for silencing. Plasmid DNA was recovered from transformants that did not regain tgm silencing, indicating the silencing function was compromised. This DNA was transformed into *E.coli* DH5α cells. The plasmid was purified through DNA miniprep (Qiagen) and reinserted into BY4741 + pMPH2 as well into MT8 in order to retest the silencing and suppression phenotypes.

Testing steady state levels of mutant proteins

Strains were grown in 200 ml of SDC-ura to an OD_{600} of 1.5. Cells were harvested and resuspended with 0.5 ml of lysis buffer 10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet (NP-40). Samples (20 µg protein) subjected to a SDS-PAGE 10% electrophoresis and electroblotted to a PVDF membrane (Millipore). Western analyses were done with anti-TAP (Open Biosystems) and anti-rabbit from donkey (GE healthcare). After exposure to detect TAP-tagged proteins blots were probed with anti-actin A5060 (Sigma) and anti-rabbit from donkey.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as described (123) except for the following adaptations. Cells were grown in 100 ml of YPD to 0.5-0.7 OD_{600} and fixed with formaldehyde at a final concentration of 1% for 1 hour at room temperature. Fixation was stopped by the addition of glycine (final concentration 400 mM) for 10 minutes.

Cell pellets were resuspended in 800 µl of lysis buffer (50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 1mM EDTA, 1% Triton, 0.1% deoxycholate, complete protease inhibitors (Roche) and transferred to 2 ml screw cap tubes. Zymolyase 20T was added to the cells (20 µl of 12 µg/µl stock) and incubated at 30°C for 30 minutes.

1 ml of glass beads (425-600 µm, Sigma) were added and chilled samples were lysed in a bead beater at 4°C (Biospec Products) 4 times at a setting of 5. Tubes were pierced at the bottom by a hot needle and the cell lysate was collected in a 1.5 ml Eppendorf tube. The extract was spun for 25 minutes at 14000 rpm and supernatants were then discarded. The cell pellet was resuspended in 800 µl of lysis buffer and was sonicated 8 times for 20 seconds at power 6 in a Branson Sonifer 250. The lysate was spun at 15,000 rpm for 20 minutes and then the supernatants were bound to 40 µl IgG Separose (GE Healthcare) for 2 hours at 4°C. The beads were washed five times with 15 ml lysis buffer. The samples were eluted as described in (123).

Polymerase Chain Reaction

DNA from chromatin immunoprecipitations (2.0 µl from 100 µl) was used as template in 50 µl reactions containing 0.20 mM dNTPs, 0.4 µg each of primers. Cycles were performed using an applied biosystems GeneAmp PCR system 9700. Cycling parameters for the amplications were (94˚C for 30 s, 63˚C for 30 s, and 72˚C for 60 s) x 21, 24, 27 cycles. The primer sequences were
5'TTATTAGCACGGTGCTTAACCAACT and
5'GCGCTTCCACCACTTAGTATGATTC for $tRNA^{Ile}$,
5'GAAAGCGGGTGTTTCTCCAATAAAT and
5'GTGGTTATCACTTTCGGTTTTGATCC for $tRNA^{Gln}$
5'CAAAGTTTCGGTGGACTTCTAGTCAAA and
5'GCTTTAAACCGAACGCATTGAAGAAAA for *ATG22* coding region.
PCR products were analyzed on a 2.5% agarose gels stained with ethidium bromide. Gels were imaged on a Syngene Bioimaging System with GeneSnap (Syngene) and bands were then quantified with GeneTools (Syngene). To determine enrichment, tRNA bands were divided by the control band at 24 cycles. Each reaction was then set to the ratio signal from the untagged strain.

Preparation of yeast soluble extracts and co-immunoprecipitations

The purification protocol was modified from previous work (153). Cells were grown in 500 ml of YPD to an OD_{600} between 0.8-1.2, and harvested cell pellets were stored at -80˚C. Pellets were resuspended in 1ml of buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet (NP-40), plus Complete protease inhibitors). Zymolyase 20T was added to the cells (20 µl of 12 µg/µl stock), incubated for 1 hr, and the cells lysed by mechanical bead disruption. Cell extracts were spun for 30 minutes at 14,000 rpm and the lysate collected. Lysates (3 ml) were added to 100 µl of IgG Sepharose bead (GE Healthcare) and incubated at 4°C for 3 hours. Beads were washed 4 times with 10 ml lysis buffer and once with TEV cleavage buffer (10 mM Tris, 150 mM NaCl, 01 % NP-40, 0.5 mM EDTA, and 1 mM DTT). Beads were resuspended with 300 µl of TEV cleavage buffer plus about 50 µg of TEV protease. Elutions were precipitated by Trichloroacetic acid ((TCA) and resolved using on SDS-PAGE gel 10% (Bio-Rad). Proteins were transferred to PVDF membrane. The membrane was probed with anti-myc antibody (Santa Cruz) and then with anti-mouse from sheep (GE Healthcare). Signals were detected through ELC Plus (GE Healthcare). In DNase treated samples, 50 units DNase I (Cooper Biomedical) were added and incubated at 4°C for 30 minutes before adding the lysate to the IgG Sepharose. PCR analysis of parallel samples determined this level to eliminate detectable DNA fragments at the tRNA genes after 35 rounds of amplification as described above.

Results

Mod5p is required for silencing near tRNA genes

Over 200 gene deletions and mutations have previously been tested to determine which proteins are necessary for the silencing phenomenon (5,12). Silencing near tRNA genes was tested using a plasmid that contained an active tRNA gene upstream of a *HIS3* coding region, in strains where the chromosomal *HIS3* gene is deleted. The plasmid *HIS3* gene was controlled by a modified GAL1 promoter with a single, consensus binding site for Gal4 protein (Fig 4.1A). Wild type cells are not able to grow in the absence of histidine (Fig 4.1B)(6,12).

All previously tested mutations that alleviated tgm silencing caused a gross mislocalization of the tRNA genes (5). Recently, the deletion of the gene *MAF1* was shown to alleviate tgm silencing (116). Since *MAF1* is known to affect both localization and function of another protein, Mod5p, a known tRNA modifying enzyme (33,34,148), we also tested *mod5*Δ for effects on tgm silencing using the reporter construct in Fig 4.1A. In wild type backgrounds, cells were not able to grow in the absence of histidine (Fig 4.1B) (6,12), but the *mod5*Δ strain was able to alleviate tgm silencing. Because this assay utilized a plasmid containing an active *SUP4* $tRNA^{Tyr}$ gene, which is a substrate for Mod5p, we then used a second construct to test if *mod5*Δ would cause a loss of tgm silencing near a tRNA gene that is not a substrate of Mod5p. In this construct the *SUP4* tRNA gene was replaced with a *SUP53* tRNA gene, encoding a variant of $tRNA^{Leu3}$ (6). Our results show that *mod5*Δ caused alleviation of silencing in both constructs (Fig 4.1C). Thus, the product of the tRNA gene did not need to be a target of Mod5p for local tgm silencing to be active.

Mod5p and Maf1p are not involved in the subnuclear distribution of tRNA genes

Previous deletions and mutations that caused alleviation of tgm silencing had been in nucleolar effectors of rRNA expression and in the chromosome architecture complex condensin. All of these mutations had dramatic effects on the subnuclear locations of the tRNA genes themselves (5,12, Haeusler *et* al, submitted). It is possible that mislocalization of tRNA genes alleviated tgm silencing in these cases simply because the removal of loci from the nucleolus made them more accessible to pol II and pol II transcription factors (5), or that mislocalization disrupted spatial relationships needed for organizing "active" silencing events. To test whether this is the case for the *MAF1* and *MOD5* gene deletions, these strains were tested for localization of the tRNA genes. These two deletions had growth rates similar to wild type cells, unlike many previous deletions or mutations that alleviate tgm silencing that grew slowly or were temperature sensitive (5 and data not shown). The clustering of the 10 linearly dispersed $tRNA^{Leu3}$ (CAA) genes new the nucleolus was tested in these deletion strains by employing fluorescent *in situ* hybridization (FISH)

as previously described (5,7). In both the *mod5*Δ and the *maf1*Δ mutant strains, the $tRNA^{Leu3}$ genes co-localized with the nucleolar marker U14, similar to the wild type (Fig 4.2). Although we are unable to rule out subtler disturbance of tRNA gene positions, other mutations giving loss of tgm silencing had displayed obvious mislocalization phenotypes (5). These data therefore suggest that the alleviation of tgm silencing caused by the *mod5*Δ and *maf1*Δ deletions are not due to by gross redistribution of the tRNA genes.

*mod5*Δ does not alleviate repression of tRNA gene transcripts

Previous studies have demonstrated that inhibition of the TOR (target of rapamycin) pathway by rapamycin caused inhibition of pol III transcription of tRNA genes. This inhibition required Maf1p (151,152,159). We tested whether Mod5p similarly affected repression of tRNA transcription by the TOR pathway, using quantitative northern blot analysis. As expected, wild type cells treated with rapamycin showed an approximate four-fold reduction in new tRNA synthesis (Fig 4.3), whereas *maf1*Δ cells showed no dramatic loss of transcription when treated with rapamycin (Fig 4.3). In contrast the *mod5*Δ cells responded to rapamycin similarly to wild type and reduced tRNA transcription three fold (Fig 4.3). These results indicate that the tgm silencing effects of both Maf1p and Mod5p may be unrelated to the other local effects of the Maf1p.

Role of Mod5p catalytic activity in tgm silencing

Mod5p is a isopentenyl transferase that catalyzes the transfer of a dimethylallyl group from dimethylallyl diphosphate (DMAPP) to the exocyclic amine on position 37 of a few tRNAs in yeast (28, 30,31,140,141). While not absolutely required for life, these modifications enhance the function of these tRNAs, which has allowed the development of a tRNA suppressor assay for translation efficiency (28). Previous studies of Mod5p's enzymatic activity have utilized a strain (MT-8) with a *MOD5* deletion and a *SUP7* ochre suppressor tRNA (141). If Mod5p modifies *SUP7* tRNAs, they will suppress nonsense mutations in the *ADE* and *LYS* genes, allowing for growth on media lacking these nutrients.

We tested whether Mod5p's previously defined catalytic activity is required for tgm silencing by depleting its substrate from which the isopentenyl group is obtained, DMAPP. This was accomplished by two different methods (136,139,160). The first method was to insert a plasmid that overexpresses the protein Erg20p, which uses DMAPP in a parallel pathway for sterol biogenesis. The overexpression of this protein from this plasmid has been shown to decrease the amount of i^6A tRNA to 30% of normal, and cause a loss of ochre suppression in the *SUP7* test system (136). The overexpression of Erg20p caused no observable loss of tgm silencing in wildtype cells (Fig 4.4A). The second method was to treat with the drug Atorvastatin. This drug inhibits HMG-CoA reductase, which produces a precursor to DMAPP (135,139). We employed the translation suppression assay to determine that 20µg/ml Atorvastatin was the minimal amount needed to inhibit Mod5p tRNA modification activity sufficiently to limit growth on SD-ade plates in strains containing *MOD5* (Fig 4.4B). We then tested Atorvastatin's ability to limit tgm silencing at 20, 50, and 100, µg/ml (higher concentrations were toxic). There was no loss of silencing at 20, 50, or 100 µg/ml Atorvastatin in the wild type compared to the *mod5*Δ strain (Fig 4.4C). The data suggests, that Mod5p's tRNA modifying activity may not be required for tgm silencing, although the result is not definitive because in each case the DMAPP is not entirely limited, nor is it known if there is differential depletion of nuclear and cytoplasmic pools.

The effects of *MOD5* mutation on tgm silencing

In order to further test if the catalytic ability of Mod5p is necessary for tgm silencing we created both site-directed and random mutations of *MOD5*. The first strategy was to produce site directed mutations in three different residues shown to have dramatic effects on K_{cat}, but little effect on K_m or overall structure of the *E. coli* (155) Mod5 ortholog, miaA. Mod5p has been conserved through many different organisms, and thus we chose to mutate only highly conserved residues. Using the programs ClustalX and Bioedit we performed our own alignment (Fig 4.5) and then compared it to previous alignments (142,143) to confirm that those residues were

conserved. The residues mutated were T23A, Y51A, and T131A (Fig 4.5). The consequences of T23A and Y51A on tgm silencing could not be determined because T23A caused Mod5p to mislocalize to the cytoplasm and T131A caused Mod5p instability (very low cellular levels). In contrast, the protein with the Y51A mutation was stable (Fig 4.6A) and located in the nucleus (data not shown). The Y51A mutation caused both loss of suppression (Fig 4.6B), and a loss of silencing (Fig 4.6C).

In order to determine whether tgm silencing by Mod5p could be separated from its ability to isopentenylate tRNAs we performed random mutagenesis of the *MOD5* gene on a plasmid and transformed the mutagenized plasmids into a test strain. We screened 600 yeast transformants for loss of silencing and isolated 18 plasmids that demonstrated a loss of silencing. The plasmids were then re-transformed into the MT-8 strain to test for the consequences on *SUP7* isopentenylation. All mutations that alleviated silencing also removed Mod5p activity in the tRNA suppression assay, suggesting these functions were not differentiated by any of the identified mutations.

Two mutations located in the putative DMAPP binding site (142) were identified twice each. These mutations eliminated tgm silencing (Fig 4.6C) as well as the ability to modify *SUP7* tRNA (Fig 4.6B). One of these mutants, R220L, was tested for protein expression and stably expressed Mod5p (Fig 4.6A).

Mod5p homologs restore tgm silencing

As stated previously Mod5p has been conserved throughout many different organisms (Fig 4.5). Two of the conserved domains are the ATP/GTP binding domain and the DMAPP binding site (142). One notable feature is that the eukaryotic versions have a longer C-terminus than the prokaryotic version, and this region has a Zn-Finger motif (142). Homologs of Mod5p have been cloned in both *Arabidopsis thaliana* and humans, and both have been shown to be active in yeast in an assay testing for isopentenylation of *SUP7* (141,142,143,157). In order to test whether the ability to confer active tgm silencing in a *mod5*Δ strain was conserved, we inserted a plasmid expressing the either the human or *Arabidopsis* homolog ORF, along with a

plasmid containing the silencing test construct. The human Mod5p homolog (TRIT1) was able to fully restore tgm silencing in yeast and the *Arabidopsis thaliana* homolog partially restored tgm silencing (Fig 4.7). The *E. coli* homolog failed to rescue the *mod5*Δ in both the isopentenylation of *SUP7* assay and with tgm silencing assay (data not shown).

Mod5p is present at tRNA genes

Since Mod5p and most tRNA genes were shown to be located in the same subnuclear compartment, the nucleolus (4,32), this led us to hypothesize that Mod5p might be directly associated with tRNA genes and acting in *cis* to exert its effect on tgm silencing. In order to determine if Mod5p is present at tRNA genes we utilized chromatin immunoprecipitation (ChIP). We created both wild type and mutant (Y51A) expression constructs for Mod5p fused to a C-terminal Tandem Affinity Purification (TAP) tag, where the gene is transcribed from its endogenous promoter. These plasmids were inserted into a *mod5*Δ strain, and the wild type *MOD5* plasmid was able to complement the *mod5*Δ phenotypes of loss of tgm silencing and modification of *SUP7* tRNA (Figs 4.4 and 4.7). We performed ChIP of both wild type and mutant Mod5-TAP and the TFIIIB subunit Brf1p, which is stably bound to tRNA genes, for occupancy at the tRNA genes $tRNA^{Ile}$ and $tRNA^{Gln}$ by semi-quantitative PCR amplification (Fig 4.8). The signals from these PCR products were compared to an internal amplification control, the coding region of *ATG22*. As shown in Figure 4.8, both wild type Mod5p and the catalytically inactive mutant are enriched three to four fold at each tRNA gene relative to an internal control. This is similar to the to the result obtained pol III transcription factor subunit Brf1p, which is known to stably associate with the tRNA genes (154). These results demonstrate that Mod5p is associated with these two tRNA genes, possibly by associating with some part of the large protein complex on the genes. It is notable that neither of these two tRNA genes produces a substrate for Mod5p (28), indicating that Mod5p is present at tRNA genes even in the absence of synthesis in *cis* of a substrate for the known tRNA gene modification activity. This is in agreement with our results that demonstrated that both *SUP4* and *SUP53* both cause tgm silencing even though only *SUP4* is a

substrate for Mod5p. Our demonstration of tRNA gene occupancy by a Mod5 mutant that is defective in tgm silencing (and translation suppression) indicates that Mod5p is required to be at the tRNA loci and catalytically active in order for tgm silencing to be active.

