View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by KnowledgeBank at OSU

Genetic and Biochemical Analysis of Trm10 and Trm10 Homologs in *S. cerevisiae*

A Senior Honors Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Biochemistry in the undergraduate colleges of The Ohio State University

By

Dylan Fortman

The Ohio State University
April 2016

Project Advisor: Dr. Jane E. Jackman, Department of Chemistry and Biochemistry

Abstract

Transfer RNAs (tRNAs) depend upon proper tRNA editing and modification, as well as proper secondary and tertiary folding in order to be stable and correctly functional within the cell. One such tRNA modification is the methylation of guanine at its N-1 position to form N-1 methylguanosine (m^1G), found at position 9 (m^1G_9) in multiple cytoplasmic and mitochondrial tRNAs from Eukarya and Archaea. In the budding yeast, *Saccharomyces cerevisiae*, m^1G_9 is produced by the enzyme tRNA methyltransferase 10 (Trm10) on at least thirteen different tRNA substrates. Interestingly, multiple (up to three) different *TRM10* homologs are encoded in metazoa (including humans), but the reason for the presence of these variants is currently unknown. However, the widespread conservation of Trm10, as well as the recent identification of disease-causing mutations in one human homolog (TRMT10A), suggests that this enzyme plays a crucial role in biology. Therefore, the overall goal of this project was to investigate the biological function of nine diverse Trm10 homologs. Deletion of *TRM10* in *S. cerevisiae* does not cause obvious growth defects on normal media, but the *trm10Δ* strain is hypersensitive to the chemical 5-fluorouracil. Taking advantage of this hypersensitive phenotype, we identified three eukaryotic *TRM10* homologs that could substitute for wild-type yeast Trm10, as measured by their ability to provide resistance to 5FU when expressed in *S. cerevisiae*. Among the six non-complementing homologs, two are of significant interest because western blots revealed that they were abundantly expressed in yeast, suggesting that the lack of complementation is not due to defects in expression in *S. cerevsisiae*. Instead, for these two genes (one each from human and mouse), we hypothesize that the resulting enzymes do not methylate the same thirteen tRNAs that are normally substrates for yeast Trm10, thus leading to the inability to complement the growth phenotype on 5FU. Preliminary primer extension data for the three eukaryotic *TRM10*

homologs that could substitute for the loss of wild-type Trm10 suggest that there are specific tRNA substrates within the cells that must modified in order to provide 5FU resistance.

Introduction

In the study of biology, there exist universal mechanisms that have been conserved across all organisms, from bacteria to humans. One such mechanism is known as the central dogma of molecular biology; namely, that the genetic information contained within DNA gets transcribed by RNA polymerase into mRNA and then mRNA is subsequently translated by the ribosome into a growing polypeptide chain which folds into its native protein structure. Often over-looked in this process is the crucial role of tRNAs. In order for the tRNAs to be universally recognized by the ribosome, yet uniquely recognized by their cognate aminoacyl-tRNA synthetases with high specificity for correct amino acid charging, they must first be extensively processed. Many tRNA post-transcriptional processing events (and the enzymes that catalyze them) are conserved across all domains of life suggesting that the nucleotide modifications play a crucial role in biology (Swinehart, et. al. 2013). Typical tRNA processing steps include 5' end maturation by RNase P cleavage, the three-nucleotide CCA addition to the 3' ends, and additional nucleotide modifications introduced into the D-loops, T-loops, anticodon loops, and variable loops (Jackman and Alfonzo, 2013). While the precise reasoning behind nucleotide modifications of tRNAs is not fully understood, it is suggested that the modifications aid in correct folding of the secondary and tertiary structures of the tRNA as well as in some cases, enhancing the decoding of the mRNA (Helm, 2006). In *S. cerevisiae*, there are three known essential tRNA modifying enzymes: the Gcd10p-Gcd14p nuclear complex, the Tad2p-Tad3p cytosolic complex, and the cytosolic Thg1p which are responsible for m^1A_{58}, I_{34}, and $tRNA^{His}_{G-1}$ respectively (Hinnebusch, et al., 1998; Keller W. and Gerber AP., 1999; Gu et al., 2003). While deletion of other single tRNA modifying enzymes do not cause phenotypic growth defects, pairwise deletion of more than one enzyme has been shown to result in lethal phenotypes, suggesting that the optimal

functionality of tRNAs in vivo is supported by a network of modifications or an overlap of functions. (Alexandrov et al., 2006; Chernyakov et al., 2008; Kotelawala et al., 2008; Phizicky and Alfonzo, 2010; Whipple et al., 2011).

One important modification found in biology is the N-1 methylation of guanine, which has been found to occur at both positions 9 and 37. The m^1G_{37} modifications that are found in all three domains of life are catalyzed by the proteins Trm5 (in Archaea and Eukarya) and TrmD (in Bacteria) and are crucial for yeast and bacterial growth (Christian and Hou, 2007). In contrast, m^1G_9 modification is only found in Eukarya and Archaea. In *S. cerevisiae*, the enzyme tRNA methyltransferase 10 (Trm10) catalyzes m^1G_9 on at least 13 different tRNA substrates by way of the *S*-adenosyl-methonine (SAM) cofactor, generating *S*-adenosyl-homocysteine (SAH) as the side-product (Figure 1). For tRNA modifying enzymes, tRNA substrate recognition is accomplished by a wide variety of mechanisms. For enzymes that modify only a single tRNA species, such as the $tRNA^{His}$-specific guanylyltransferase (Thg1), the anticodon nucleotides act as a critical sequence element to be recognized by the enzyme (Jackman and Phizicky, 2006). However, for enzymes similar to Trm10 that act on more than one tRNA species, a challenge exists to recognize the correct tRNA substrates among the many species that exist at a given time within the cell. It is apparent that Trm10 activity requires tRNAs to have more than just a G residue at position 9, due to the observation that of the 19 tRNA species with G_9 that have been investigated and whose modification sequences are fully known, approximately half are modified in vivo (Swinehart, et. al., 2013). Nonetheless, additional sequence elements in the tRNA that specify recognition are not known. It is therefore critical to understand the structural determinants for Trm10 recognition in order to completely understand its mechanism of action.

Trm10 is solely responsible for modification of all m^1G_9-containing tRNAs in vivo in S. cerevisiae, since the *trm10Δ* strain loses detectable m^1G_9 modification on all 13 tRNAs that are known to be modified (Swinehart, et. al. 2013). Despite this, the *trm10Δ* in *S. cerevisiae* is viable, although a phenotype of hypersensitivity to the presence of low concentrations of 5-fluorouracil (5FU), which are otherwise not toxic to the *TRM10* wild-type strain, was associated with the *trm10Δ* strain (Jackman et al., 2003; Alexandrov et al., 2006; Gustavsson and Ronne, 2008). In this case, we hypothesize that specific tRNA substrate(s) are defective when they lack m^1G_9 modification, thus causing the 5FU phenotype. It is known that some modifications to tRNAs are important for function and/or folding of the tRNA, and these effects could explain the role of Trm10 in 5FU hypersensitivity.

In order to understand the role of Trm10 in this 5FU phenotype and its potential role in tRNA function/folding, we chose to use a genetic approach to examine the biological functions of Trm10 in *S. cerevisiae* (yTrm10), as well as its homologs found in Archaea and metazoan eukaryotes. In particular, we focused on investigating the roles of Trm10 enzymes from animals, which, unlike *S. cerevisiae*, encode more than one Trm10 gene. For example, in *Homo sapiens*, there are three homologs of yTrm10 known as: TRMT10A, TRMT10B, and TRMT10C. The importance of this enzyme for human biology is evident from a familial mutation of the human TRMT10A identified by us and another group that causes an absence of the TRMT10A mRNA and protein. This absence leads to glucose metabolic defects, short stature, and a smaller brain size in children (Igoillo-Esteve et al., 2013; Gillis et al., 2014). However, as with the 5FU hypersensitive phenotype in *S. cerevisiae*, the mechanism by which the presumed loss of m^1G_9 modification causes the observed biological defects in humans is unknown.

