Analysis of Bacterial and Human Epitranscriptomic Writers and RNA Modifications

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Analysis of Bacterial and Human Epitranscriptomic Writers and RNA Modifications

An honors thesis presented to the Department of Biological Sciences, University at Albany, State University of New York in partial fulfilment of the requirements for graduation with Honors in Biological Sciences and graduation from The Honors College

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Research Advisor: Cara Pager, Ph.D.

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Abstract

Translation is a key step in protein synthesis in which the codons in messenger RNA (mRNA) are decoded by the corresponding anticodon in transfer RNA (tRNA). Prior to translation, tRNAs are modified by epitranscriptomic writers in order to ensure accurate decoding. During the previous semesters, different strains of *Escherichia coli* (*E. coli*) were used where genes corresponding to a tRNA modification enzyme were deleted from the genome. These strains were from the Keio *E. coli* gene deletion library and included the epitranscriptomic writers: *CmoA, CmoB, Tgt, MnmE, QueA, ThiL, MnmC* and *TtcA*. These strains were used to test the role of tRNA modifications in protein synthesis during the stress response to the antibiotic chloramphenicol (CAM). *E. coli* cells lacking the tRNA modification writers: *selU, cmoA, cmoB, tgt, queA, thiL, mnmC ttcA* and *mnmE* showed CAM sensitivity. The database Modomics was also used to identify RNA modifications and epitranscriptomic writers specific to the anticodon loops of tRNA from *E. coli* and humans. Then BLAST analysis was used to identify human homologs. Lastly, 27 human writers were analyzed for their links to cancer using The Cancer Genome Atlas and cBioportal database. Various cancers were identified that have amplifications in 5 or more writers and could be predicted to have changes in their epitranscriptome and may be addicted to these RNA modifications to promote translation. The human epitranscriptomic modifications: ALRYEF, CDKAL1, FTSJ1, GTPBP3, METTL11B, METTL18, METTL23 and METTL24 were found to have high levels of alteration frequencies for specific cancer types, with Bladder/Urinary Tract cancer and Uterine Serous Carcinoma/Uterine Papillary Serous Carcinoma cancers the most pronounced.

**Keywords:** *Escherichia coli, RNA modifications, Epitranscriptomic writers, Cancer, Chloramphenicol*
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Introduction

Transcription occurs when DNA is accessed and converted into mRNA. For actively decoded mRNA species, translation can next occur in which the codons in mRNA are decoded by the corresponding anticodon in tRNA to make a protein. In this process, mRNA and the fMet-tRNA, the initiator tRNA that brings in the first amino acid in proteins: methionine, binds to the small subunit of ribosomal RNA (rRNA) with proper codon-anticodon pairing. The large ribosomal subunit rRNA with three binding sites: A, P and E sites, joins the complex and the fMet-tRNA sits on the P site. The tRNA specific for a codon in the A site enters the ribosome. A peptide bond is formed between fMet-tRNA of P site and the amino acid of A site. As ribosomes move in a 5’ to 3’ direction on the mRNA, the peptidyl-tRNA relocates to the P site. A new codon is exposed in the A site once again. This process continues until the ribosome reaches a termination codon, which causes the peptidyl transferase to cleave the completed polypeptide chain from the last tRNA (Griffiths et al., 2015).

During the pairing of the codon to the anticodon, the third position of codons has relaxed pairing rules compared to the first two codon bases, due to a phenomenon known as wobble codon-anticodon pairing (Griffiths et al., 2015). This breaks the Watson-Crick rules of base pairing as the third base of the anticodon is capable of base pairing with more than one type of base (Griffiths et al., 2015). In general, the anticodon base guanine (G) can interact with uridine (U) or cytosine (C) in the codon; the C anticodon base can interact with G in the codon; an adenine (A) anticodon base interacts with U in the codon; U anticodon base can interact with A or G in the codon; lastly, the anticodon base inosine (I) can interact with A, U or C in the codon. This is important since 64 different tRNAs are not available in most cells to translate the different mRNA codons (Griffiths
et al., 2015). Meanwhile, Inosine (I), a modified base found in tRNA, is one of many that is written into tRNA by enzyme writer systems (Griffiths et al., 2015).