Mod5p associates with RNA polymerase III transcription complex components

After a multi-step affinity and ion-exchange purification of Mod5-TAP, associated proteins were analyzed using MudPIT (161), and results indicated that Mod5p may interact with the chromosome condensing complex condensin (data not shown). This complex has recently been found to associate with all tRNA genes in the yeast genome and to be required for the nucleolar clustering of tRNA genes (D'Ambrosio *et* al, submitted, and Haeusler *et* al, submitted). Using co-immunoprecipitation we tested directly whether Mod5p-myc could be pulled down with the condensin subunit Smc4-TAP. We also tested TFIIIB, TFIIIC, and RNA polymerase III subunits (each with a TAP tag) because of their known presence at sites where tRNA genes are transcribed. Each of the tested TAP tagged proteins were able to pull down the myc-tagged Mod5p compared to a negative control strain without any TAP-tag (Fig 4.9). Since these components are not thought to associate with each other away from the genes, these results are consistent with the data that indicated that Mod5p is located in the nucleolus (4) and at the tRNA genes (Fig 4.8). To determine whether the interactions of Mod5p with these proteins required the continued presence of DNA, we repeated the co-immunoprecipitations in the presence of sufficient DNase to completely remove any PCR signal from the immunoprecipitated fractions. DNase treatment did not cause the total loss of any interaction, however a reproducible decrease in signal was seen in RNA pol III TAP tagged samples (Fig 4.9). These results were repeated in a reciprocal manner, where Tfc1-myc, Smc2-myc, and Smc4-myc were all able to co-immunoprecipitate Mod5-TAP (data not shown).

Discussion

The mechanism of tgm silencing is unlike any other characterized form of transcriptional silencing in yeast, and our attempts to characterize this mechanism have revealed surprising requirements. Previously all identified mutants that disrupted tgm silencing also mislocalized tRNA genes in the cell (5,12). Here we demonstrate that while localization of tRNA genes in the nucleolus is necessary for silencing near tRNA genes, it is not sufficient, and additional mechanisms must come into play. We identified two genes, *MOD5* and *MAF1*, which have previously been found to be genetically linked (33), that are required for tgm silencing. The Mod5p and Maf1p gene products are both found associated with tRNA genes, but their absence does not disrupt the localization of tRNA genes to the nucleolus, indicating that they perform their crucial activity in *cis* at tRNA loci.

Mod5p is conserved from *E.coli* to *H.sapiens* (Fig 4.5). Besides the conservation of the ATP/GTP binding domain and the DMAPP binding domain there are several other areas of high conservation. The mutation (Y51A) that we found to be non-functional in both assays, yet continues to show wild-type levels of protein expression and remains bound to tRNA genes, is in one such patch of conservation outside the identifiable motifs in the primary sequence. Studies of bacterial Mod5p homologs have found this residue to be critical for catalytic function (152), and structural analysis suggests that the residue may play a part in the DMAPP substrate binding site (137). The eukaryotic isopentenyl transferases possess a 100 amino acid C-terminal tail not found in the prokaryotic version. The function of this tail is currently unknown, although it has a Zn-finger motif (142) and has been implicated in nucleolar localization (4). It seemed possible that mutations in this region might affect only tgm silencing, but aside from truncations that removed the entire C-terminal region and abrogated both tested functions, no point mutations in this region were identified in our screen that selectively eliminated tgm silencing.

We attempted several methods to determine if Mod5p's catalytic activity is necessary for tgm silencing. Both site-directed and random mutagenesis of the *MOD5* ORF produced a large number of non-functional mutations, but all of these

removed both tgm silencing and tRNA modification. Most also compromised protein stability or localization, so that the effects were not interpretable. However, we were able to confirm that at least one mutation (Y51A) in the active site was both expressed at wild-type levels and found in the nucleus. These results are consistent with a role for Mod5p's catalytic activity in *cis* at tRNA gene site, although a more subtle structural explanation cannot be ruled out.

We also used two different methods to deplete Mod5's known substrate for tRNA modification, DMAPP. Neither of these methods alleviated tgm silencing, although they do decrease Mod5p tRNA modification to the point where an *in vivo* assay for Mod5p-dependent function of an ochre suppressor tRNA shows insufficient function to allow growth. On the surface this would seem to contradict the loss of tgm silencing due to mutations in the active site, but there are several possible explanations. First, depletion of DMAPP by both methods is known to be incomplete, and it is possible that the silencing function uses DMAPP, but does not require concentrations as high as for tRNA modification in the cytoplasm. A similar explanation would be that the cytoplasmic pool of DMAPP is depleted selectively, with a remaining nuclear pool still sufficient for whatever is needed for tgm silencing. It would be surprising if this nuclear function was due to modification of the few known tRNA substrates, since those substrates do not need to be encoded in *cis* by the tRNA gene causing the silencing. However, it is possible that the known tRNA substrates (or some other RNA substrate) synthesized at other gene locations are modified by Mod5p bound in *cis* at the silenced locus, and that these modified RNAs are somehow used locally in the silencing mechanism. If DMAPP is a requirement for translational suppression by Mod5 but not tgm silencing, then we can speculate that for the tgm silencing activity Mod5p may use a different small molecule substrate, and may even target different macromolecules. We made several attempts to determine Mod5p's targets at tRNA genes. For example, tRNA isopentenyl transferases from some eukaryotes can utilize oligonucleotides, including oligo (A), as substrates (146,157), and it would be theoretically possible for amino groups from proteins to serve as the isopentenyl acceptor in lieu of the exocyclic amine of the A37 in tRNA substrates. We were unable to modify recombinant histones or

oligonucleotides with TAP-isolated yeast Mod5, using DMAPP or endogenous small molecules found in soluble cell extracts (data not shown), but the negative results in these experiments are inconclusive.

Mod5p exists in both the cytoplasm and nucleus, with a concentration in the nucleolus (4,32). Mod5p's previously characterized activity in modifying tRNAs is thought to occur in the cytoplasm, but the function of Mod5p activity in the nucleus is currently unknown (32). The results presented here indicate that Mod5p possesses a previously uncharacterized function. There are other examples of tRNA modifying enzymes possessing novel functions. One example of this is the yeast protein Nam2p, a tRNA synthetase, which is involved in tRNA aminoacylation and also participates in RNA splicing of group I introns (145,149). A second example is the Elongator protein subunit ELP3. This protein is part of a complex that has H3 and H4 acetyltransferase activity (162). However, this same protein has also been shown to be necessary for a modification of the wobble position in tRNA (147). These two activities seem to use separate substrates (147), providing a precedent for a similar possibility for Mod5p. A third example is the Pus1 protein, which modifies uridine to pseudouridurine in tRNAs. However, this protein is also a co-activator that cooperates with Steroid Receptor RNA Activator to enhance mRARgamma-mediated transcription (164). While it remains possible that Mod5p modifies tRNAs in both the nucleus and the cytoplasm (32,141), its localization in both compartments, and specifically the nucleolus, is consistent with its additional function at tRNA genes.

The human homolog of Mod5p, TRIT1, is shown here to have retained whatever activity is necessary to complement the yeast enzyme in tgm silencing. This is of particular interest because previous studies have suggested that TRIT1 might be a tumor suppressor in specific types of human cancers. It is downregulated or alternatively spliced in lung adenocarcinoma, and when transfected into a human lung carcinoma line that had low expression of TRIT1, it is able to significantly reduce tumor development in a nude mouse test system (156). It has also been shown that the other product of its isopentenylation reaction, N6-isopentenyladenosine, a naturally occurring cytokinin, has potent anti-proliferative activity for multiple types of tumors (150,179).

These results suggest a previously unsuspected role for Mod5p in the nucleus. Although Mod5p does not appear to be involved in Maf1p-mediated regulation of tRNA gene transcription, it is associated with the tRNA gene complexes and contributes to local silencing of pol II transcription, a process that also requires Maf1p. The precise mechanism by which Mod5p affects local transcription is still not clear, but our observations are consistent with a mechanism involving Mod5p-mediated catalysis at the sites of the tRNA genes.

Acknowledgments

I would like to thank David Engelke, Anita Hopper, and Rebecca Haeusler for their assistance in writing this chapter. I would like to acknowledge the work done for Figure 4.2 was done by Rebecca Haeusler. I would like to thank Olivier Lefebvre strains and Bjorn Nicander for plasmids. I would also thank May Tsoi, Sara Neill, and Ravi Patel for their technical assistance. I would like to thank Dan Bochar for advice on chromatin immunoprecipitation. This work was supported by National Institutes of Health predoctoral training grants T32 GM007315 for M.P.H. and T32 GM07544 for D.A.P; P41 RR011823 (to J. Yates) for the support of I.X.M.; RO1 GM063142 to D.R.E.;and RO1 GM 27930 (to A.K. Hopper).

Table 4.1 – Strains and Plasmids used

Name	Genotype, description, or sequence	Reference
BY4741	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80*	Brachmann *et* al
BY4741 *mod5Δ*	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, mod5Δ*	Winzeler *et* al
BY4741 MOD5-TAP	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, MOD5-TAP:HIS5*	Ghaemmaghami *et* al
W3031A	*MAT□, ura3-1, ade2-1, his3-11,15 trp1-1, can1-100 GAL4, GAL80*	Rothstein *et* al
MT-8	*MAT□,SUP7, ura3-1, his5-2, leu2-3,112, ade2-1, trp1, lys1-1, lys2-1, can1-100, mod5:TRP1*	Gillman *et* al.
YPH500□	*MAT□, ade2-101, his3- Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52*	Sikorski and Hieter
MPHY6	*MAT□, ade2-101, his3- Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52, MOD5-13Myc:kanMX6*	This study
MPHY7	*MAT□, ade2-101, his3- Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52, SMC2-13Myc:kanMx6*	Haeusler *et* al
MPHY8	*MAT□, ade2-101, his3- Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52, SMC4-13Myc:kanMx6*	Haeusler *et* al
MPHY9	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, BRF1-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
MPHY10	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, RPC160-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
MPHY11	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, TFC1-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
MPHY12	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, SMC4-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
MPHY13	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, BDP1-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
MPHY14	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, RPC53-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
MPHY15	*MAT□, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GAL4, GAL80, RPC82-TAP:HIS5, MOD5-13Myc:kanMX6*	This work
yOH1	*MAT□, ade2-101, his3- Δ200, leu2-Δ1, lys2-801, trp1-Δ63, ura3-52, TFC1-13Myc:kanMX6*	Harismendy *et* al.
p*SUP4*^{o}	tRNA gene mediated (tgm) silencing construct	Hull *et* al
p*SUP53*^{o}	tRNA gene mediated (tgm) silencing construct	Hull *et* al
pMPH1	*MOD5* in pRS316	This work
pMPH2	TGM silencing construct in pRS315	This work
pMPH3	*MOD5-TAP* in pRS316	This work
pMPH4	*MOD5-TAP-1* in pRS316	This work

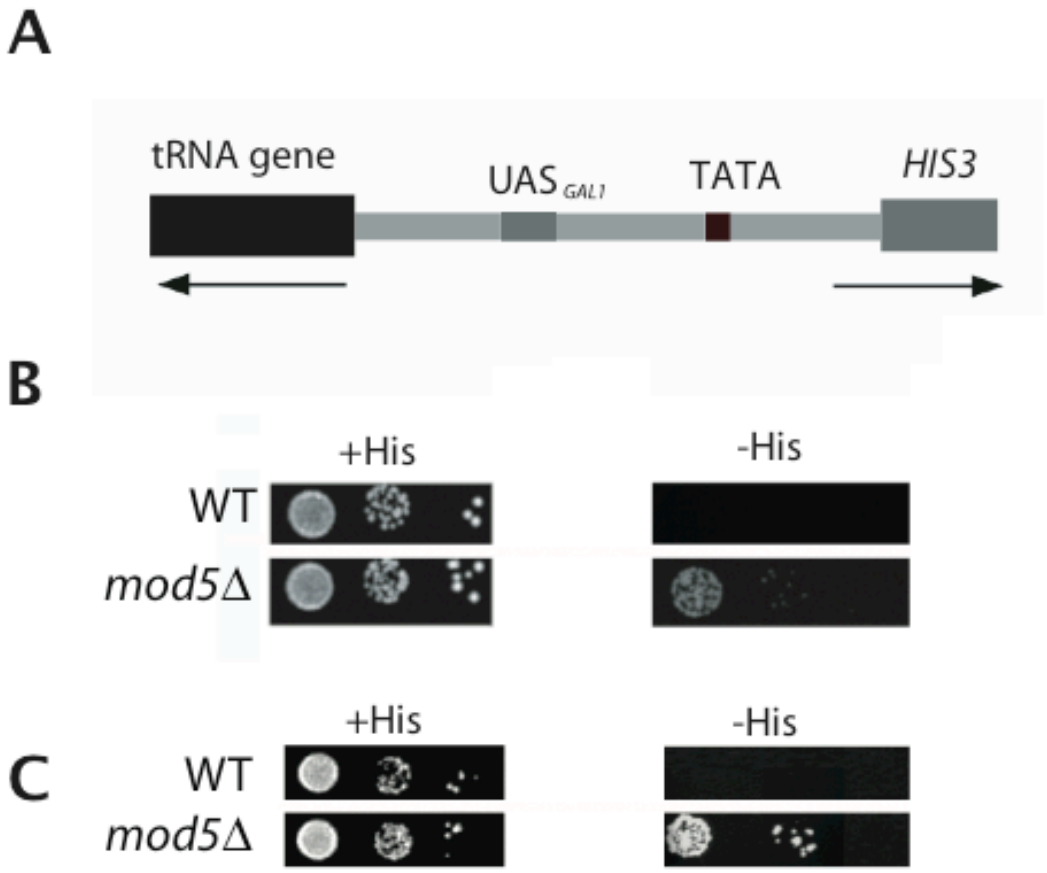


Figure 4.1. Deletion of *MOD5* releases tgm silencing. (A) Schematic representing construct used for testing for tgm silencing. The tRNA gene in wild type cells silences the *HIS3* reporter gene under the control of the Gal4 UAS (6). (B) Cells in which tgm silencing is disrupted express *HIS3* and are able to grow on media lacking histidine (SGR-ura-his). (C) tgm silencing still requires *MOD5* when the *SUP4* $tRNA^{Tyr}$ gene is replaced on the silencing reporter plasmid by the *SUP53* $tRNA^{Leu3}$ gene, whose tRNA product is not a substrate for Mod5p modification.