Among the three human homologs of Trm10, TRMT10A is most similar to yTrm10 and TRMT10C functions in the mitochondria as part of an unusual protein-only version of the 5'-end tRNA maturation enzyme RNase P (Vilardo, et al. 2012). However, the function(s) and location of action for TRMT10B are unknown and methyltransferase activity of TRMT10C is apparently not strictly required for the ribonuclease activity of RNaseP, raising questions about the role of this enzyme in the complex (Vilardo, et al. 2012). The existence of the familial disease-associated mutation in human TRMT10A described above provides further evidence for the distinct nature of the three Trm10 homologs in humans, since the presence of wild-type copies of TRMT10B and TRMT10C is apparently not able to substitute for the defect in TRMT10A in these patients.

Besides in *H. sapiens*, Trm10 homologs have been identified in the mouse, *Mus musculus* (also encoding 3 homologs), the fruit fly, *Drosophila melanogaster* (2 homologs), the plant, *Arabidosis thaliana* (1 homolog), and many archaeal species, including *Pyrococcus furiosus* and *Thermococcus kodakarensis*, each of which encode one gene with similarity to *TRM10*. Therefore, an evolutionary argument can be made for the importance of this protein since it is highly conserved throughout eukaryotes and many archaea, which suggests that a significant selective pressure ensured its maintenance throughout the evolutionary timescale. It is for these reasons that the function of Trm10 enzymes is important to determine. Revealing the functions and activities of these homologs has been the primary focus of this project. We present the results of those studies here.

Part 1: Molecular Biology and Trm10 Functionality

The *S. cerevisiae* Trm10 enzyme that produces m^1G_9 on at least 13 different tRNA species (Swinehart, et. al. 2013) is highly conserved in both Eukarya and Archaea (Figure 2). The phylogenetic analysis reveals a group of Trm10 homologs with sequences that are most similar to the yTrm10 (such as human TRMT10A). Our hypothesis is that these enzymes exhibit the same biological function as yTrm10 to methylate cytosolic tRNA(s). Other homologs cluster separately into groups that may also imply shared functions, but much less is known about the role of these enzymes. Several TRMT10C enzymes share sequence similarity, suggesting that they may share the function that has been associated with the human TRMT10C, which is to act in the mitochondria as part of the RNaseP complex along with a required cofactor SDR5C1 (Vilardo et al., 2012). TRMT10B enzymes are found in many animals, but their biological function(s) have not been addressed in any system. Besides the conservation of the Trm10 protein, which is suggestive of a crucial catalytic role in biology, the human TRMT10A enzyme has been linked to disease in children (Figure 3) (Igoillo-Esteve et al., 2013; Gillis et al., 2014). Thus, understanding the function of these enzymes is important and here we have chosen to use *S. cerevisiae* as a model organism to analyze the function of Trm10 homologs from a wide variety of species. Specifically, my project seeks to answer the question on whether any of the Trm10 homologs exhibit the same biological function as yTrm10 and to analyze tRNA modification patterns in strains expressing various homologs to identify possible differences in tRNA substrates used by each enzyme. In order to tackle the first objective, the powerful tools of molecular biology have been enlisted.

The yTrm10 protein is non-essential for the cells, as evident by the cells' survival on normal media when the gene is deleted. Yet, it has been found that this *trm10Δ* strain is hypersensitive to concentrations of 5FU (Figure 4) which do not normally inhibit the wild-type strain (Jackman et al., 2003; Alexandrov et al., 2006; Gustavsson and Ronne, 2008). This knowledge can be utilized for identifying if the Trm10 homologs function similarly to the yTrm10 when expressed within the *trm10Δ* yeast strain and grown on similar concentrations of 5FU; in other words, if the homolog can provide resistance to 5FU, it complements (makes up for) the loss of the wild-type *TRM10*. Here we investigate exactly which Trm10 homologs under study have functionality comparable to that of yTrm10 through the use of coloning, complementation assays and western blots.

Results

The Cloning Vector. *Escherichia coli* shuttle vectors were used for easier manipulation and have been engineered to include four key components: ampicillin resistance (*ampR*), self-production of the amino acid leucine (*LEU2* selection marker), a galactose promoter by which the *TRM10* homolog can be induced, and a HA/FLAG epitope tagging of the Trm10 proteins for eventual western blot visualization. The galactose inducible (P_{Gal}) promoter was placed directly upstream of a multiple cloning site within the vector, allowing expression of the tested *TRM10* homolog by inclusion of galactose in the growth media. The specific primer design for the *TRM10* homologs incorporated restriction sites that allowed both the DNA vector and *TRM10* homologs to be digested with the same REases and then ligated together by DNA Ligase (Figure 5). Upon transformation of the ligated DNA into chemically competent *E. coli* XL-1 Blue cells, potential colonies were screened by restriction enzyme analysis and candidates were

subsequently verified by DNA sequencing. The fully verified clones included: *S. cerevisiae* Trm10 (as a positive control), *Mus musculus* TrmT10B and TrmT10C, *Drosophila melanogaster* TrmT10A, *Homo sapiens* TRMT10A, TRMT10B, and TRMT10C, *Arabidopsis thaliana* Trm10, *Thermococcus kodakarensis* Trm10, and *Pyrococcus furiosus* Trm10.

Complementation Assay Analysis. The fundamental basis of yeast complementation assays is to conclude whether gene variants can substitute for the loss of the wild-type gene. With the use of two yeast strains (wildtype and *trm10Δ*) and the knowledge of the deletion strain's hypersensitivity to 5FU, we were able to elucidate which Trm10 homologs could make-up for (complement) the loss of wild-type yTrm10 functionality. The verified clones listed above were transformed into the two yeast strains and subsequently grown on selective media (synthetically designed minimal media excluding leucine: SD-leu). The colonies which formed were then plated on a master SD-leu plate in patches for subsequent replica plating. The technique of replica plating is important for this experiment because it allows for the cells that originated from a single colony to be transferred onto numerous plates with varying media in order to qualitatively make distinguishes on their growth patterns. The various agar plates that were used, and in the order of replica plating, are as follows: SD-leu, SGal-leu, SGal-leu + 0.1µg/ml 5FU, SGal-leu + 1.0µg/ml 5FU, SGal-leu + 25µg/ml 5FU, and SD-leu. This particular ordering of plates is important for two reasons: the first being that the SD-leu plates were at the beginning and end where growth on both plates signifies that the transfer worked and that cells were not lost upon transfer; the second is that the SGal-leu plates prove that there is no observable phenotypic discrepancy between the two strains even though the wild-type strain's Trm10 was induced for transcription by the addition of galactose within the media. In addition to the order of replica plating, the concentrations of 5FU that were used are crucial as well. The Ronne group

originally showed that a 5FU concentration of 1.0μg/ml was enough to inhibit the *trm10Δ* strain in growth (Gustavsson and Ronne, 2008).We also chose two other 5FU concentrations (0.1μg/ml and 25μg/ml) to allow us to detect a range of expected growth phenotypes. At the low end of 0.1μg/ml, both *TRM10* and *trm10Δ* strains are expected to grow well on media while at a high concentration of 25μg/ml both strains should be completely inhibited. From this technique of replica plating and the important incorporation of 5FU, were we able to identify three strains that complemented the loss of wild-type yTrm10, and six that failed to complement. Of the three strains, not including the successful positive control of reintroducing *S. cerevisiae* Trm10 into the deletion strain, *D. melanogaster* TrmT10A, *H. sapiens* TRMT10A, and *A. thaliana* Trm10 all showed growth comparable to that of the wild-type strain when grown on SGal-leu + 1.0μg/ml. The six strains that did not complement – *M. musculus* TrmT10B and TrmT10C, *H. sapiens* TRMT10B and TRMT10C, *P. furiosus* Trm10, and *T. kodakarensis* Trm10 – were all inhibited of growth on SGal-leu + 1.0μg/ml, similar to the vector control strain (Table 1).