Prior to translation, tRNAs are modified by epitranscriptome writers in order to ensure proper decoding. Below, I describe the enzymes I examine. The figures show where the chemical moiety is deposited and the extent to which the nucleotide within the tRNA is modified.

The tRNA modification enzymes: 5-carboxymethoxyuridine methyltransferase (CmoM), 5-methylaminoamethyl-2-thiouridine (MnmC), 5-carboxymethyl aminomethyl uridine (MnmE), carboxymethyltransferase (CmoB), and carboxy-S-adenosyl-L-methionine synthase (CmoA) are involved in wobble uridine modification (U34) in the bacteria *E. coli* (Keseler et al., 2017). The U34 wobble base of tRNAs (Ala, Ser, Pro and Thr) is modified by the enzyme *CmoM*, as it catalyzes the final step of 5-methoxycarbonylmethoxyuridine (mcmO5U) modification through the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to 5-methoxycarbonyl methyl uridine (mcm5U) (Kim et al., 2013).

![Chemistry of the CmoM Modification in tRNAs. (The UniProt Consortium, 2021)](image-url)
Figure 2

CmoM Modifies tRNAs Ala and Ser at the U34 position (circled in red)

Note. The black dots and semi circles represent all of the areas where tRNAs modifications occurs as well as the frequency. The extent of the filled in circle represents how often the nucleotide is modified at the respective position (Boccaletto et al., 2017).

CmoA and CmoB are required for the formation of 5-oxyacetyl uridine (cmo^5U) modification through the transfer of carboxymethyl group (Keseler et al., 2017). CmoA specifically catalyzes the conversion of SAM to carboxy-S-adenosyl-L-methionine (Cx-SAM), to facilitate the formation of a modified base at the U34 wobble position (Kim et al., 2013).

Figure 3

Chemistry of CmoA modification of SAM (The UniProt Consortium, 2021).
Meanwhile, *CmoB* catalyzes the transfer of the carboxymethyl group from carboxy-\(S\)-adenosyl-L-methionine (Cx-SAM) to 5-carboxymethoxyuridine (cmo\(^5\)U) at U34 wobble base (Figure 4) (Kim et al., 2013).

![Figure 4: Chemistry of CmoB modification in tRNAs (The UniProt Consortium, 2021).](image)

*MnmC* catalyzes the formation of 5-methylaminomethyl-2-thiouridine (mnm\(^5\)s\(^2\)U) from 5-carboxymethylaminomethyl-2-thiouridine (cmnm\(^5\)s\(^2\)U) for tRNAs (Glu, Lys and Gly) (Figure 5) (Keseler et al., 2017).

![Figure 5: Chemistry of MnmC modification in tRNAs (The UniProt Consortium, 2021).](image)
*MnmE* adds a carboxymethylaminomethyl (cmnm) group at the wobble position (U34) for tRNAs (Lys, Leu, Glu, Gln, Gly and Arg) and is necessary for the biosynthesis of 5-methylaminomethyl-2-thiouridine (mmn\(^5\)s\(^2\)U) modification (Figure 6 and Figure 7) (Elseviers et al., 1984).

**Figure 6**

*Chemistry of MnmE modification in tRNAs. (Boccaletto et al., 2017).*

**Figure 7**

*MnmE modifies the tRNAs Lys, Leu, Glut, Gln, Gly and Arg at the U34 position (circled in red).*

*Note:* The black dots and semi circles represent all of the areas where tRNAs modifications occurs as well as the frequency. The extent of the filled in circle represents how often the nucleotide is modified at the respective position (Boccaletto et al., 2017).
Similarly, the enzymes: S-adenosylmethionine ribosyltransferase-isomerase (*QueA*) and guanine transglysylase (*Tgt*) are involved in wobble G modification. *Tgt* catalyzes the post-transcriptional base exchange of guanine (G) with queuine precursor (*PreQ1*) in tRNAs (Asp, Asn, His and Tyr) (Figure 8 & Figure 9) (Keseler et al., 2017; The UniProt Consortium, 2021).