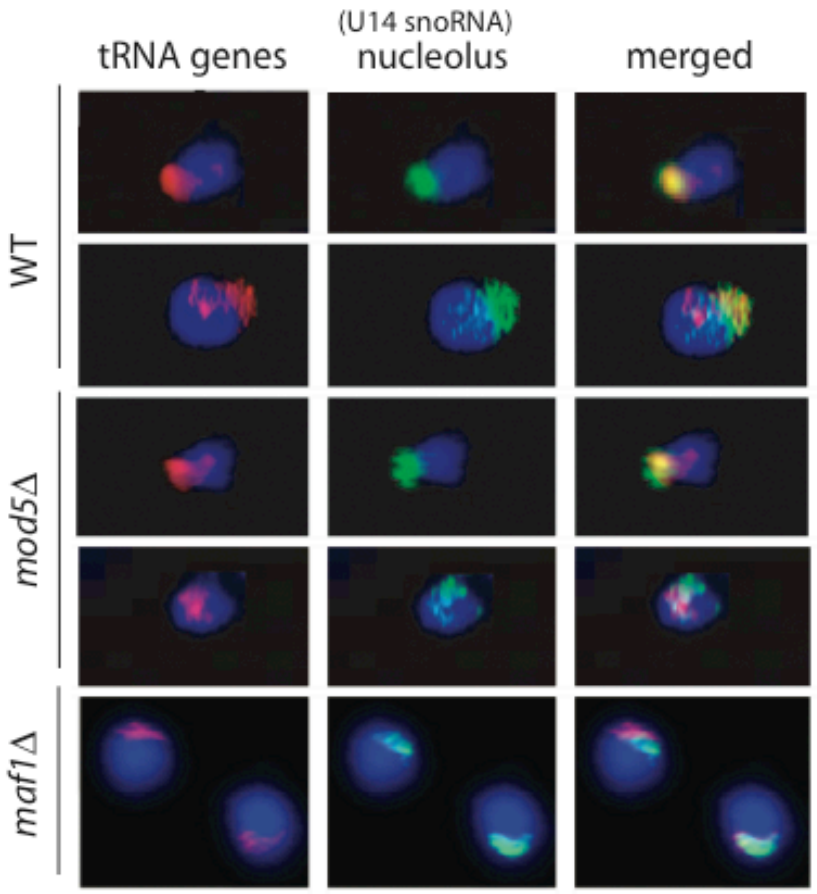


Figure 4.2. *mod5*Δ and *maf1*Δ do not cause mislocalization of tRNA genes. 10 $tRNA^{Leu}$(CAA) genes (red) and the U14 snoRNA nucleolar marker (green) were detected in fixed nuclei by *in situ* hybridization with fluorescent oligonucleotides, and representative cells are shown. Blue represents DAPI staining of nucleoplasmic DNA. The two mutant strains, *mod5*Δ and *maf1*Δ, maintain the tRNA gene localization seen in the wild type (135,12).

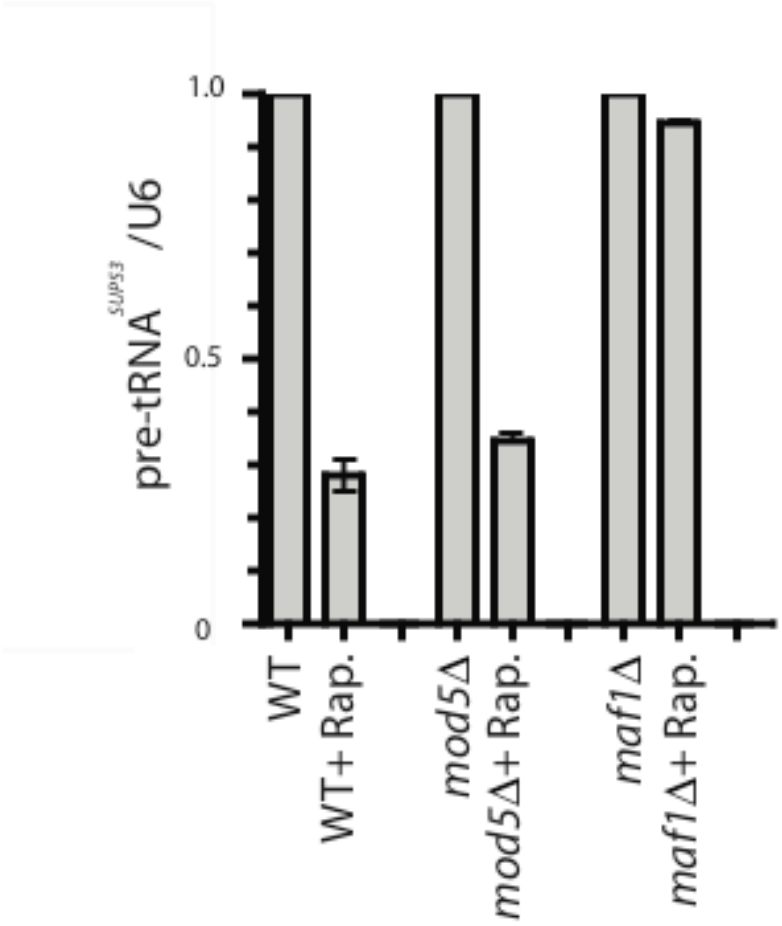


Figure 4.3. *mod5*Δ does not alleviate repression of pol III genes mediated by Maf1p. Three strains (wildtype, mod5Δ, and maf1Δ) were treated with 0.2 μg/ml of rapamycin for 40 minutes. Total RNA was purified from duplicate samples, run on a 12% polyacrylamide gel and analyzed by northern blot for pre-tRNA^{Leu3}, using the stable U6 snRNA pool as an internal normalization control. For each duplicate sample the signal from the precursor tRNA was divided by the U6 signal. This normalized signal in the treated sample is expressed as a ratio to the normalized signal from the untreated sample.

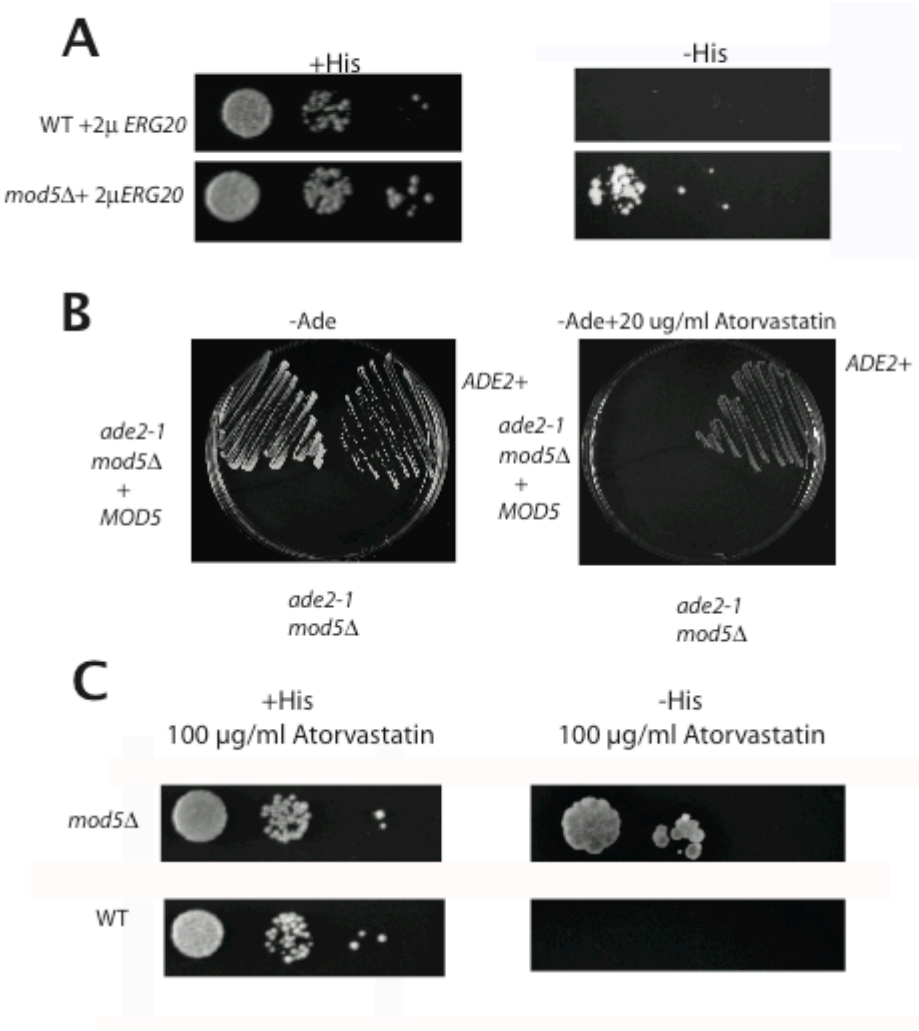


Figure 4.4. DMAPP depletion does not affect tgm silencing. (A) A high-copy plasmid expressing *ERB20* (YepERG20) was inserted into both wildtype and *mod5*Δ strains. Overexpression from this plasmid has been shown to limit Mod5p's ability to modify tRNAs by depleting cellular pools of DMAPP into a competing pathway (33, not shown). Dilutions of these strains were plated in duplicate to selective and non-selective plates to test for tgm silencing. Overexpression of *ERG20* does not alleviate tGM silencing. (B) A second method to deplete DMAPP was to use the drug Atorvastatin, which prevents its formation by inhibiting HMG-CoA reductase. Concentrations of 5, 10, 20, and 40 μg/ml Atorvastatin were used with strain MT-8 (12) to test growth on SDC-ade plates, which is dependent on modification of a $tRNA^{SUP7}$ by Mod5p. At 20 μg/ml of the drug, Mod5p modification is insufficient to allow for growth. (C) Atorvastatin could not alleviate tgm silencing (as in Figure 1) even at 100 μg/ml of the drug. On plates with lacking histidine (-his) the *mod5*Δ allows growth strain but the wildtype strain the function of Mod5 in tgm silencing remains.

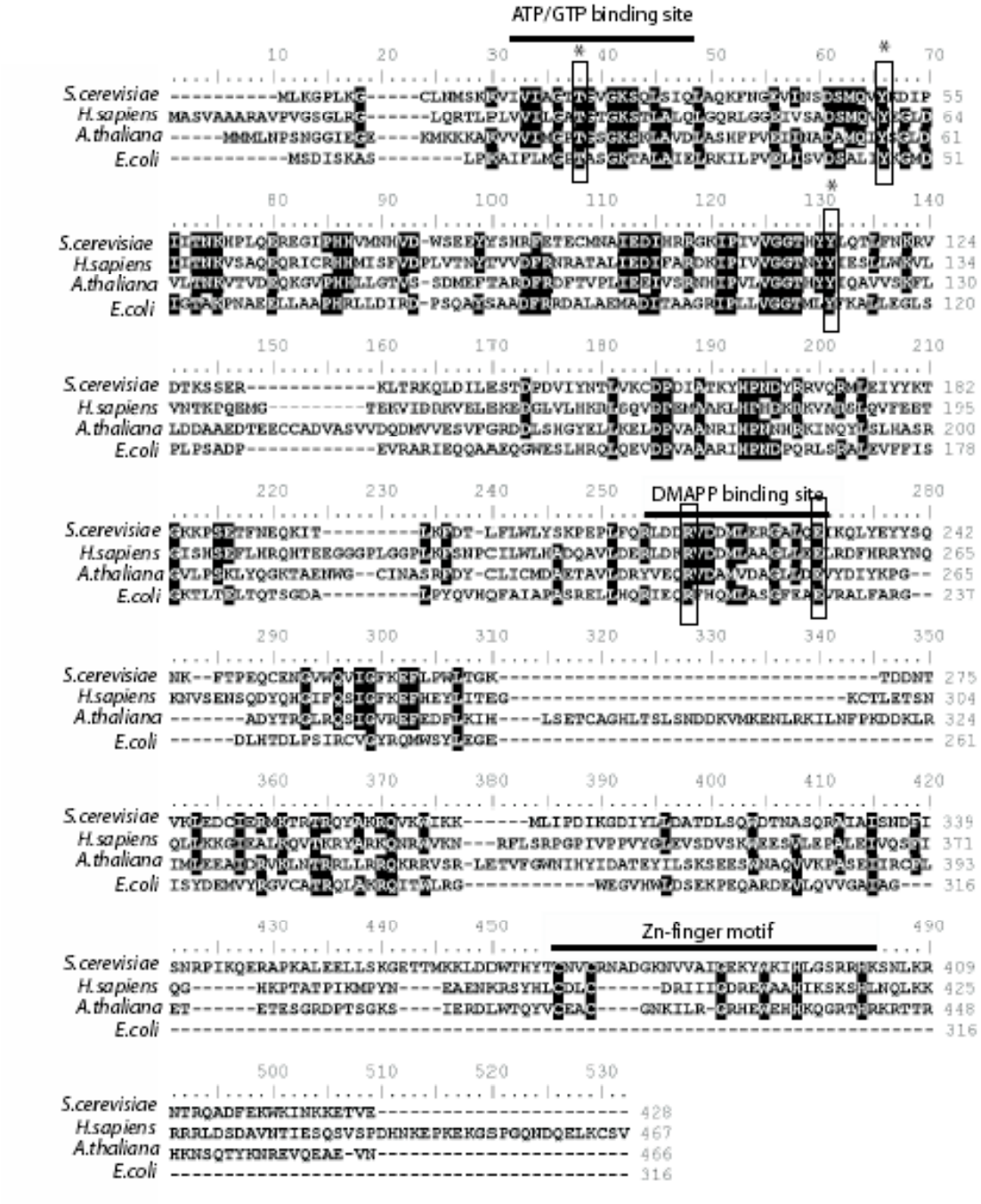


Figure 4.5. Alignment of homologs of Mod5p. The alignment of four Mod5p proteins is depicted with areas of conservation indicated by black boxes. All variants contain motifs for ATP/GTP and DMAPP binding, whereas the eukaryotic enzymes also have a zinc finger in the C-terminal extensions (139). Five different mutants of Mod5p (indicated by boxed residues) were produced by site-directed (asterisks) and random mutagenesis that caused loss of activity.