Western Blot – Visualizing Trm10 Expression. Western blot is a technique used to visualize proteins with the use of antibodies exclusive to the protein of interest. Specifically, a crude protein extract is run on a denaturing gel which separates the proteins based on size. After transferring the proteins to a nitrocellulose membrane, antibodies are used to specifically recognize a protein of interest, allowing visualization of the sizes and relative concentrations of the proteins under study. With the incorporation of the HA/FLAG epitopes engineered within our original cloning vector, we were able to test for Trm10 homolog expression in the strains, and used this information in conjunction with the complementation assay analysis to make predictions about conservation of function of the various homologs. We expected to see high levels of expression of a protein similar to the predicted size for the three Trm10 homologs

which complemented 5FU hypersensitive phenotype of the *trm10Δ* strain. For the cloned strains which failed to complement the *trm10Δ* strain, observing a protein band of expected size would suggest that the Trm10 homolog must have a function different than that of yTrm10 since its expression failed to provide resistance to 5FU. However, seeing no protein band could mean that the levels of Trm10 expression were weak or that the protein was unstable; either of these possibilities would predictably result in an inability of the homolog to complement the 5FU phenotype.

First, we tested *D. melanogaster* TrmT10A, *M. musculus* TrmT10B, and the *S. cerevisiae* Trm10 positive control strains in both SD-leu and SGal-leu media to ensure that the galactose inducible promoter (P_{Gal}) was effective in the up-regulation of *TRM10* expression. These clones were transformed into the *trm10Δ* strain and grown on SD-leu media. The resultant colonies were transferred to SD-leu and SGal-leu broth and grown in bulk culture. After growth, the cell lysates were resolved by denaturing gels and probed for the presence of the epitope-tagged proteins. (Figure 6). In the western blots derived from yeast extracts, two prominent background bands were routinely observed but were relatively consistent across all samples, allowing us to distinguish the protein bands of interest from the background bands based on their expected sizes and differences in the samples grown under different conditions. In Figure 6, all samples grown in SD-leu only displayed the background signal, indicative that the Trm10 homolog was not expressed, as expected due to glucose-based repression of the P_{Gal} promoter. However, with the addition of galactose to the growth media (SGal-leu), we observed robust signals separate from the background, which suggests that they represent the Trm10 homolog and that *TRM10* was successfully expressed by the P_{Gal} promoter. In each case, the bands are roughly consistent with the expected size of the expressed protein (based on the cloned gene sequence), although the *M.*

musculus TrmT10B protein band may migrate at a slightly higher apparent molecular weight than predicted. In this case, post-translational modifications (such as glycosylation) may explain the added size. Of these three strains, all showed considerable over-expression of the apparent Trm10 homolog in SGal-leu, including notably the *M. musculus* TrmT10B strain which did not complement the loss of wild-type *TRM10*.

After validating the expected galactose-dependence of *TRM10* expression, the remaining clones (*M. musculus* TrmT10C, *H. sapiens* TRMT10A, TRMT10B, and TRMT10C, *A. thaliana* Trm10, *P. furiosus* Trm10, and *T. kodakarensis* Trm10) were all transformed into the *trm10Δ* strain on SD-leu media and then grown in only SGal-leu broth culture for bulk growth. Following the same exact procedure as outlined above, extracts derived from these cells were all tested by western blot (Figure 7). The image again shows the consistent background bands across all samples, with several variable bands that were evident in individual lysates. From this western blot, we observed high intensity protein bands for both *H. sapiens* TRMT10A and *A. thaliana* Trm10, both of which are consistent with the observed complementation assay results that demonstrated each of these proteins are able to complement the lack of yTrm10. In addition, a high intensity band was seen for *H. sapiens* TRM10TB and a weaker band for *T. kodakarensis* Trm10, while no bands were observed for either *H. sapiens* TRMT10C or *M. musculus* TrmT10C. We believe that the potential smeared observed in the lane containing extracts from *P. furiosus* Trm10 is in fact bleed-over from the adjacent *A. thaliana* signal, and does not reflect actual *P. furiosus* Trm10 expression. As in Figure 6, there is general agreement between the migration of the bands and their expected molecular weights, with the possible exception of human TRMT10A, which is observed to migrate at a molecular weight that is slightly higher than what would be expected, and could again be due to post-translational modifications.

Discussion. Here we demonstrated that three eukaryotic Trm10 homologs (human and fruit fly TrmT10A and *A. thaliana* Trm10) are able to complement the 5FU hypersensitive phenotype of the yeast *trm10Δ* strain in a manner that is consistent with their robust expression in the engineered strains. Thus, we believe that these homologs share at least some common biological function(s) with yTrm10. In contrast, the two homologs (*M. musculus* TrmT10B and *H. sapiens* TRMT10B) are expressed well in *S. cerevisiae* but do not complement the *trm10Δ* phenotype, suggesting that they may have a different biological function when compared to yTrm10 or that they may potentially modify a subset of tRNA species that are different from those of yTrm10. The latter case is particularly interesting since human TRMT10B has been shown to catalyze in vitro m^1G_9 methyltransferase on at least one yeast tRNA substrate, but its overall substrate specificity is unknown (Vilardo et al., 2012). Therefore, a particular tRNA substrate (or substrates) may be critical in providing 5FU resistance, but modified differently by TrmT10B compared to the more closely related TrmT10A enzymes. This could explain the observed growth phenotype, as has been observed in several other cases, such as for the heat-sensitive phenotype from the loss of t^6A that is complemented by the expression of $tRNA^{Lys}_{UUU}$ but not other tRNA species that are targets of the t^6A modification machinery (Crecy-Lagard, et. al., 2015). *S. cerevisiae* Trm10 exhibits a selectivity toward certain tRNA substrates in vivo (Swinehart, et. al., 2013); if any of this subset of tRNA substrates are conserved in the complemented strains, but differently modified in the non-complementing strains, it is possible that those species are involved in providing 5FU resistance. Our results for the *P. furiosus* and *T. kodakarensis* Trm10 strains are less clear, but possibly interesting. The moderate-weak signal observed for *T. kodakarensis* Trm10 and its lack of complementation could simply mean that not enough Trm10 was present to rescue the 5FU phenotype. Another possibility, and one that

includes the lack of expression for *P. furiosus* Trm10, is that these proteins are not naturally found in *S. cerevisiae* and expressed at a temperature of 30 ℃; as thermophiles, their natural habitats are in much higher temperatures than that which was tested, with the result being potentially unstable protein product upon expression in *S. cerevisiae*. Alternatively, the possibility of alternative substrate specificity exhibited by *T. kodakarensis* Trm10 could also contribute to its inability to complement the phenotype. Addressing these possibilities could require either quantifying the amount of the Trm10 homolog protein that was expressed as compared to those homologs which were able to complement (purify the protein and conduct a Bradford assay or if antibodies are available conduct an ELISA assay) or identifying which substrates *T. kodakaresnsis* Trm10 acts on within the cells. Lastly, the lack of *H. sapiens* TRMT10C and *M. musculus* TrmT10C protein expression and complementation we observed in our assays can be readily explained by the requirement of the SDR5C1 cofactor for proper activity (Vilardo et. al., 2012) which was not co-expressed within our cloning vector because the essential nature of this cofactor for TrmT10C stability was not yet known at the time we began constructing the yeast strains; had the SDR5C1 cofactor been co-expressed, we would most likely have observed TrmT10C expression by western blot due to stability of TrmT10C . However, complementation of the loss of wild-type yTrm10 by the TrmT10C homologs, whose activities are within the mitochondria, remains questionable.

Part 2: Identifying Trm10 tRNA Substrates

We identified three eukaryotic Trm10 homologs which can be expressed to high levels in *S. cerevisiae* and appear to function similarly to yTrm10 type yTrm10 based on their ability to complement the 5FU phenotype of the *trm10Δ* strain: *D. melanogaster* TrmT10A, *A. thaliana* Trm10, and *H. sapiens* TRMT10A. Based on this data, we hypothesize that they may catalyze similar methylation activities on a subset of tRNA species, and that this pattern of tRNA methylation is required for cells to be resistant to 5FU. Interestingly, however, although there is somewhat limited information about actual tRNA species that contain m^1G_9 modification in these organisms (*D. melanogaster*, *A. thaliana*, and *H. sapiens*), if this were to be valid, it suggests that those tRNA substrates are crucial to be modified within the cell as the modification has been conserved across an evolutionary timescale.

Similarly, we have identified six Trm10 homologs which failed to complement the loss of wild-type yTrm10, two of which (*M. musculus* TrmT10B and *H. sapiens* TRMT10B) were strongly expressed within the *trm10Δ* strain as detected by western blot; these two non-complementing enzymes therefore must be catalyzing different reactions within the cells and will ultimately require further study. Alternatively, they do catalyze the same reaction, but need a cofactor or need to be modified which may not happen in yeast. Interestingly, there is already evidence for distinct functions of the TRMT10A and TRMT10B homologs in humans. In the context of the human TRMT10A mutation that leads to the inherited disease syndrome, wild-type TRMT10B is still presumably functional in the affected individuals and therefore its presence is not sufficient to complement the process that are involved in human pathology. One potential activity of these two Trm10 homologs is that they are methylating a completely different set of tRNA substrates

within the *trm10Δ* strain and this set includes none of the normal targets of yTrm10 and its TRMT10A orthologs. The biochemical activities of TRMT10B enzymes have not been extensively determined in any system, although there is one report of methyltransferase activity of the human TRMT10B on a singly human cytosolic $tRNA^{Arg}$, which is interestingly not one of the typical substrates of yeast Trm10 in vivo (Vilardo et al., 2012). Thus, it is possible that they are modifying a set of tRNA species that are different than those modified by the complementing enzymes listed above and therefore cannot provide resistance to 5FU. While there is no current data to support this hypothesis, another potential activity of the *M. musculus* TrmT10B and *H. sapiens* TRMT10B homologs is that they are simply not methylating tRNAs but rather are acting on different substrates within the cell, such as DNA or other RNA species.

In order to test the first hypothesis for elucidating whether the complementing Trm10 enzymes and the non-complementing Trm10 enzymes are methylating different tRNAs, it is crucial to identify specific tRNA substrates for the homologs under study. One way to carry out this process is by the technique known as primer extension. After isolating low molecular weight RNA from cells grown in galactose media (expressing our Trm10 homologs), specific tRNA substrates are selected by the addition of a radioactively labeled DNA oligo nucleotide primer that is complementary in sequence to a specific area of the tRNA substrate of interest. The annealed primer is extended by Reverse Transcriptase (RT), creating a cDNA strand according to the primary sequence of the target tRNA. However, if the tRNA of interestwere to be modified with m^1G_9, in which methylation occurs on the Watson-Crick face of the template nucleotide, the RT cannot efficiently incorporate the incoming cytosine (C) nucleotide; the result is a cDNA fragment that is shorter in length than the full-length fragment resulting from primer extension of

an unmodified tRNA. This size difference is visualized on a gel with the radioactive phosphate acting as our source of detection.

With this technique at our disposal, we decided to first test the complementing Trm10 enzymes (*D. melanogaster* TrmT10A, *H. sapiens* TRMT10A, and *A. thaliana* Trm10) on their ability to modify $tRNA^{Gly}_{GCC}$ which is a known substrate for *S. cerevisiae* Trm10 in vivo (Figure 8) (Swinehart, et. al., 2013). Typically, using this assay yTrm10 is observed to modify m^1G_9 to the $tRNA^{Gly}_{GCC}$ substrate with almost 100% efficiency. We sought to compare the levels of modification from our complementing Trm10 enzymes after isolating the RNA and carrying out primer extension (Figure 9). In the assay that was performed on RNA isolated from the complemented strains, m^1G_9 modification was seen only weakly, if at all, for both *A. thaliana* Trm10 and *H. sapiens* TRMT10A $tRNA^{Gly}_{GCC}$, while *D. melanogaster* TrmT10A had low, but slightly more detectable activity on $tRNA^{Gly}_{GCC}$ when compared to the *trm10Δ* vector control.

Discussion. Based on this result, it is clear that m^1G_9 is not to full efficiency as seen most often in *S. cerevisiae* for $tRNA^{Gly}_{GCC}$ in vivo. The first possible explanation for the primer extension results could be that because these Trm10 homologs were expressed in the *trm10Δ* strain while being grown on a rich, non-selective medium, the cells lost the selective pressure to maintain the plasmid. By losing this selection, it is conceivable that a portion of the cells within the culture slowly lost the plasmid as it was no longer beneficial for cell survival. This result would imply that there was less of the *TRM10* homologs expressed and therefore less m^1G_9 modification.

A second possible explanation for significantly lower levels of modification when compared to yTrm10 returns to the concept of a single tRNA in high abundance being able to rescue a phenotype observed with other tRNA modifying enzyme deficiencies, as seen previously (Crecy-

Lagard, et. al., 2015). It is possible that for these complementing Trm10 homologs, even though preliminary data shows low methylation of $tRNA^{Gly}_{GCC}$, the "essential" tRNA that must be modified to restore cell viability is not $tRNA^{Gly}_{GCC}$. As stated previously, *S. cerevisiae* modifies 9 tRNA substrates in vivo; by continuing primer extension and identifying the tRNA substrates of these complementing enzymes, determining the complete set of specific tRNAs that are modified by each complementing enzyme could help narrow the gap of possible targets in determining a single tRNA that could be responsible in restoring the 5FU phenotype.

While these two possibilities could help explain the primer extension data, our results are still preliminary and will require further testing to validate what we have observed. What is clear, however, is that we have identified three Trm10 homologs whose function replaces that of wild-type yTrm10 as measured by our previous yeast complementation assays. In addition, *M. musuclus* TrmT10B and *H. sapiens* TRMT10B have been characterized to have a biological role different than that of yTrm10 due to their inability to rescue the 5FU phenotype even under conditions where the proteins are in high abundance. Future work for this project will include repeating the current primer extension experiments, identifying tRNA substrates for the complementing Trm10 homologs, and characterizing the activity of *M. musculus* TrmT10B and *H. sapiens* TRMT10B to ascertain if they modify specific tRNA substrates or are potentially acting on a different substrate (DNA, RNA species) within the cells.

Materials and Methods

Cloning Plasmid Preparation. Trm10 genes (*S. cerevisiae TRM10, A. thaliana TRM10, D. melanogaster TRMT10A, H. sapiens TRMT10A, TRMT10B,* and *TRMT10C, M. musculus TRMT10B* and *TRMT10C, P. furiosus TRM10,* and *T. kodakarensis TRM10*) were PCR amplified from cDNA constructs previously designed within the lab. These genes were cloned into both a 2μ LEU2 vector downstream of the galactose promoter and N-terminal HA/FLAG epitope tag respectively. Clones were verified by single restriction enzyme digestion and DNA sequencing. Transformation of the clones was carried out in *E. coli* XL-1 Blue competent cells.

Yeast Complementation Assay. Cloned plasmids were transformed into both *trm10Δ* and wildtype yeast strains following a standard protocol as described in (Abad et al., 2010) and grown on SD-leu media at 30 ℃. The resultant colonies were replica plated as described previously at 30 ℃.

Western Blot. Cloned plasmids were transformed into both *trm10Δ* and wildtype yeast strains following a standard protocol as described in (Abad et al., 2010) and grown on SD-leu media at 30 ℃. The resultant colonies were grown in 5ml SD-leu for overnight growth at 30 ℃. This was then used to inoculate 5ml SGal-leu at an OD 0.1. Cells were harvested at an OD 2.0. Lysis of the cells included 50μl lysis buffer (50μl of 1M Tris-Cl pH 7.5, 2μl of 500mM EDTA, 250μl of 2% TritonX-100, 1μl of 1M DTT, 200μl of 5M NaCl, 2.5μl of 1000μg/ml pepstatin, 2.5μl of 1000μg/ml leupeptin, and 10μl of 100mM PMSF) and the addition of zirconium beads. This mixture was vortexed at 4 ℃ 10 times for 30sec each with a minute of ice bath between vortexing. After cell lysis, 10μl of SDS 2X loading dye + BME was added and the mixture boiled for 5 minutes at 100°C. The extracts were spun down and 10μl was loaded on a SDS-

PAGE gel (12% resolving and stacking gel). The gel was run for approximately 1 hour at 180V and then transferred to a nitrocellulose membrane. Subsequent staining with Ponceau to ensure transfer of proteins was conducted. The membrane was blocked with 5% milk overnight. Exposure to the primary antibody (anti-FLAG) and then to the secondary antibody (anti-Rabbit conjugated with horseradish peroxidase) followed blocking with milk. The image was developed with X-ray film.