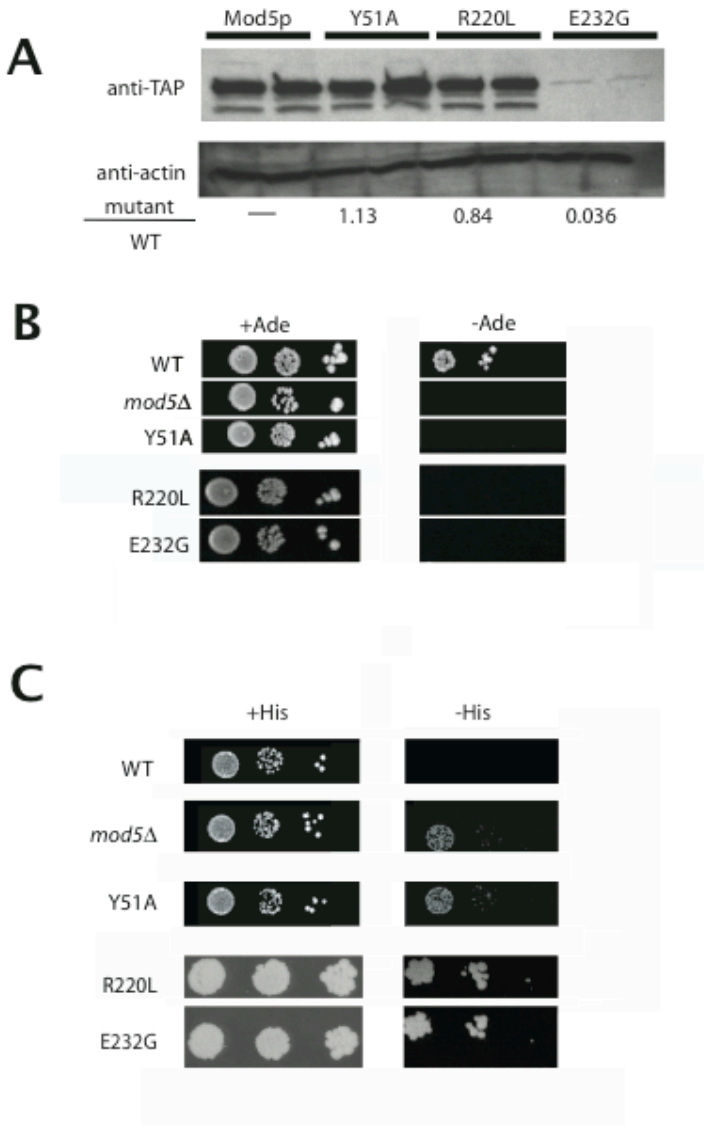


Figure 4.6. Mod5p mutants. (A) *MOD5* wild type and mutants, expressed as functional C-terminal TAP tag fusions, were grown in duplicate to an OD_{600} of 1.5. Protein (20µg) was run out on a 10% SDS-PAGE and analyzed by western blot with primary antibodies to the TAP tags, using subsequent probing with anti-actin as an internal normalization control.. The average TAP/actin ratio in the mutant is expressed as a ratio to the wildtype TAP/actin ratio. (B) Plasmids expressing either a wildtype or the Y51A *mod5* mutant were tested for the ability to restore suppression of an *ade2* ochre mutation through modification of $tRNA^{SUP7}$. (C) Plasmids expressing either the wildtype or the Y51A *mod5* mutant were tested for the ability restore tgm silencing in a *mod5*Δ strain.

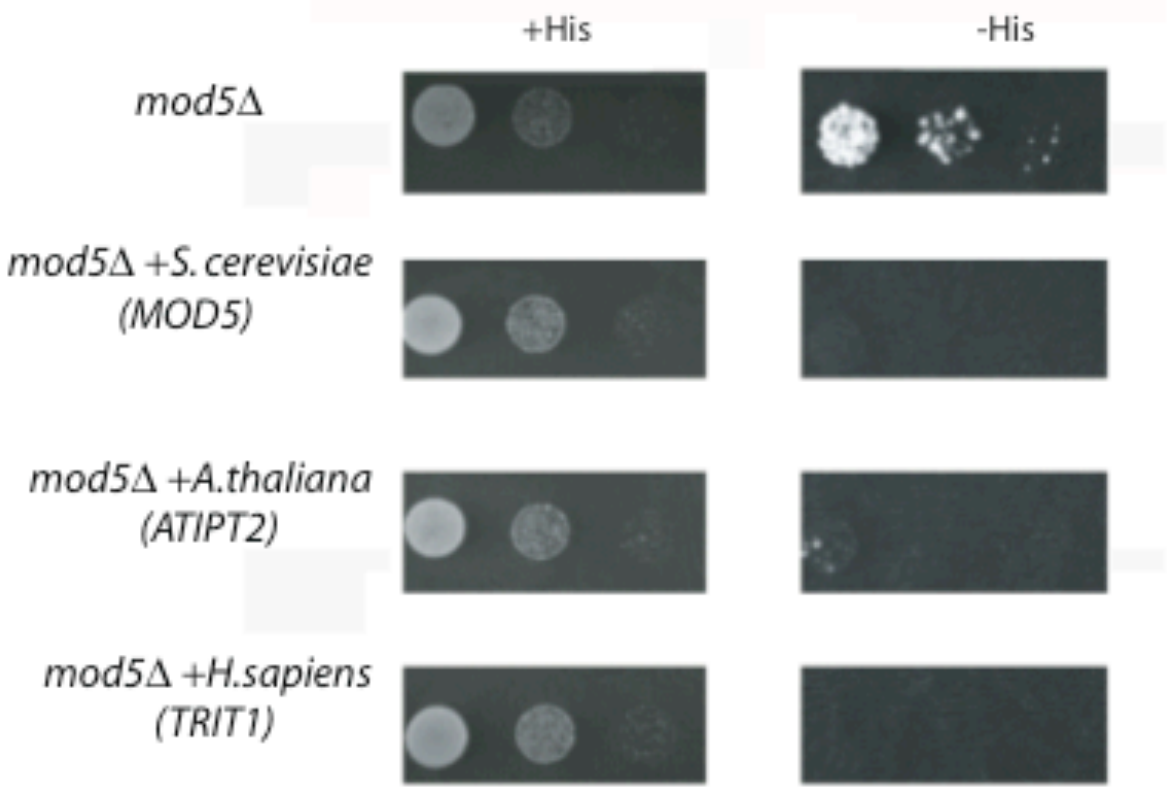


Figure 4.7. Silencing functions of Mod5p are conserved in plant and human. It has been previously shown that human and *Arabidopsis* Mod5p homologs are able to restore i^6A modification of tRNAs in yeast in a *mod5*Δ strain (31,139). We inserted plasmids expressing these ORFs into a mod5Δ strain containing a tgm silencing reporter plasmid. Growth on media lacking histidine (-his) indicates a loss of tgm silencing. While both homologs restore silencing, the *H.sapien* version of Mod5p (TRIT1) is consistently more robust.

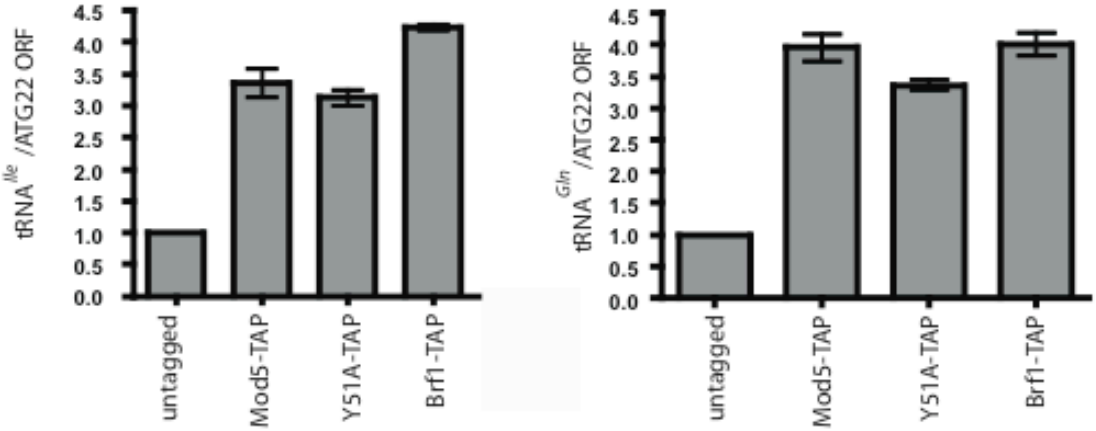


Figure 4.8. Mod5p is physically associated with tRNA genes. Chromatin immunoprecipitations were performed using TAP-tagged versions of Mod5p, the Mod5p mutant Y51A, and a positive control, the Brf1p subunit of the continuously bound TFIIIB transcription factor. PCR detection used primers flanking $tRNA^{Ile}$ and $tRNA^{Gln}$ genes, with the coding region of *ATG22* as an internal negative control because the nearest tRNA genes is >5000 bp away. Triplicate reactions from duplicate experiments are expressed as ratios of linear range PCR signal at the tRNA gene/ATG22 control. Ratios are normalized to parallel controls using an untagged strain.

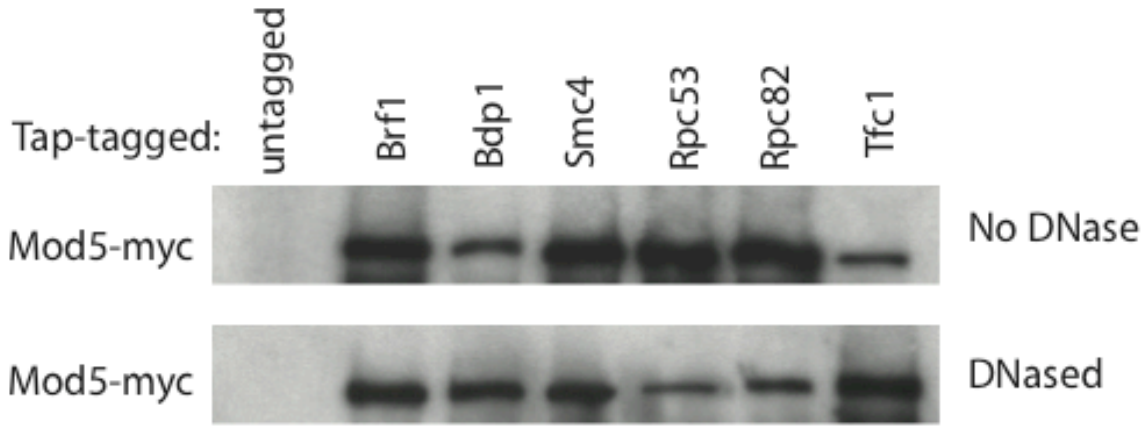


Figure 4.9. Mod5-myc is co-immunoprecipitated with proteins bound at tRNA genes. *MOD5* was chromosomally C-terminally tagged with 13xmyc in strains where the indicated other proteins are TAP-tagged, as well as a strain with no secondary TAP tag. 1 L of each strain was grown to an OD600 of 0.8-1.0 and harvested. Cell lysates of these were divided and half of the samples were treated with excess DNase I in order to eliminate DNA-dependent results. Complexes containing TAP tags were isolated in single affinity steps and western blots were probed for co-isolation of Myc-tagged Mod5p. These results indicate that Mod5-myc is present in affinity pulldowns of TAP-tagged Brf1p, Bdp1, Smc4, Rpc53, Rpc82, and Tfc1, but not when a TAP tag is absent. Although formation of these complexes is likely to be DNA mediated on the tRNA genes, the DNase-insensitivity indicates that they are not due to isolation of large chromatin fragments.

Chapter V

Conclusion

tRNA transcription and recombination

In *Saccharomyces cerevisiae* and most other eukaryotic organisms the tRNA genes are spread throughout the genome (13). The research described in this dissertation gives insight into how these tRNA genes affect DNA stability and transcription in the cell. These influences include increasing recombination, organization, and silencing of nearby RNA polymerase II genes

As depicted in Chapter II of this dissertation the homologous recombination between identical tRNA genes is increased when two identical tRNA genes are transcriptionally active. These results may be very pertinent since Alu elements, which have a pol III promoter, are numerous in the human genome and may lead to deleterious recombination as seen in the BRCA1 gene (41,65).

The mechanisms behind the increase in recombination have yet to be elucidated. There are many different possible explanations for this increase in recombination. One of these explanations involves two active tRNA genes increasing the amount of DNA strand breaks at the site. Previous studies have shown that tRNA gene transcription causes an increase in replication fork pauses as well as genomic instability (18-21). However, the data presented here do not seem to support this argument, since having one active tRNA gene does not cause an incremental increase in the recombination rate over having no active tRNA genes.

A second explanation is that two active tRNA genes are brought in spatial proximity, thus increasing their chance of recombining. There is some evidence in

mammalian cells of two genes being brought into the same vicinity causing an increase in recombination. Previous studies have discovered the existence of RNA polymerase II (pol II) foci, termed transcription factories, where many different genes are being transcribed simultaneously (182-184). A recent study has demonstrated that the proto-oncogene *Myc* and the gene that it translocate with, *Igh*, co-localize when both are being transcribed (185). This may explain why most tumors caused by a juxtaposition of these two genes arise in tissues where both are transcribed (185).

There was no increase in the homologous recombination rate between repetitive elements near tRNA genes. The assay did not detect any difference caused by the presence or absence of an active tRNA gene on deleterious recombination between the repetitive elements.

Future experiments should be done to gain insight into how the transcription of tRNA genes increases their homologous recombination. One possible experiment is to use capturing chromosome conformation (3C) to determine if the tRNA genes are brought into closer proximity by being transcribed (189). Rebecca Haeusler attempted this experiment in our lab, however she was unable to get reliable positive controls to work so her results were inconclusive. Another series of experiments that should be attempted is to insert site-specific HO endonuclease site to create a double stranded break between the two tRNA genes when expressing the HO endonuclease(186). This construct would be useful to answer the question whether tRNA gene transcription increases recombination through the induction of breaks. If strand breakage by the tRNA genes is to blame, the frequency of recombination should be equal in both the strains with active or inactive tRNA strains because the induction of breaks by HO would out compete any breaks caused by the tRNA gene transcription.

tRNA gene nucleolar localization

tRNA clustering may also play a part in the global organization of tRNA genes. Arrangement of many genes could be instituted by the localization of tRNA

genes at the nucleolus and other silenced loci located at the nuclear periphery (13). Understanding the mechanisms that maintain these localizations could be important in understanding the regulation of transcription in the cell.

The data presented here provides insight into understanding these mechanisms. This data illustrate that distinct mechanisms assist in the localizing of the tRNA genes to the nucleolus and in the clustering of tRNA genes. As seen in Chapter III, when nocodazole is used to depolymerize the microtubules the tRNA genes become divorced from the nucleolus. However, the tRNA gene clustering is not abated, leading to the conclusion that microtubules are necessary for bringing the tRNA genes to the nucleolus but not their clustering. The finding that microtubules are important for localization of the tRNA genes is not entirely surprising since microtubules have previously been shown to be involved with chromosomal movement in the nucleus (114) and clustering of centromeres (74). Further studies must be done in order to gain a better understanding of how microtubules assist in this localization. It is unlikely that microtubules are binding directly to the tRNA genes, thus raising the question which protein, or more likely proteins, associates with microtubules in order to give them specificity and what residues of these proteins assist in their interactions with microtubules. Tubulin subunits have been shown in previous studies to be associated with purified RNA polymerase III (95,96), suggesting the polymerase itself might make tight contact. Attempts were made to address these interactions biochemically, but it was found that tubulin is a major contaminant, so the studies were inconclusive. A more thorough examination of whether there are specific associations of the large pol III complexes with the tubulin complexes will be needed to approach the mechanism of nucleolar association.

Since microtubules are necessary for proper of localization of the tRNA genes, but not for the clustering of the tRNA genes, additional studies were done to identify factors necessary for the clustering. The discovery that the protein complex condensin, in *Saccharomyces cerevisiae,* is preferentially bound to tRNA genes by the Uhlmann lab using a ChIP assay (166) led to a better understanding of the mechanism of clustering of tRNA genes. The localization of tRNA genes was visualized in temperature sensitive mutants of condensin subunits and in wildtype

cells. The strains with the mutant condensin subunits exhibited a loss of both the clustering and silencing near the tRNA genes. One hypothesis of how condensin assists with the clustering is through interactions with RNA polymerase III transcription factors. The Uhlmann data indicates that condensin binds to loci previously indicated to be TFIIIC binding sites (166) and along with the finding illustrated here that condensin physically interacts with the pol III transcription factors TFIIIC and TFIIIB, indicates that condensin may use these interactions to cluster the tRNA genes.

Additional studies should be performed to determine which specific transcription factors condensin interacts with. One experiment could use Glutathione-S-Transferase (GST) tagged in vitro translated pol III transcription factors and perform a GST-pulldown assay with tagged condensin subunits (187).