Primer Extension. DNA oligo primer (Gly^{GCC}) was labeled with γ-^{32}P ATP by heating 1µl of 25µM primer, 2.5µl 10X PNK buffer, 2µl T4 PNK, 2µl γ-^{32}P ATP, and 17.5µl ddH_2O at 37 ℃ for 30 minutes and then subsequently at 72 ℃ for 10 minutes. BioGelP6 spin columns were used to remove excess ATP. Labeled primers were annealed to $tRNA^{Gly}_{GCC}$ (8µg/µl) by heating to 95°C and then cooling to room temperature (RT). Extension of the annealed DNA oligo primer was done with a final concentration of 0.4mM dNTPs; 2µl 5X Promega buffer, 0.8µl 5X dNTP, 0.6µl AMV-Reverse Transcriptase, and 1.6µl ddH_2O were combined with annealing reaction and set to RT for 5 minutes, followed by 37 ℃ for 1 hour. The reaction was combined with 10µL 2X loading dye and 5µL loaded onto a 10%PA/4M urea gel. The gel was run at 55W for approximately 2 hours, transferred to filter paper, exposed overnight, and imaged on a Typhoon Imager.

Figures

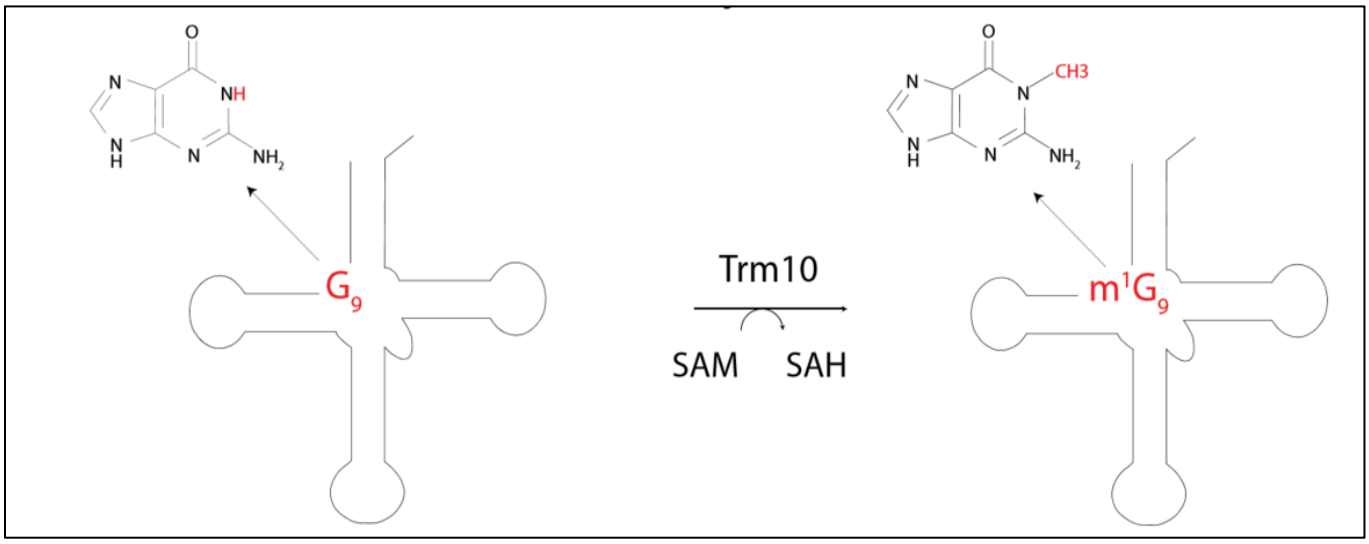


Figure 1. Trm10 Mechanism of Action. tRNA substrates with G_9 get methylated by Trm10 use of a SAM cofactor, producing m^1G_9 and SAH as the byproduct.

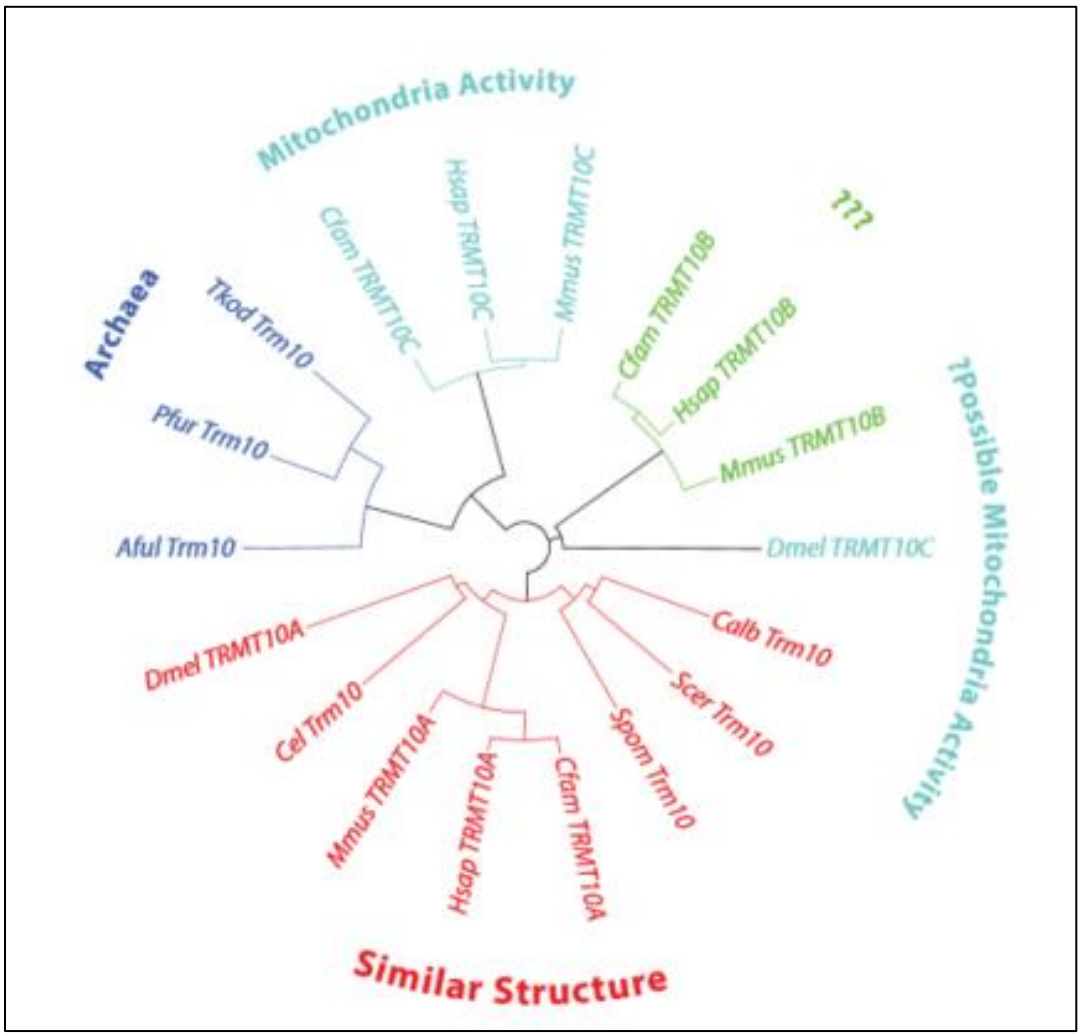


Figure 2. Phylogenetic Representation of *TRM10* Homologs. Protein alignment of the Trm10 homologs results in an observable phylogenetic trend. Trm10 homologs with similar structure to wild-type yTrm10 have been shown to complement the loss of wild-type yTrm10 in the *trm10Δ* strain. Several Trm10 homologs have been found to have mitochondrial activities and the localization and activity of a few other Trm10 homologs remains unknown.

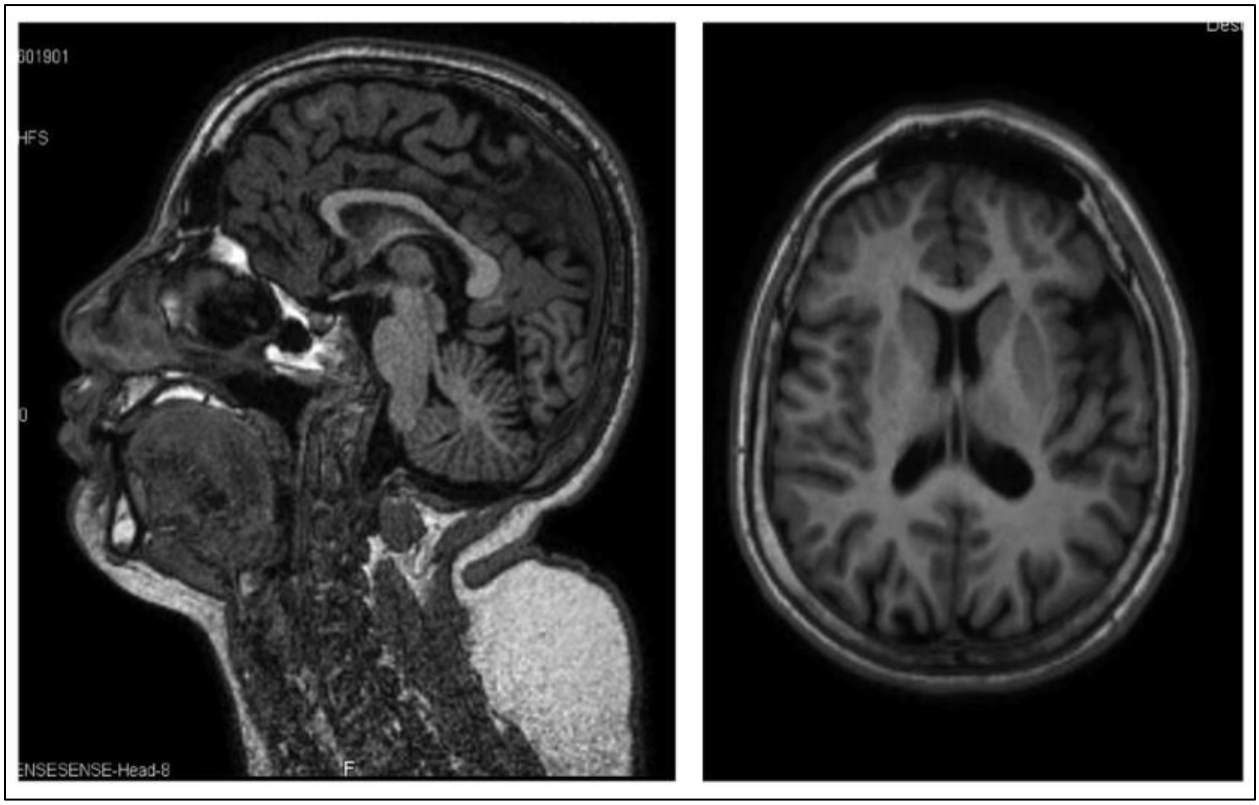


Figure 3. Pleiotropic Disease Associated with Absence of Human TRMT10A. A familial mutation of *H. sapiens* TRMT10A resulted in children with microcephaly, short stature, and glucose metabolic defects. Figure adapted from Igoillo-Esteve, 2013.

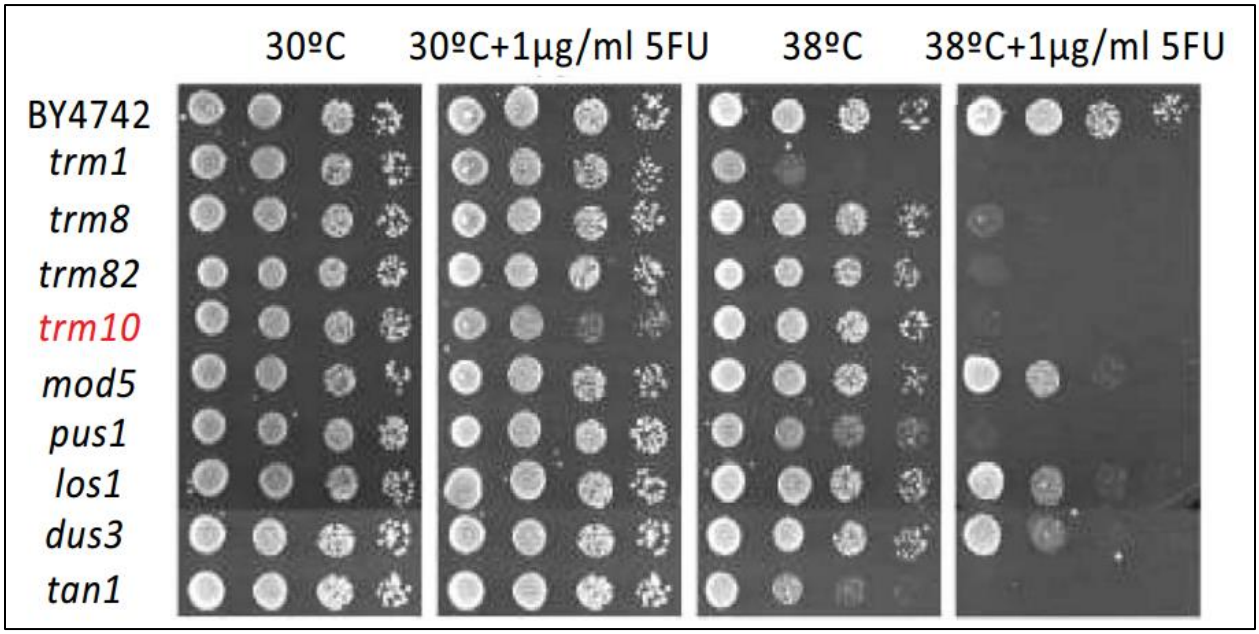


Figure 4. 5FU Hypersensitivity. An absence of *TRM10* in *S. cerevisiae* results in inhibition of cell viability when grown on media with 1µg/ml 5FU. Figure adapated from Ronne, 2008.