Mod5p is necessary for tgm silencing

Until recently little was known about the mechanisms behind tRNA mediated (tgm) silencing of pol II genes. Deletions of most genes necessary for other type of silencing were tested in an assay for silencing of a *HIS* gene reporter by a neighboring tRNA gene (5). This demonstrated that tgm silencing was maintained by an uncharacterized mechanism. Recent studies have demonstrated that mutations that cause a disruption of tRNA gene localization cause an alleviation of the tgm silencing effect (5). This was further proven by the condensin mutants that cause an alleviation of tgm silencing through disruption of the clustering.

This dissertation examines two gene deletions that cause an alleviation of the tgm silencing phenomenon without a gross redistribution of the tRNA genes. This finding suggests that tRNA gene clustering is required for the silencing effect, but is not sufficient. Chapter IV describes the characterization of one of these two genes, Mod5p. Mod5p is a conserved protein from *E.coli* to humans (142-143) and possesses a previously characterized activity involving the isopentenylation of the exocyclic amine of position 37 of a subset of tRNAs (28,30,31,140,141). Through mutations of the *MOD5* gene it was shown that Mod5p's catalytic activity is

necessary for tgm silencing. It was also demonstrated through chromatin immunoprecipitation assays that Mod5p is located at tRNA genes, even those that do not produce a substrate for Mod5p. Finally Mod5p was shown to physically interact with tRNA gene transcription machinery, namely TFIIIC, TFIIIC, and pol III.

These results seem to indicate that Mod5p has a secondary activity separate from modification of tRNA genes. Other recent discoveries of tRNA modifying enzymes possessing secondary functions have recently been published. The protein Nam2p, a tRNA synthetase, is involved with tRNA aminoacylation and also participates in RNA splicing of group I introns (145). The tRNA modification enzyme Pus1p has been shown to be a co-activator for mRARgamma-mediated transcription (164), and the protein ELP3 modifies the wobble position of a subset of tRNAs, but using a second substrate to acetyltransferase the histone subunits H3 and H4 (147,162).

There is also evidence that the human version of Mod5p (TRIT1) may be involved in silencing. Certain human tumors were found to display downregulation or have an alternatively spliced version of TRIT1 mRNA. Using cell lines possessing low levels of TRIT1 the study demonstrated reduced tumor development by transfection of the gene (156). These results indicate that Mod5p plays a role in silencing and may be modifying a target in *cis* at the tRNA loci.

There are many different experiments that need to be performed to better understand Mod5p's role in tgm silencing. The first of these is to attempt to identify a target. Previous attempts to identify a target for Mod5p have been inconclusive. Two separate attempts that involved using purified Mod5p in an *in vitro* reaction with suspected targets were performed. Small molecules such as adenosine mono-,di, and tri-phosphate versions were attempted to be modified by Mod5. The purified protein and target were incubated with and without the know Mod5p substrate DMAPP (see appendices for additional details). In a substrate independent manner the purified Mod5p was able to modify adenosine monophosphate and diphosphate, but not tri-phosphate. A mass spectrum was taken of deoxyadenosine monophosphate incubated with the purified Mod5p. The spectrum indicated a 72 Dalton mass shift from 330 Daltons to 402 Daltons. It was undetermined if this modification was performed by

Mod5p or a protein that co-purified with Mod5p. Recombinant histones were also used as potential substrates for Mod5p. Recombinant histones were incubated with purified Mod5p with and without DMAPP. No modifications were seen by mass spectrometry of the histones. Since Mod5p may require prior modifications to the histones, or DMAPP may not be the appropriate substrate these results were viewed as inconclusive.

Four methods that may help to find targets of Mod5p are yeast two hybrid analysis, GST-pulldown assays, RNA microarray, and analysis of modification to histones. Yeast two hybrids is a useful tool for the identification of potential protein interactions. The downside of this experiment is that it may provide false positive results. GST-pulldown assay are a useful tool to determine if certain proteins interact. These experiments could potentially give less background than the TAP pulldowns described in Chapter IV.

The downsides of this approach is that the proteins may not be expressible in yeast, it takes time to set up, and candidate proteins need to be known in advance. There are also many different subunits to test. Proteins that should be tested for an interaction with Mod5 should be the pol II subunits, pol III subunits, TFIIIB subunits, TFIIIC subunits and histones. The third method that could be used to identify Mod5p is RNA microarray. Since, Mod5p's previously characterized activity is the modification of tRNAs it is conceivable that Mod5p may modify other RNAs in *cis* in order to maintain the silencing. Previous studies identified RNA substrates through protein-RNA interactions detected through the use of microarray analysis (188). One final method may be to compare modifications of histones on a genomic scale in plus or minus Mod5p strains. One disadvantage of this type of experiment is that the modifications may be to a small subset of histones and would be hard to detect. A possible, but labor intensive variant of this approach might be to immunoprecipitate crosslinked chromatin bearing tRNA transcription complex regions, and subject all of the proteins to tandem mass spec analysis in wild type of *mod5*Δ strains. There are a large number of caveats to this approach, but done on sufficient scale it might be able to identify mass shifts in specific proteins, that can then be approach in a more directed fashion.

Appendix

Appendix

Modification of small molecules using purified Mod5p

Rationale

The work in Chapter V indicates that Mod5p may be maintaining tgm silencing at tRNA genes through a modification of a target compound in *cis*. Plant isopentenyltransferases have been shown to modify molecules containing either adenine or adenosine for the formation of cytokinins (157). Cytokinins are important for the regulation of many different plant processes such as cell differentiation and division (190,191).

The isopentenyltransferase from *Arabidopsis, AtIPT2*, has been shown to compensate for Mod5p activities in tRNA modification (143) and tgm silencing. This leads to the possibility that Mod5p may also modify small molecules containing adenine or adenosine. The experiments detailed in this appendix were undergone to test if Mod5p could also modify small molecules similar to its *Arabidopsis* homolog.

Materials and Methods

Purification of Mod5-TAP

Nine liters of cells that possessed a genomic Mod5-TAP were grown in YPD. These cells were disrupted by use of a microfluidizer cell disruption system (Divtech equipment) using cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.9, 0.5% NP-40) and complete inhibitors (Roche). 150 ml of extract was then bound to 2 ml of IgG Sepharose (GE Healthcare) at 4ºC. After washing 5 times with 50 ml of lysis buffer (minus complete inhibitors) beads were incubated with 10 ml of TEV elution buffer (50 mM Tris-HCl pH 7.9, 0.5 mM EDTA, 1 mM DTT) with 200 μg TEV protease and incubated overnight at 4ºC.

After incubation supernatant was collected and saved. Beads were then subjected to an additional 10 ml of TEV buffer. The supernatant was then added to

the previous 10 ml. NaCl was added to the sample to a final concentration of 50 mM. The sample was then added to 4 ml of DE-52 resin and incubated for 15 minutes at 4ºC. The sample was washed two times with 40 ml of 150-Wash buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.9, 0.5% NP-40). Samples were eluted with 400-Elution buffer (400 NaCl, 50 mM Tris-Cl pH 7.9, 0.5% NP-40) and collected in 2 ml aliquots. Westerns using the TAP antibody (open biosystems) were performed to determine which aliquots possessed the Mod5p. Four elution aliquots were pooled for a total of 8 ml. 10 ml of calmodulin binding buffer minus NaCl (25 mM pH 7.9, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP40) was added to the sample. This sample was then added to 700 μl of calmodulin beads (GE Healthcare) and incubated for 3 hr at 4ºC. Beads were then washed 4x15 ml with calmodulin binding buffer (150 mM NaCl). Samples were serial eluted with 1.4 ml elutions with calmodulin elution buffer (10 mM β–mercaptoethanol, 10 mM Tris-Cl pH 7.9, 150 NaCl, 1 mM Mg-acetate, 1 mM imidazole, 20 mM EGTA, 0.1% NP40). Elutions were then tested by western analysis for the presence of Mod5-TAP. Three elutions were pooled together for a total of 4.5 ml of protein sample. (Figure 6.1)

In vitro reaction

In vitro buffer contained 10 mM of possible target molecule (adenine (Sigma), adenosine (Sigma) deoxyadenosine monophosphate (dAMP) (Sigma), adenosine diphosphate (ADP) (Sigma), adenosine triphosphate (ATP) (Pharmacia), or cytosine monophosphate (CMP) (Sigma)), 20 mM MgCl, and 40 mM Tris-HCl pH 7.9. Variables included H_20, 5 μl Mod5 sample, 10 mM dimethylallyl pyrophosphate (DMAPP), or 10 mM isopentenyl pyrophosphate (IPP). *In vitro* reactions were incubated at 37ºC for 2 hr in 50 μl reactions.

Thin layer chromatography

Reactions were spotted (5x1μl) on thin-layer chromatography cellulose with fluorescent indicator sheets (Kodak). These sheets were developed in a developing

chamber with eluent (0.05 N HCl). Sheets were visualized by 254 nm short wave UV lamp (Ultra Violet Products).

Results

Purified Modp5 was incubated with small molecules containing either adenine adenosine to examine whether Mod5p could modify them. The small molecules tested were: adenine, adenosine, dAMP, AMP, ADP, ATP, and CMP. Purified Mod5p (5 μl) was incubated in 50 μl reactions for 2 hours at 37 ^{o}C. These reactions were then spotted (5 μl) on cellulose TLC plates and developed with 0.05 N HCl. Upon treatment with Mod5p the spots from the small molecules dAMP, AMP, and ADP exhibited a single shift upward (greater migration) on TLC plates (Figure 6.2). The small molecules adenine, adenosine, ATP, and CMP displayed no shift when treated with Mod5p compared to controls. The purified Mod5p also did not modify single or double stranded DNA or RNA molecules. Recombinant Mod5p and MiaA from the Dr. Huang lab, University of Illinois-Champaign, were unable to modify any of the small molecules.

The purified Mod5p sample seemed to completely convert the small molecules to product since the spot was completed shifted to the new position. The catalytic activity of the Mod5p sample seemed to be necessary because if the sample was incubated at 95 ^{o}C for 5 minutes prior to addition to the sample the shift was ablated.

Mod5p's uses the substrate DMAPP to modify the adenosine at position 37 of three tRNAs. The shifts caused by the purified Mod5p were independent on the presence of additional DMAPP. This seemed to indicate that Mod5p was using a different substrate from the buffer to modify the small molecules.

Several experiments were attempted to uncover what product was formed when dAMP was incubated with the purified Mod5p. The modified product was proved not to be hypoxanthine since then did not run the same distance on the TLC plate. The substrate utilized was not coming from Tris or another dAMP molecule. This was deduced by replacing Tris with Hepes in the reaction and the fact that there was only one spot in the product. If Mod5p was adding the phosphate from one

dAMP to another there would have been two spots. However, only one spot was observed in any of the products. The product of the dAMP/Mod5p reaction a portion was analyzed by electrospray mass spectrometry by Dr. James Windak at the University of Michigan department of Chemistry Mass Spectrometry Core. It was determined that the new product (Figure 6.3) was 402 Daltons, which is 71 Daltons larger than dAMP.

Discussion

It is currently unclear what the products were produced in the reactions. If 71 Daltons were being added to dAMP the most likely modification would be two chloride molecules. It is also uncertain if Mod5p is performing the activity since recombinant Mod5p was unable to modify dAMP. It is possible that a protein co-purified with Mod5p is responsible for the activity.

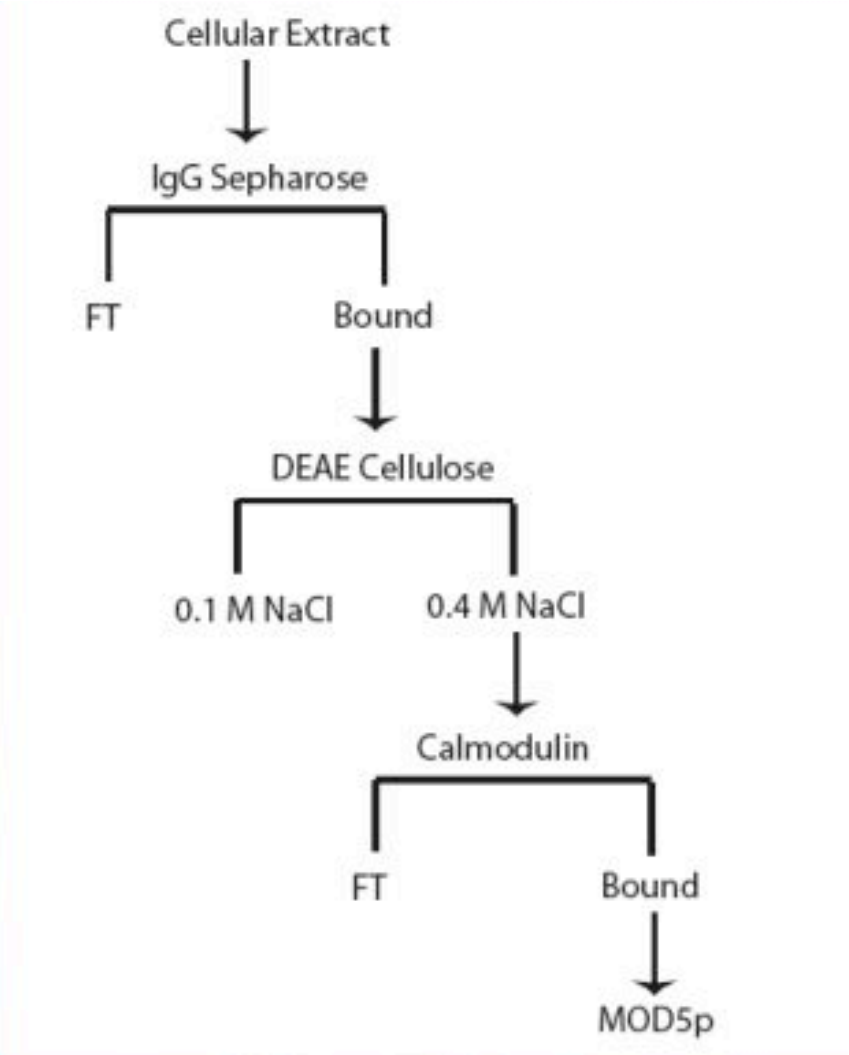


Figure 6.1 Purification of Mod5p. Nine liters of cells expressing the MOD5-TAP protein were grown. Extract was first bound to IgG Sepharose, washed, and then eluted with TEV protease. TEV elution was bound to DEAE cellulose and eluted with 400 mM NaCl. CaCl was added to this elution and then bound to Calmodulin. The protein was then eluted by EGTA.

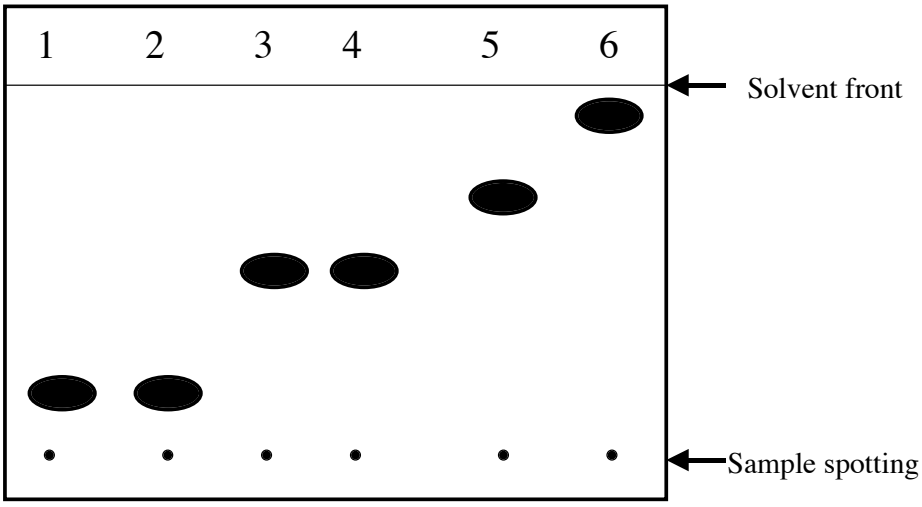


Figure 6.2 The ability of purified Mod5p to modify certain small molecules was tested in an *in vitro* reaction. These reactions were then spotted on cellulose TLC plates and developed in 0.5 N HCl. The small molecules indicated in this diagram are adenosine (lanes 1 and 2), adenine (lanes 3 and 4) and dAMP (lanes 5 and 6). Purified Mod5p was added to *in vitro* reactions spotted on lanes 2, 4, and 6. As observed in lane 6 the purified Mod5p modified dAMP causing the product to run faster than the unmodified sample. AMP was converted approximately as well as dAMP.