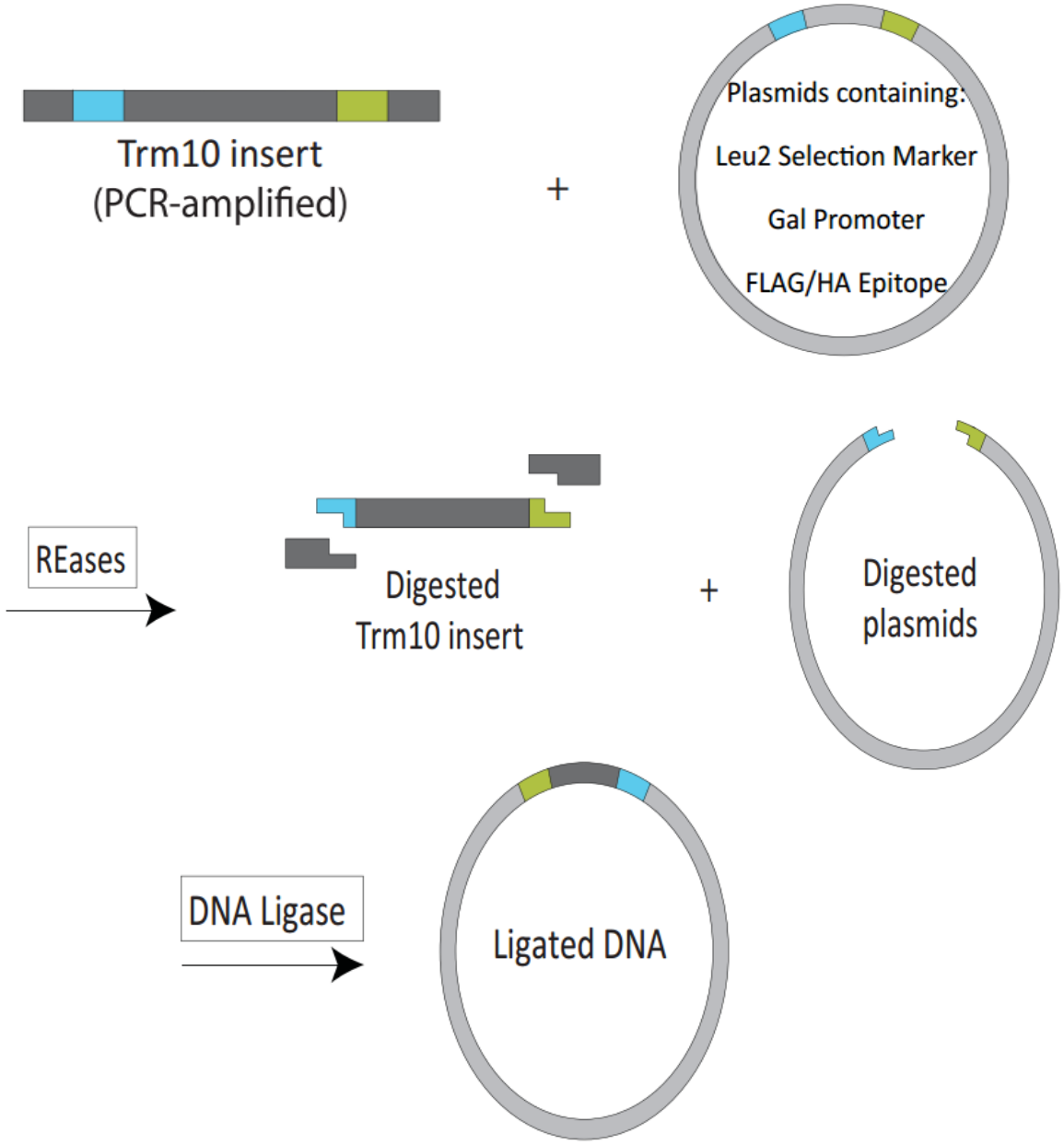
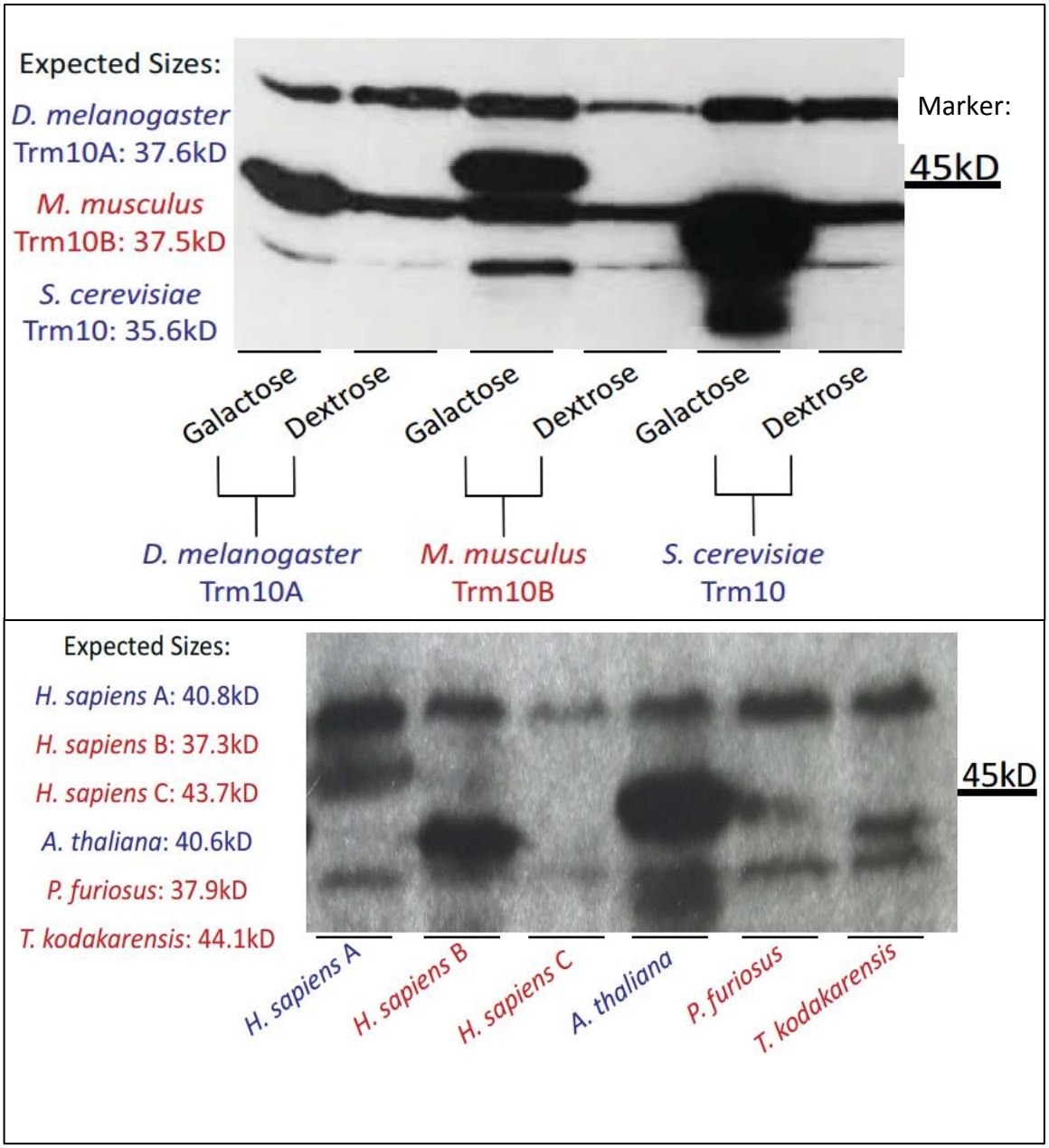


Figure 5. Cloning Procedure. The *TRM10* gene was PCR amplified to include restriction enzyme sites similar to those within the original cloning vector. This vector includes the crucial Leu2 selection marker, gal promoter, and FLAG/HA epitope. After digestion with REases, the *TRM10* genes were ligated into the digested vector.



Figures 6 and 7. Visualizing Trm10 Homolog Expression. (Figure 6): Western Blot of 3 previously analyzed homologs (visualized by FLAG epitope within the plasmids), demonstrating the necessity of galactose within the media for expression of the gene. (Figure 7): Western Blot analysis of previously unstudied Trm10 homologs grown in the presence of galactose, visualized by specific epitopes (FLAG) that were engineered within the plasmids.

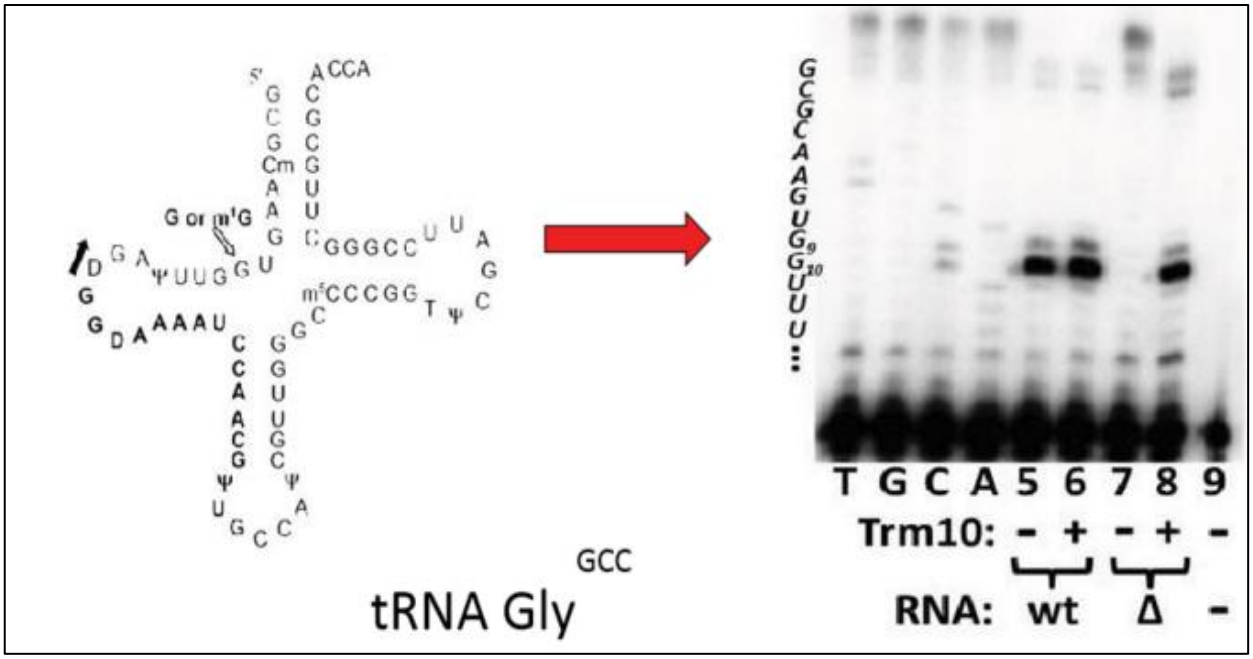


Figure 8. tRNA$^{Gly}_{GCC}$ Example Primer Extension. Bold nucleotides in cloverleaf tRNA structure indicate primer binding for exenstion by reverse transcriptase. When visualized on a gel, m1G9 modification can be visualized. Figures adapted from Jackman and Phizicky (2003) and Swinehart (2013).