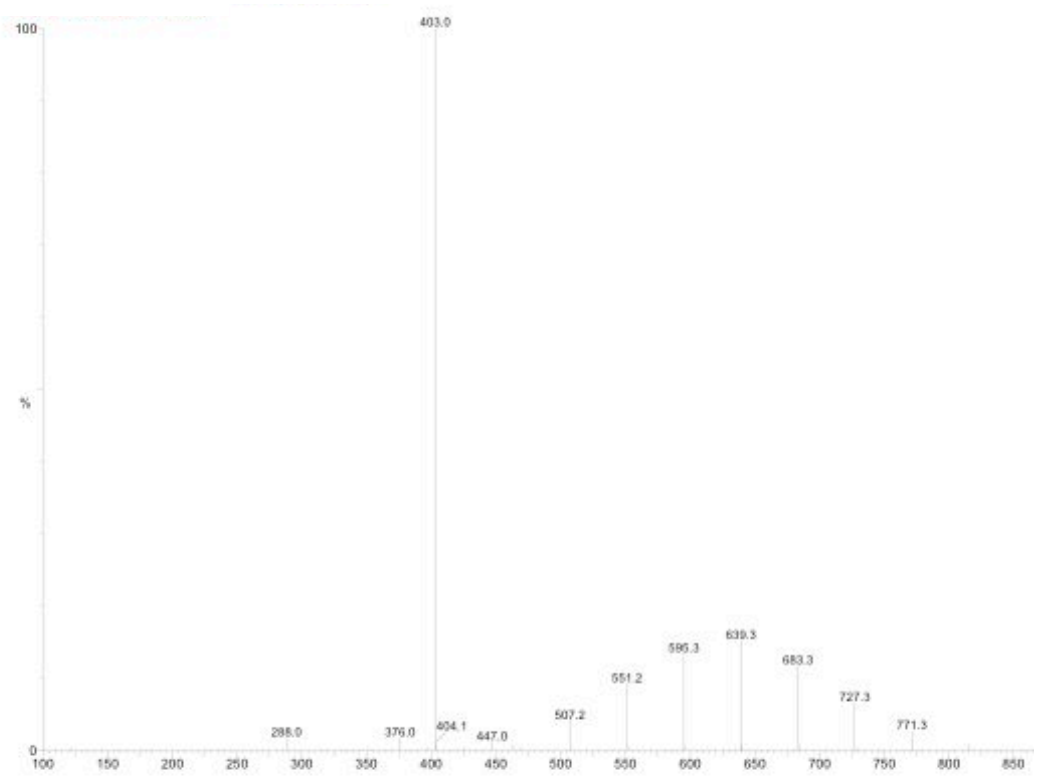


Figure 6.3 Mass Spectrum of modified dAMP by Mod5p purified protein. Purified Mod5p was used in an *in vitro* reaction that modified dAMP. The median value for this molecular weight is 402, which is 71 Daltons more than unmodified dAMP.

Bibliography

1. White R. J.(1998) *RNA Polymerase III transcription*. R.G. Landes, Austin.
2. Donze, D. and Kamakaka, R.T. (2001) RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in Saccharomyces cerevisiae. *EMBO J.*, **20**, 520-531.
3. Bertrand E., Houser-Scott, F., Kendall, A., Singer, R. H. and Engelke D.R. (1998) Nucleolar localization of early tRNA processing. *Genes Dev.*, **12**, 2463-2468.
4. Tolerico, L.H., Benko, A.L., Aris, J.P., Stanford, D.R., Martin, N.C. and Hopper, A.K. (1999) Saccharomyces cerevisiae Mod5p-II contains sequences antagonistic for nuclear and cytosolic locations. *Genetics*, **151**, 57-75.
5. Wang, L, Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S. and Engelke, D.R. (2005) Silencing near tRNA genes requires nucleolar localization. *J. Biol. Chem.*, **280**, 8637-8639.
6. Hull, M.W., Erickson, J., Johnston, M. and Engelke D.R. (1994) tRNA genes as transcriptional repressor elements. *Mol. Cell. Biol.*, **14**, 1266-1277.
7. Thompson, M., Haeusler, R.A., Good, P.D. and Engelke, D.R. (2003) Nucleolar clustering of dispersed tRNA genes. *Science*, **302**, 1399-1401.
8. Zou, S., Ke, N., Kim, J.M. and Voytas, D.F. (1996) The Saccaromyces retrotransposon Ty5 integrates preferentially into regions of silent chromatin at the telomeres and mating loci. *Genes Dev.*, **10**, 634-645.
9. Kinsey, P.T. and Sandmeyer, S.B. (1991) Adjacent pol II and pol III promoters: transcription of the yeast retrotransposon Ty3 and a target tRNA gene. *Nucleic Acids Res*. **19**, 1317-24.
10. Bolton, E. C., and Boeke, J.D. (2003) Transcriptional interactions between yeast tRNA genes, flanking genes and Ty elements: a genomic point of view. *Genome Res*. **13**, 254-263.
11. Bilanchone, V.W., Claypool, J. A., Kinsey, P. T., and Sandmeyer S.B. (1993) Positive and negative regulatory elements control expression of the yeast retrotransposon Ty3. *Genetics*. **136**, 685-700.
12. Kendall, A., Hull, M.W., Bertrand, E., Singer, P.D., and Engelke, D.R. (2000) A CBF5 mutation that disrupts nucleolar localization of early tRNA biosynthesis in yeast also suppresses tRNA gene-mediated transcriptional silencing. *Proc. Natl. Acad. Sci*. **97**, 13108-13113.
13. Haeusler, R.A. and Engelke D.R. (2006) Spatial organization of transcription by RNA polymerase III. *Nucleic Acids Res*. **34**, 4826-4836.
14. Goffeau A., Barrell, B.G., Bussey H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philppsen, P., Tettelin, H., Oliver, SG. (1996) Life with 6000 genes. *Science*. **274**, 563-567.
15. Hani, J. and Feldmann, H. (1998) tRNA genes and retroelements in the yeast genome. *Nucleic Acids Res*. 26, 689-696
16. Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., De Montigny, J., Marck, C., Neuveglise, C., Talla, E. Goffard, N., Frangeul, L., Aigle, M., Anthouard, V., Babour, A., Barbe, V., Barnay, S., Blanchin, S., Beckerich, J.M., Beyne, E., Bleykasten, C., Boisrame, A., Boyer, J., Cattolico,

L., Confanioleri, F. De Daruvar, A., Despons, L., Fabre, E., Fairhead, C., Ferry-Dumazet, H., Groppi, A., Hantraye, F., Hennequin, C., Jauniaux, N., Joyet, P., Kachouri, R., Kerrest, A., Koszul, R., Lemaire, M., Lesur, I., Ma, L., Muller, H., Nicaud, J.M., Nikolski, M., Oztas, S., Ozier-Kalogeropoulos, O., Pellenz, S. Potier, S., Richard, G.F., Straub, M.L., Suleau, A., Swennen, D., Tekaia, F., Wesolowski-Louvel, M., Westhof, E., Wirth, B., Zeniou-Meyer, M., Zivanovic, I., Bolotin-Fukuhara, M., Thierry, A., Bouchier, C., Caudron, B., Scarpelli, C., Gaillardin, C., Weissenbach, J., Wincker, P., and Souciet, J.L. (2004) Genome evolution in yeast. *Nature*. **430**, 35-44.

17. Admire A., Shanks L., Danzl, N., Wang M., Weier, U., Stevens, W., Hunt, E. and Weinert T. (2006) Cycles of chromosome instability are associated with a fragile site and are increase by defects in DNA replication and checkpoint controls in yeast. *Genes Dev.* **20**, 159-173.
18. Deshpande, A.M., and Newlon, C.S. (1996) DNA replication fork pause sites dependent on transcription, *Science,* **272**, 1030-1033.
19. Liu, B., Wong, M.L, Tinker, R.L., Geiduschek, E.P., and Alberts B.M. (1993) The DNA replication fork can pass RNA polymerase without displacing the nascent transcript. *Nature,* **366**, 33-39.
20. B. Liu and Alberts B.M. (1995) Head on Collision between a DNA replication apparatus and RNA polymerase transcription complex. *Science,* **267**, 1131-1137.
21. Dunham M.J., Badrane H., Ferea, T., Adams, J., Brown, P.O., Rosenzweig, F., and Botstein, D. (2002) Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *PNAS*, **99**, 16144-16149.
22. Thrower, D.A., Stemple, J., Yeh, E., and Bloom, K. (2003) Nuclear oscillations and nuclear filament formation accompany single-strand annealing repair of a dicentric chromosome in *Saccharomyces cerevisiae*. *J Cell Sci*, **116**: 561-569.
23. Sirlin, J.L. Jacob, J., and Tandler, C.J. (1963) Transfer of the Methyl Group of Methionine to Nucleolar Ribonucleic Acid. *Biochem J*, **89**, 447-452.
24. Sirlin, J.L. Jacob, J. and Birnstiel, M.L. (1966) Synthesis of transfer RNA in the nucleolus of Smittia. *Natl Cancer Inst Monogr*, **23**, 255-270.
25. Hopper A.K. and Phizicky E.M. (2003) tRNA transfer to the limelight. *Genes Dev*. **17**,162-180.
26. Thompson, M., Haeusler, R.A., Good, P.D. and Engelke, D.R. (2003) Nucleolar clustering of dispersed tRNA genes. *Science*, **302**, 1399-1401.
27. Chalker, D.L. and Sandmeyer, S.B. (1992) Ty3 integrates within the region of RNA polymerase III transcription initiation. *Genes Dev*. **6**,117-128.
28. Laten, H., Gorman, J., and Bock, R.M. (1978) Isopentenyladenosine deficient tRNA from an antisuppressor mutant of Saccharomycs cerevisiae. *Nucleic Acids Res*. **5**,4329-4342.
29. Dirheimer, G., Keith G., Sibler, A.P. and Martin, R.P. (1979) *The primary structure of tRNAs and their rare nucleosides*. P 19-41. In P.R. Schimmel D. Soll, and J.N. Abelson (ed) Transfer RNA: structure, properties and recognition. Cold Spring Harbor laborartory, Cold Spring Harbor, N.Y.

30. McCloskey, J.A., and Nishimura, S. (1977) Modified nucleosides in transfer RNA. *Acc. Chem. Res*. **10**, 403-410.
31. Dihanich, M.E., Najarian, D., Clark, R., Gillman, E.C., Martin, N.C., and Hopper, A.K. (1987) Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae*. *Mol. Cell. Biol*. **7**, 177-184.
32. Boguta M., Hunter, L.A., Shen, W.C., Gillman, E.C., Martin, N.C., and Hopper, A.K. (1994) Subcellular locations of MOD5 proteins: mapping of sequences suffienct for targeting to mitochondria and demonstration that mitochondrial and nuclear isoforms commingle in the cytosol. *Mol Cell. Biol*. **14**,2298-2306.
33. Murawski, M., Szczesniak, B., Zoladek, T., Hopper, A.K., Martin, N.C., and Boguta, M. (1994) *maf1* mutation alters the subcellular localization of the Mod5 protein in yeast. *Acta Biochem Pol*. **41**, 441-448.
34. Boguta, M., K. Czerska, T. Zoladek. 1997. Mutation in a new gene Maf1 affects tRNA suppressor efficiency in *Saccharomyces cerevisiae*. *Gene* **185**,291-296.
35. Tolerico, L.H., Benko, A.L., Aris, J.P., Stanford, D.R., Martin, N.C., and Hopper, A.K. (1999) *Saccharomyces cerevisiae* Mod5p-II Contains Sequences Antagonistic for Nuclear and Cytosolic Locations. *Genetics*. **151**, 57-75.
36. Aguilera, A. (2002) The connection between transcription and genomic instability. *EMBO J*. **21**, 195-201.
37. Allison, D.S., Goh, S.H., and Hall, B.D. (1983) The promoter sequence of a yeast tRNA tyr gene. *Cell*. **34**, 655-664.
38. Elder, J.T., Pan, J., Duncan, C.H., and Weissman, S.M. (1981) Transcriptional analysis of interspersed repetitive polymerase III transcription units in human DNA. *Nucleic Acids Res*. **9**, 1171-1189.
39. Fishman-Lobell, J., Rudin, N., and Haber, J.E. (1992) Two alternative pathways of double-strand break repair that are kinetically separable and independently modulated. *Mol. Cell. Biol*. **12**, 1291-1303.
40. Fornace JR, A.J. and Mitchell, J.B. (1986) Induction of B2 RNA polymerase III transcription by heat shock: enrichment for heat shock induced sequences in rodent cells by hybridization subtraction. *Nucleic Acids Res***. 14**, 5793-5811.
41. Gilbert, N. and Labuda, D. (2000) Evolutionary inventions and continuity of CORE-SINEs in mammals. *J Mol Biol*. **5**, 365-377.
42. Haber, J.E. and Leung, W.Y. (1996) Lack of chromosome territoriality in yeast: promiscuous rejoining of broken chromosome ends. *Proc. Natl. Acad. Sci*. **93**, 13949-13954.
43. Huibregtse, J.M. and Engelke, D.R. (1989) Genomic footprinting of a yeast tRNA gene reveals stable complexes over the 5'-flanking region. *Mol. Cell. Biol*. **9**, 3244-3252.
44. Ivanov, E.L., Sugawara, N., Fishman-Lobell, J., and Haber, J.E. (1996) Genetic requirements for the single-strand annealing pathway of double-strand break repair in Saccharomyces cerevisiae. *Genetics*. **142**, 693-704.
45. Jackson, J.A. and Fink, G.R. (1981) Gene conversion between duplicated genetic elements in yeast, *Nature* **292**, 306-311.

46. Jurka, J. (2004) Evolutionary impact of human Alu repetitive elements. *Curr. Opin. Genet. Dev*. **14**, 603-608.
47. Koski, R.A. and Clarkson, S.G. (1982) Synthesis and maturation of Xenopus laevis methionine tRNA transcripts in homologous cell-free extracts. *J Biol. Chem*. **257**, 4514-4521.
48. Liu, W.M. and Schmid, C.W. (1993) Proposed roles for DNA methylation in Alu transcriptional repression and mutational inactivation. *Nucleic Acids Res*. **21**, 1351-1359.
49. Liu, W.M., Chu, W.M., Choudary, P.V., and Schmid, C.W. (1995) Cell stress and translational inhibitors transiently increase the abundance of mammalian SINE transcripts. *Nucleic Acids Res*. **23**, 1758-1765.
50. Longtine, M.S., McKenzie III, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, P., Philippsen, A., and Pringle, J.R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast*. **14**, 953-961.
51. Lukasova, E., Kozubek, S., Kozubek, M., Kjeronsak, J., Ryznar, L., Horakova, J., Krahulcova, E., and Horneck, G. (1997) Localization and distance between ABL and BCR genes in interphase nuclei of bone marrow cells of control donors and patients with chronic myeloid leukemia. *Hum. Genet*. **100**, 525-535.
52. McDonald, J.P. and Rothstein, R. (1994) Unrepaired heteroduplex DNA in Saccharomyces cerevisiae is decreased in RAD1, RAD52-independent recombination. *Genetics*. **137**, 393-405.
53. Munz, P., Amstutz, H., Kohli, J., and Leupold, U. (1982) Recombination between dispersed serine tRNA genes in Schizosaccharomyces pombe. *Nature*. **300**, 225-231.
54. Neves, H., Ramos, C., da Siva, M.G., Parreira, A., and Parreira L. (1999) The nuclear topography of AMB, BCR, PML, and RAR genes: evidence for gene proximity in specific phases of the cell cycle and stages of hematopoietic differentiation. *Blood*. **93**, 1197-1207.
55. Newman, A. J., Ogden, R.C., and Abelson, J. (1983) tRNA gene transcription in yeast: effects of specified base substitutions in the intragenic promoter. *Cell*, **35**, 117-125.
56. Ozenberger, B.A. and Roeder, G.S. (1991) A unique pathway of double-strand break repair operates in tandemly repeated genes, *Mol. Cell. Biol*. **11**, 1222-1231.
57. Pavlicek, A., Noskov, V.N., Kouprina, N., Barrett, J.C., Jurka, J., and Larionov, V. (2004) Evolution of the tumor suppressor BRCA1 locus in primates: implication for cancer predisposition. *Hum. Mol. Genet*. **13**, 2727-2751.
58. Prado, F. and Aguilera, A. (1995) Role of reciprocal exchange, one ended invasion crossover and single-strand annealing on inverted and direct repeat recombination in yeast: different requirements for the RAD1, RAD10. *Genetics* **139**, 109-123.
59. Prado, F., Cortes-Ledesma, F., Huertas, P., and Aguilera, A. (2003) Mitotic recombination in Saccharomyces cerevisiae. *Curr. Genet*. **42**, 185-198.

60. Puget, N., Stoppa-Lyonnet, D., Sinilnkova, O.M., Page, S., Lynch, H.T., Lenoir, S., and Mazoyer G.M. (1999) Screening for germ-line rearrangements and regulatory mutations in BRCA1 led to the identification of four new deletions. *Cancer Res*. **59**, 455-46.
61. Rattray, A.J. and Symington, L.S. (1995) Multiple pathways for homologous recombination in Saccharomyces cerevisiae. *Genetics*. **139**, 45-56.
62. Rohlfs, E.M., Puget, N., Graham, M.L., Weber, B.L., Garber, J.E., Skrzynia, C., Halperin, J.L., Lenoir, G. M., Silverman, L. M., and Mazoyer, S. (2000) An Alu-mediated 7.1 kb deletion of BRCA 1 exon 8 and 9 in breast and overian cancer families that results in alternative splicing of exon 10. *Genes Chromosomes Cancer*. **28**, 300-307.
63. Roix, J.J., McQueen, P.G., Munson, P.J., Parada, L.A., and Misteli, T. (2003) Spatial proximity of translocation-prone gene loci in human lymphomas. *Nat. Genet*. **34**, 287-291.
64. Rose, M.D., Winston, F., and Hieter. P. (1990) *Methods in yeast genetics: a laboratory course manual*. (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.)
65. Smith, T.M., Lee, M.K., Szabo, C.I,, Jerome, N., McEuen, M., Taylor, M., Hood, L., and King, M.C. (1996) Complete genomic sequence and analysis of 117 kb of human DNA containing the gene BRCA1. *Genome Res*. **6**, 1029-1049.
66. Tournier, I., Paillerets, B.B., Sobol, H., Stoppa-Lyonnet, D., Lidereau, R., Barrois, S., Mazoyer, M., Coulet, F., Hardouin, A., Chompret, A., Lortholary, A., Chappuis, P., Bourdon, V., Bonadona, V., Maugard, C., Gilbert, B., Nogues, C., Frebourg, T., and Tosi, M. (2004) Significant contribution of germline BRCA2 rearrangements in male breast cancer families. *Cancer Res*. **64**, 8143-8147.
67. Wilson, E.T., Larson, D., Young, L.S., and Sprague, K.U. (1985) A large region controls tRNA transcription. *J Mol Biol*, **183**, 153-163.
68. Bartholomew, B., Kassavetis, G.A., and Geiduschek, E.P. (1991). Two components of Saccharomyces cerevisiae transcription factor IIIB (TFIIIB) are stereospecifically located upstream of a tRNA gene and interact with the second-largest subunit of TFIIIC. *Mol Cell Biol* **11**, 5181-5189.
69. Bartova, E., and Kozubek, S. (2006). Nuclear architecture in the light of gene expression and cell differentiation studies. *Biol Cell* **98**, 323-336.
70. Brickner, J.H., and Walter, P. (2004). Gene recruitment of the activated INO1 locus to the nuclear membrane. *PLoS Biol* **2**, e342.
71. Brown, J.M., Leach, J., Reittie, J.E., Atzberger, A., Lee-Prudhoe, J., Wood, W.G., Higgs, D.R., Iborra, F.J., and Buckle, V.J. (2006). Coregulated human globin genes are frequently in spatial proximity when active. *J Cell Biol* **172**, 177-187.
72. Brown, K.E., Baxter, J., Graf, D., Merkenschlager, M., and Fisher, A.G. (1999). Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Mol Cell* **3**, 207-217.
73. Buchenau, P., Saumweber, H., and Arndt-Jovin, D.J. (1997). The dynamic nuclear redistribution of an hnRNP K-homologous protein during Drosophila

embryo development and heat shock. Flexibility of transcription sites in vivo. *J Cell Biol* **137**, 291-303.
74. Bystricky, K., Heun, P., Gehlen, L., Langowski, J., and Gasser, S.M. (2004). Long-range compaction and flexibility of interphase chromatin in budding yeast analyzed by high-resolution imaging techniques. *Proc Natl Acad Sci U S A* **101**, 16495-16500.
75. Casolari, J.M., Brown, C.R., Drubin, D.A., Rando, O.J., and Silver, P.A. (2005). Developmentally induced changes in transcriptional program alter spatial organization across chromosomes. *Genes Dev* **19**, 1188-1198.
76. Casolari, J.M., Brown, C.R., Komili, S., West, J., Hieronymus, H., and Silver, P.A. (2004). Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. *Cell* **117**, 427-439.
77. Chuang, C.H., Carpenter, A.E., Fuchsova, B., Johnson, T., de Lanerolle, P., and Belmont, A.S. (2006). Long-range directional movement of an interphase chromosome site. *Curr Biol* **16**, 825-831.
78. Cremer, T., Cremer, M., Dietzel, S., Muller, S., Solovei, I., and Fakan, S. (2006). Chromosome territories--a functional nuclear landscape. *Curr Opin Cell Biol* **18**, 307-316.
79. Faro-Trindade, I., and Cook, P.R. (2006). Transcription factories: structures conserved during differentiation and evolution. *Biochem Soc Trans* **34**, 1133-1137.
80. Feuerbach, F., Galy, V., Trelles-Sticken, E., Fromont-Racine, M., Jacquier, A., Gilson, E., Olivo-Marin, J.C., Scherthan, H., and Nehrbass, U. (2002). Nuclear architecture and spatial positioning help establish transcriptional states of telomeres in yeast. *Nat Cell Biol* **4**, 214-221.
81. Foster, H.A., and Bridger, J.M. (2005). The genome and the nucleus: a marriage made by evolution. Genome organisation and nuclear architecture. *Chromosoma* **114**, 212-229.
82. Freeman, L., Aragon-Alcaide, L., and Strunnikov, A. (2000). The condensin complex governs chromosome condensation and mitotic transmission of rDNA. *J Cell Biol* **149**, 811-824.
83. Fuchs, J., and Loidl, J. (2004). Behaviour of nucleolus organizing regions (NORs) and nucleoli during mitotic and meiotic divisions in budding yeast. *Chromosome Res* **12**, 427-438.
84. Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. *Nature* **425**, 737-741.
85. Goodenbour, J.M., and Pan, T. (2006). Diversity of tRNA genes in eukaryotes. *Nucleic Acids Res* **34**, 6137-6146.
86. Guacci, V., Hogan, E., and Koshland, D. (1994). Chromosome condensation and sister chromatid pairing in budding yeast. *J Cell Biol* **125**, 517-530.
87. Hagstrom, K.A., and Meyer, B.J. (2003). Condensin and cohesin: more than chromosome compactor and glue. *Nat Rev Genet* **4**, 520-534.
88. Harismendy, O., Gendrel, C.G., Soularue, P., Gidrol, X., Sentenac, A., Werner, M., and Lefebvre, O. (2003). Genome-wide location of yeast RNA polymerase III transcription machinery. *EMBO J* **22**, 4738-4747.

89. Harnicarova, A., Kozubek, S., Pachernik, J., Krejci, J., and Bartova, E. (2006). Distinct nuclear arrangement of active and inactive c-myc genes in control and differentiated colon carcinoma cells. *Exp Cell Res* **312**, 4019-4035.
90. Hediger, F., Neumann, F.R., Van Houwe, G., Dubrana, K., and Gasser, S.M. (2002). Live imaging of telomeres: yKu and Sir proteins define redundant telomere-anchoring pathways in yeast. *Curr Biol* **12**, 2076-2089.
91. Heun, P., Laroche, T., Raghuraman, M.K., and Gasser, S.M. (2001a). The positioning and dynamics of origins of replication in the budding yeast nucleus. *J Cell Biol* **152**, 385-400.
92. Heun, P., Taddei, A., and Gasser, S.M. (2001b). From snapshots to moving pictures: new perspectives on nuclear organization. *Trends Cell Biol* **11**, 519-525.
93. Hirano, T. (2005). Condensins: organizing and segregating the genome. *Curr Biol* **15**, R265-275.
94. Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutilier, K.*, et al.* (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. *Nature* **415**, 180-183.
95. Hu, P., Wu, S., and Hernandez, N. (2003). A minimal RNA polymerase III transcription system from human cells reveals positive and negative regulatory roles for CK2. *Mol Cell* **12**, 699-709.
96. Hu, P., Wu, S., Sun, Y., Yuan, C.C., Kobayashi, R., Myers, M.P., and Hernandez, N. (2002). Characterization of human RNA polymerase III identifies orthologues for Saccharomyces cerevisiae RNA polymerase III subunits. *Mol Cell Biol* **22**, 8044-8055.
97. Huang, C.E., Milutinovich, M., and Koshland, D. (2005). Rings, bracelet or snaps: fashionable alternatives for Smc complexes. *Philos Trans R Soc Lond B Biol Sci* **360**, 537-542.
98. Hurst, L.D., Pal, C., and Lercher, M.J. (2004). The evolutionary dynamics of eukaryotic gene order. *Nat Rev Genet* **5**, 299-310.
99. Jacobs, C.W., Adams, A.E., Szaniszlo, P.J., and Pringle, J.R. (1988). Functions of microtubules in the Saccharomyces cerevisiae cell cycle. *J Cell Biol* **107**, 1409-1426.
100. Kaplan, F.S., Murray, J., Sylvester, J.E., Gonzalez, I.L., O'Connor, J.P., Doering, J.L., Muenke, M., Emanuel, B.S., and Zasloff, M.A. (1993). The topographic organization of repetitive DNA in the human nucleolus. *Genomics* **15**, 123-132.
101. Kassavetis, G.A., Riggs, D.L., Negri, R., Nguyen, L.H., and Geiduschek, E.P. (1989). Transcription factor IIIB generates extended DNA interactions in RNA polymerase III transcription complexes on tRNA genes. *Mol Cell Biol* **9**, 2551-2566.
102. Kosak, S.T., Skok, J.A., Medina, K.L., Riblet, R., Le Beau, M.M., Fisher, A.G., and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science* **296**, 158-162.

103. Lavoie, B.D., Hogan, E., and Koshland, D. (2002). In vivo dissection of the chromosome condensation machinery: reversibility of condensation distinguishes contributions of condensin and cohesin. *J Cell Biol* **156**, 805-815.
104. Lavoie, B.D., Tuffo, K.M., Oh, S., Koshland, D., and Holm, C. (2000). Mitotic chromosome condensation requires Brn1p, the yeast homologue of Barren. *Mol Biol Cell* **11**, 1293-1304.
105. Legagneux, V., Cubizolles, F., and Watrin, E. (2004). Multiple roles of Condensins: a complex story. *Biol Cell* **96**, 201-213.
106. Liu, J., and Francke, U. (2006). Identification of cis-regulatory elements for MECP2 expression. *Hum Mol Genet* **15**, 1769-1782.
107. Liu, Z., and Garrard, W.T. (2005). Long-range interactions between three transcriptional enhancers, active Vkappa gene promoters, and a 3' boundary sequence spanning 46 kilobases. *Mol Cell Biol* **25**, 3220-3231.
108. Loidl, J. (2003). Chromosomes of the budding yeast Saccharomyces cerevisiae. *Int Rev Cytol* **222**, 141-196.
109. Long, R.M., Elliott, D.J., Stutz, F., Rosbash, M., and Singer, R.H. (1995). Spatial consequences of defective processing of specific yeast mRNAs revealed by fluorescent in situ hybridization. *RNA* **1**, 1071-1078.
110. Losada, A., and Hirano, T. (2005). Dynamic molecular linkers of the genome: the first decade of SMC proteins. *Genes Dev* **19**, 1269-1287.
111. Machin, F., Paschos, K., Jarmuz, A., Torres-Rosell, J., Pade, C., and Aragon, L. (2004). Condensin regulates rDNA silencing by modulating nucleolar Sir2p. *Curr Biol* **14**, 125-130.
112. Machin, F., Torres-Rosell, J., Jarmuz, A., and Aragon, L. (2005). Spindle-independent condensation-mediated segregation of yeast ribosomal DNA in late anaphase. *J Cell Biol* **168**, 209-219.
113. Maillet, L., Boscheron, C., Gotta, M., Marcand, S., Gilson, E., and Gasser, S.M. (1996). Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. *Genes Dev* **10**, 1796-1811.
114. Marshall, W.F., Straight, A., Marko, J.F., Swedlow, J., Dernburg, A., Belmont, A., Murray, A.W., Agard, D.A., and Sedat, J.W. (1997). Interphase chromosomes undergo constrained diffusional motion in living cells. *Curr Biol* **7**, 930-939.
115. Misteli, T. (2004). Spatial positioning; a new dimension in genome function. *Cell* **119**, 153-156.
116. Moir, R.D., Lee, J., Haeusler, R.A., Desai, N., Engelke, D.R., and Willis, I.M. (2006). Protein kinase A regulates RNA polymerase III transcription through the nuclear localization of Maf1. *Proc Natl Acad Sci U S A* **103**, 15044-15049.
117. Nasmyth, K., and Haering, C.H. (2005). The structure and function of SMC and kleisin complexes. *Annu Rev Biochem* **74**, 595-648.
118. Okada, N. (1991). SINEs. *Curr Opin Genet Dev* **1,** 498-504.
119. Parada, L.A., Roix, J.J., and Misteli, T. (2003). An uncertainty principle in chromosome positioning. *Trends Cell Biol* **13**, 393-396.
120. Parada, L.A., Sotiriou, S., and Misteli, T. (2004). Spatial genome organization. *Exp Cell Res* **296**, 64-70.