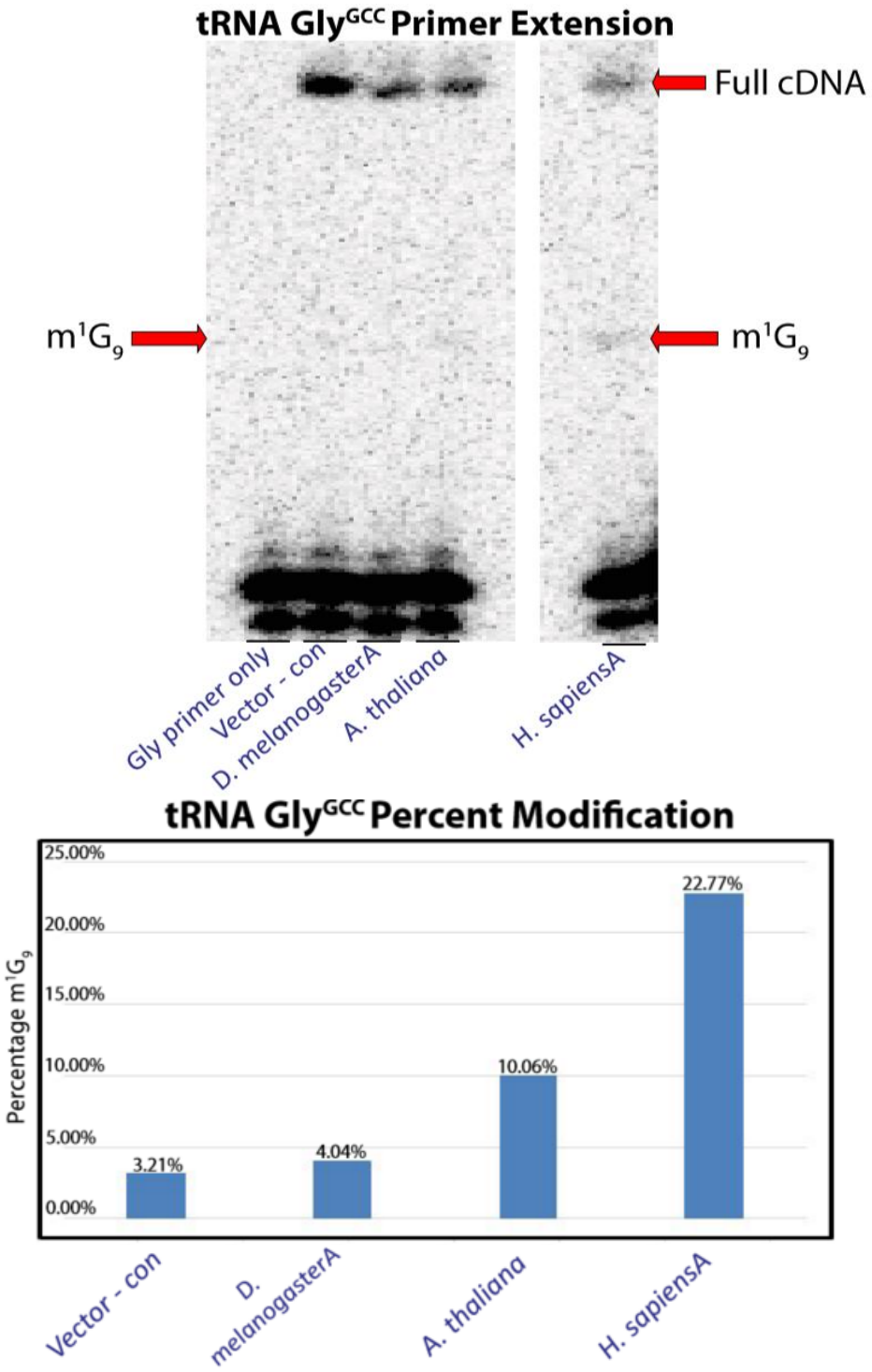


Figure 9. Trm10 Homolog Primer Extension of tRNA$^{Gly}_{GCC}$. Visualization of m^{1}G$_9$ modification for Trm10 homologs which complemented loss of wild-type yTrm10 and quantification of percent modification.

References

Abad, et al. Template-dependent 3'-5' nucleotide addition is a shared feature of tRNA-His guanylyltransferase enzymes from multiple domains of life. *PNAS*. 2010;107(2): 674 679.

Alexandrov A, et al. Rapid tRNA decay can result from lack of non-essential modifications. *Mol Cell*. 2006;21: 87–96.

Chernyakov, et al. Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5′–3′ exonucleases Rat1 and Xrn1. *Genes & Dev.* 2008; 22: 1369-1380.

Christian T and Hou YM. Distinct Determinants of tRNA Recognition by the TrmD and Trm5 Methyl Transferases. *J Mol Biol*. 2007;373: 623-632.

Crecy-Lagard, et al. Global translational impacts of the loss of the tRNA modification t6A in yeast. *Microbial Cell*. 2015.

Gillis D, et al. TRMT10A dysfunction is associated with abnormalities in glucose homeostasis, short stature and microcephaly. *J Med Genet*. 2014;51: 581-586.

Gustavsson M and Ronne H. Evidence that tRNA modifying enzymes are important in vivo targets for 5-fluorouracil in yeast. *RNA*. 2008;14: 666–674.

Helm, Mark. Post-transcriptional nucleotide modification and alternative folding of RNA. *Nucleic Acids Research*. 2006;34(2): 721-733.

Hinnebusch, et al. The essential Gcd10p-Gcd14p nuclear complex is required for 1 methyladenosine modification and maturation of initiator methionyl-tRNA. *Genes & Dev*. 1998; 12: 2650-3662

Igoillo-Esteve M, et al. tRNA Methyltransferase Homolog Gene TRMT10A Mutation in Young Onset Diabetes and Primary Microcephaly in Humans. *PLoS Genet*. 2013;9(10).

Jackman JE and Alfonzo JD. Transfer RNA modifications: Nature's combinatorial chemistry playground. *RNA*. 2013;4: 35-48.

Jackman JE; et al. Identification of the yeast gene encoding the tRNA m1G methyltransferase responsible for modification at position 9. *RNA*. 2003;9: 574-585.

Keller, Walter and Andre P. Gerber. An adenosine deaminase that generates inosine at the wobble position of tRNAs. *Science*. 1999;286(5442): 1146-1149.

Kotelawala, et al. Identification of yeast tRNA Um_{44} 2′-O-methyltransferase (Trm44) and demonstration of a Trm44 role in sustaining levels of specific $tRNA^{Ser}$ species. *RNA*. 2008;14: 158-169.

Phizicky EM, and Alfonzo JD. Do all modifications benefit all tRNAs? *FEBS Lett*. 2010;584: 265-271.

Swinehart WE, Henderson JC, and Jackman JE. Unexpected expansion of tRNA substrate recognition by the m1G9 methyltransferase Trm10. *RNA*. 2013.

Vilardo E, et al. A subcomplex of human mitochondrial RNase P is a bifunctional methyltransferase – extensive moonlighting in mitochondrial tRNA biogenesis. *Nucleic Acids Res.* 2012;40: 11583-11593.

Weifeng Gu, et al. tRNA-His maturation: An essential yeast protein catalyzes addition of a guanine nucleotide to the 5' end of tRNA-His. *Genes & Dev.* 2003;17: 2889-2901.

Whipple, et al. The yeast rapid tRNA decay pathway primarily monitors the structural integrity of the acceptor and T-stems of mature tRNA. *Genes & Dev.* 2011;25: 1173-1184.