121. Paule, M.R., and White, R.J. (2000). Survey and summary: transcription by RNA polymerases I and III. *Nucleic Acids Res* **28**, 1283-1298.
122. Pratt-Hyatt, M.J., Kapadia, K.M., Wilson, T.E., and Engelke, D.R. (2006). Increased recombination between active tRNA genes. *DNA Cell Biol* **25**, 359-364.
123. Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., Simon, I., Zeitlinger, J., Schreiber, J., Hannett, N., Kanin, E*., et al.* (2000). Genome-wide location and function of DNA binding proteins. *Science* **290**, 2306-2309.
124. Schmid, M., Arib, G., Laemmli, C., Nishikawa, J., Durussel, T., and Laemmli, U.K. (2006). Nup-PI: the nucleopore-promoter interaction of genes in yeast. *Mol Cell* **21**, 379-391.
125. Shaw, P.J., Highett, M.I., Beven, A.F., and Jordan, E.G. (1995). The nucleolar architecture of polymerase I transcription and processing. *Embo J* **14**, 2896-2906.
126. Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27.
127. Spilianakis, C.G., Lalioti, M.D., Town, T., Lee, G.R., and Flavell, R.A. (2005). Interchromosomal associations between alternatively expressed loci. *Nature* **435**, 637-645.
128. Stray, J.E., Crisona, N.J., Belotserkovskii, B.P., Lindsley, J.E., and Cozzarelli, N.R. (2005). The Saccharomyces cerevisiae Smc2/4 condensin compacts DNA into (+) chiral structures without net supercoiling. *J Biol Chem* **280,** 34723-34734.
129. Su, J.S., Tsai, T.F., Chang, H.M., Chao, K.M., Su, T.S., and Tsai, S.F. (2006). Distant HNF1 site as a master control for the human class I alcohol dehydrogenase gene expression. *J Biol Chem* **281**, 19809-19821.
130. Taddei, A., Hediger, F., Neumann, F.R., and Gasser, S.M. (2004). The function of nuclear architecture: a genetic approach. Annu Rev Genet *38*, 305-345.
131. Thanbichler, M., and Shapiro, L. (2006). Chromosome organization and segregation in bacteria. *J Struct Biol* **156,** 292-303.
132. Volpi, E.V., Chevret, E., Jones, T., Vatcheva, R., Williamson, J., Beck, S., Campbell, R.D., Goldsworthy, M., Powis, S.H., Ragoussis, J*., et al.* (2000). Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. *J Cell Sci* **113** *(Pt 9)*, 1565-1576.
133. Wang, B.D., Eyre, D., Basrai, M., Lichten, M., and Strunnikov, A. (2005). Condensin binding at distinct and specific chromosomal sites in the Saccharomyces cerevisiae genome. *Mol Cell Biol* **25**, 7216-7225.
134. Weiner, A.M. (2002). SINEs and LINEs: the art of biting the hand that feeds you. *Curr Opin Cell Biol* **14**, 343-350.
135. Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980) Mevinolin: a highly potent competitive

inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc. Natl. Acad. Sci. USA*. **77**, 3957–3961.

136. Benko, A.L., Vaduva, G. Martin, N.C. and Hopper, A.K. (2000) Competition between a sterol biosynthetic enzyme and tRNA modification in addition to changes in the protein synthesis machinery causes altered nonsense suppression. *Proc. Natl. Acad. Sci. USA*. **97**, 61-66.
137. Chen, J., Anderson, J.B., Deweese-Scott, C. Fedorova, N.D., Geer, L.Y., He, S., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C., Madej, T., Marchler-Bauer, A., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Rao, B.S., Panchenko, A.R., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y., Yamashita, R.A., Yin, J.J., and Bryant, S.H. (2003). MMDB: Entrez's 3D-structure database. *Nucleic Acids Res*. **31**, 474-477.
138. Desai, N., Lee, J., Upadhya, R., Chu, Y., Moir, R.D., and Willis, I.M. (2005) Two steps in Maf1-dependent repression of transcription by RNA polymerase III. *J. Biol. Chem*. **280**, 6455-6462.
139. Dimster-Denk, D., Rine, J., Phillips, J., Scherer, S., Cundiff, P., Debord, K., Gilliland, D., Hickman, S., Jarvis, A., Tong, L., and Ashby, M. (1999) Comprehensive evaluation of isoprenoid biosynthesis regulation in *Saccharomyces cerevisiae* utilizing the Genome Reporter Matrix. *J. Lipid Res*. **40**, 850-860.
140. Gefter, M.L., and Bikoff, E. (1971) Studies on synthesis and modification of transfer RNA. *Cancer Res*. **31**, 667-70.
141. Gillman, E.C., Slusher, L.B., Martin, N.C., and Hopper, A.K. (1991) *MOD5* Translation Initiation Sites Determine N^6-Isopentenyladenosine Modification of Mitochondrial and Cytoplasmic tRNA. *Mol. Cell. Biol*. **11**, 2382-2390.
142. Golovko, A., Hjalm, G., Sitbon, F., and Nicander, B. (2000) Cloning of a human tRNA isopentenyl transferase. *Gene*. **258**, 85-93.
143. Golovko, A., Sitbon, F., Tillberg, E., and B. Nicander. (2002) Identification of a tRNA isopentenyltransferase gene from *Arabidopsis thaliana. Plant Mol. Biol.* **49**, 161-169.
144. Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids. Symp. Ser*. **41**, 95-98.
145. Herbert, C.J.. Labouesse, M., Dujardin, G., and Slonimski, P.P. (1988) The NAM2 proteins from *S. cerevisiae* and *S.douglasii* are mitochondrial leucyl-tRNA synthetases and are involved in mRNA splicing. *EMBO J*. **7**, 473-483.
146. Holtz, J., and Klambt, D. (1978) tRNA isopentenyltransferase from Zea mays L. Characterization of the isopentenylation reaction of the tRNA, oligo (A) and other nucleic acids. Hoppe Seylers Z. *Physiol Chem*. **359**, 89-101.
147. Huang B., Johansson, M.J.O., and Bystrom, A.S. (2005) An early step in wobble uridine tRNA modification requires the Elongator complex. *RNA*. **11**, 424-436.
148. Kaminska J., Grabinska, K., Kwapisz, M., Sikora, J., Smagowicz, W.J., Palamarczyk, G., Zoladek, T., and Boguta, M. (2002) The Isoprenoid

biosynthetic pathway in *Saccaromyces cerevisiae* is affected in a maf1-1 mutant with altered tRNA synthesis. *FEMS Yeast Res*. **2**, 31-37.

149. Labouesse, M. (1990) The yeast mitochondrial leucyl-tRNA synthetase is a splicing factor for the excision of several group I introns. *Mol Gen Genet*. **224**, 209-221.
150. Laezza, C., Notarnicola, M., Caruso, M.G., Messa, C., Macchia, M., Bertini, S., Minutolo, F., Portella, G., Fiorentino, L. Stingo, S., and Rifulco, M. (2006) N6-isopentenyladenosine arrests tumor cell proliferation by inhibiting farnesyl diphosphate synthase and protein prenylation. *FASEB* J. **20**, 412-418
151. Oficjalska-Pham, D., Harismendy, O., Smagowicz, W.J., Gonzalez de Peredo, A., Boguta, M., Sentenac, A., and Lefebvre, O. (2006) General repression of RNA polymerase III transcription is triggered by protein phsphatase type 2A-mediated dephosphorylation of Maf1. *Mol. Cell*. **22**, 623-632.
152. Pluta, K., Lefebvre, O., Martin, N.C., Smagowicz, W.J., Stanford, D.R., Ellis, S.R., Hopper, A.K., Sentenac, A., and Boguta, M. (2001) Maf1p, a negative effector of RNA polymerase III in *Saccharomyces cerevisiae*. *Mol. Cell. Biol*. **21**, 5031-5040.
153. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods*. **24**, 218-229.
154. Roberts, D.N., Stewart, A.J., Huff, J.T., and Cairns, B.R. (2006) The RNA polymerase III transcriptome revealed by genome-wide localization and activity-occupancy relationships. *Proc. Natl. Acad. Sci. USA*. **100**, 14695-14700.
155. Soderberg, T., and Poulter, C.D. (2001) *Escherichia Coli* Dimethylallyl Diphosphate: tRNA Dismethyltransferase: Site-Directed Mutagenesis of Highly Conserved Residues. *Biochemistry*. **40**, 1734-1740.
156. Spinola M., Galvan, A., Pignatiello, C., Conti, B., Pastorino, U., Nicander, B., Paroni, R., and Dragani, T.A. (2005) Identification and functional characterization of the candidate tumor suppressor gene TRIT1 in human lung cancer. *Oncogene*. **18**, 5502-5509.
157. Takei, K., Sakakibara, H., and Sugiyama, T**.** (2001). Identification of genes encoding adenylate isopentltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J. Biol. Chem*. **13**, 26405-26410.
158. Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*. **25**, 4876-4882.
159. Upadhya, R., Lee, J., and Willis, I.M**.** Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Mol. Cell*. **10**, 1489-1494.
160. Warner G.J., Berry, M.J., Moustafa, M.E., Carlson, B.A., Hatfield, D.L., and Faust, J.R. (2000). Inhibition of Selenoprotein Synthesis by Selenocysteine tRNA$^{[Ser]Sec}$ Lacking Isopentenyladenosine. *J. Biol. Chem*. **275**, 28110-28119.

161. Washburn, M.P. **(**2004) Utilisation of proteomics datasets generated via multidimensional protein identification technology (MudPIT). *Brief Funct. Genomic Proteomic*. **3**, 280-286.
162. Winkler, G.S., Kristjuhan, A., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J.Q. (2002) Elongator is a histone H3 and H4 acetyltransferase important for normal histone acetylation levels in vivo. *Proc. Natl. Acad. Sci*. **99**, 3517-3522.
163. Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., Chu, A.M., Connelly, C., Davis, K., Dietrich, F., Dow, S.W., El Bakkoury, M., Foury, F., Friend, S.H., Gentalen, E., Giaever, G., Hegemann, J.H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D.J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J.L., Riles, L., Roberts, C.J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R.K., Véronneau, S., Voet, M., Volckaert, G., Ward, T.R., Wysocki, R., Yen, G.S., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M., Davis, R.W.
(1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science*. **285**, 901-906.
164. Zhao X, Patton, J.R.,. Davis, S.L, Florence, B., Ames, S.J., Spanjaard, R.A. **(**2004). Regulation of nuclear receptor activity by a pseudouridine synthase through posttranscriptional modification of steroid receptor RNA activator. Mol. *Cell*. **15**, 549-558.
165. Bhalla, N., Biggins, S., and Murray, A.W. (2002). Mutation of *YCS4*, a budding yeast condensin subunit, affects mitotic and nonmitotic chromosome behavior. *Mol Biol Cell* **13**, 632-645.
166. Ambrosio, C.D., Katou, Y., Kelly G., Itoh, T., Shirahige, K., and Uhlmann, F. (2008) Characterization of cis-acting sites for cohesin and condensin loading onto budding yeast chromosomes. Submitted
167. Gao, L., and Gross, D.S. (2006) Using genomics and proteomics to investigate mechanisms of transcriptional silencing in *Saccharomyces cerevisiae*. *Brief Funct Genomics Proteomic*. **5**, 280-288.
168 Triolo, T., Sternglanz, R. (1996) Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. *Nature*. **381**,251-253.
169. Rine, J., Herskowitz, I. (1987) Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae*. *Genetics*. **116**, 9-22.
170. Valenzuela,L., and Kamakaka, R.T. (2006) Chromatin insulators. *Annu. Rev. Genet*. **40**, 107-138.
171. Fingerman, I.M. Wu, C.L., Wilson, B.D., and Briggs, S.D. (2005) Global loss of Set1-mediated H3 Lys4 trimethylation is associated with silencing defects in *Saccharomyces cerevisiae*. *J Biol Chem*. **280**, 28761-28765.
172. Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D., Rine, J. (2004) A protein complex

containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLos Biol* **2**,E131.
173. Mizuguchi, G, Shen X, Landry J, Wu WH, Sen S, Wu C. (2004) ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science*. **16**, 343-348.
174. Li, X., and Heyer, W.D. (2008) Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res*. **18**, 99-113.
175. Shrivastav, M., Leyma, P.D., and Nickoloff, J.A. (2008) Regulation of DNA double-strand break repair pathway choice. *Cell Res*. **18**, 134-147.
176. Malkova, A., Ivanov, E.L., and Haber, J.E. (1996) Double-strand break repair in the absence of RAD51 in yeast: a possible role for break induced DNA replication, *Proc. Natl. Acad. Sci. U.S.A*. **93**, 7131-7136.
177. Ira, G., and Haber, J.E. (2002) Characterizaion of RAD51-independent break-induced replication that acts preferentially with short homologous sequences, *Mol. Cell. Biol*. **22**, 6384-6392.
178. Vas, A.C., Andrews, C.A., Kirkland Matesky, K., and Clarke, D.J. (2007) In vivo analysis of chromosome condensation in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*. **18**, 557-568.
179. Spinola, M., Columbo, F., Falvella, S., and Dragani, T.A. (2007) N6-isopentenyladenosine: a potential therapeutic agent for a variety of epithelial cancers. *Int. J. Cancer*. **120**, 2744-2748.
180. Devine, S.E., and Boeke, J.D. (1996) Integration of the yeast retrotransposon Ty1 is targeted to regions upstream of genes transcribed by RNA polymerase III. *Genes Dev*. **10**, 620-633.
181. Kim, J.M. Vanguri, S., Boeke, J.D., Gabriel, A., and Voytas, D.F. (1998) Transposable elements and genome organization: a comprehensive survey of retrotransposons revealed by the complete Saccharomyces cerevisiae genome sequence. *Genome Res*. **8**, 464-478.
182. Iborra, F.J., Pombo, A., Jackson, D.A., and Cook, P.R. (1996) Active RNA polymerases are localized within discrete transcription "factories" in human nuclei. *J Cell Sci*. **109**, 1427-1436.
183. Grande, M.A. van der Kraan, I., de Jong, L., and van Driel, R. (1997) Nuclear distribution of transcription factors in relation to sites of transcription and RNA polymerase II. *J Cell Sci*. **110**, 1781-1791.
184. Osborne, C.S., Chakalova, L., Brown, K.E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J.A. Lopes, S., Reik, W., and Fraser, P. (2004) Active genes dynamically colocalize to shared sites of ongoing transcription. *Nat Genet*. **36**, 1065-1071.
185. Osborne, C.S., Chakalova, L., Mitchell, J.A., Horton, A., Wood, A.L., Bolland, D.J., Corcoran, A.E., and Fraser, P. (2007) Myc dynamically and preferentially relocates to a transcription factory occupied by Igh. *PLos Biol*. **5**, e192.
186. Haber, J.E. (2000) Lucky breaks: analysis of recombination in Saccharomyces. *Mutation Research*. **451**, 53-69.

187. Einarson, M. and Orlinick, J.R. (2002) *Identification of Protein-Protein Interactions with Glutathione-S-Transferase Fusion Proteins*. Cold Spring Habor Laboratory Press: Cold Spring Harbor, NY.
188. Inada, M. and Guthrie, C. (2004) Identification of Lhp1p-associated RNAs by microarray analysis in Saccharomyces cerevisiae reveals association with coding and noncoding RNAs. *PNAS.* **101**, 434-9.
189. Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002) Capturing chromosome conformation. *Science*. **295**, 1306-1311.
190. Miller, C.O., Skoog, F., von Saltza, M.H., and Strong, F. (1955) *J Am Chem Soc*. **77**, 1392.
191. Skoog, F. and Miller C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp Soc Exp Biol*. **11**, 118-131.