![preQ1 base](image1)

![7-aminomethyl-7-deazaguanosine](image2)

**Figure 8**

*Chemistry of Tgt modification in tRNAs (Boccaletto et al., 2017).*

![Aspartate tRNA](image3)
![Asparagine tRNA](image4)
![Histidine tRNA](image5)
![Tyrosine tRNA](image6)

**Figure 9**

*Tgt modifies the tRNAs Asp, Asn, His, and Tyr at the U34 position (circled in red).*

*Note.* The black dots and semi circles represent all of the areas where tRNAs modifications occurs as well as the frequency. The extent of the filled in circle represents how often the nucleotide is modified at the respective position (Boccaletto et al., 2017).
The formation of the anticodon loop modification in tRNA requires \textit{QueA} for the addition of the 2,3-epoxy-4,5-dihydroxycyclopentane ring to epoxyqueuosine in tRNAs (Asp, Asn, His and Tyr), (Keseler et al., 2017).

\textbf{Figure 10}

\textit{Chemistry of QueA modification in tRNAs (Boccaletto et al., 2017).}

\textbf{Figure 11}

\textit{QueA modifies the tRNAs Asp, Asn, His, and Tyr at the U34 position (circled in red).}

\textit{Note.} The black dots and semi circles represent all of the areas where tRNAs modifications occurs as well as the frequency. The extent of the filled in circle represents how often the nucleotide is modified at the respective position (Boccaletto et al., 2017).
2-selenouridine synthase (SelU) catalyzes the conversion of 2-thiouridine (s²U-RNA) to 2-selenouridine (Se²U-RNA) at the U34 wobble base of three different tRNAs (Lys, Glu, Gln) (Figure 12 & Figure 13) (Keseler et al., 2017).

**Figure 12**

*Chemistry of SelU modification in tRNAs (Boccaletto et al., 2017).*

**Figure 13**

*SelU modifies the tRNAs Lys, Glu and Gln at the U34 position (circled in red).*

*Note.* The black dots and semi circles represent all of the areas where tRNAs modifications occurs as well as the frequency. The extent of the filled in circle represents how often the nucleotide is modified at the respective position (Boccaletto et al., 2017).
Lastly, the enzymes thiamine monophosphate kinase (ThiL) and cytidine(32) 2-sulfurtransferase (TtcA) are involved in thio-modification of tRNAs (Keseler et al., 2017). ThiL catalyzes the phosphorylation of thiamine monophosphate to produce thiamine diphosphate (Imamura et al., 1982; Nakayama et al., 1972).

![Chemistry of ThiL modification in tRNAs](The UniProt Consortium, 2021).

TtcA catalyzes the ATP-dependent 2-thiolation of cytidine in position 32 of tRNA (Arg ad Ser) to produce 2-thiocytidine (s\(^2\)C32) (Bouvier et al., 2014).

![Chemistry of TtcA modification in tRNAs](Boccaletto et al., 2017).
TtcA modifies the tRNAs Arginine and Serine at the C32 position (circled in red).

Note. The black dots and semi circles represent all of the areas where tRNAs modifications occurs as well as the frequency. The extent of the filled in circle represents how often the nucleotide is modified at the respective position (Boccaletto et al., 2017).

Chloramphenicol (CAM) is a lipid soluble, broad spectrum antibiotic that reversibly binds to the 50S subunit of ribosomes. It diffuses through the bacterial cell membrane and acts by preventing the formation of peptide bonds by suppressing peptidyl transferase activity (Bartlett, 1982). Chloramphenicol was first derived from *Streptomyces venezuelae* in 1947 but is now synthetically made (Bartlett, 1982). It can cause severe side effects such as damage to bone marrow and aplastic anemia and thus is only used to treat serious bacterial infections such as cholera, typhoid fever, meningitis caused by bacteria. It is also used in eye drops and ointments to treat bacterial conjunctivitis. Chloramphenicol falls under the amphenicol class of antibiotics due to its phenylpropanoid structure.

In my work, I used *E. coli* mutants (part of the Keio gene deletion library) deficient in specific epitranscriptomic writer enzymes. The Keio gene deletion library is a collection of K-12 strains in the BW25113 background of *E. coli*, each being a mutant with a single gene deleted and
replaced with a kanamycin resistance gene through homologous recombination (Baba et al., 2006). These mutants can be used to assess and analyze the functions of different genes (Baba et al., 2006). Results from my work showed that strains of *E.coli* cells lacking the tRNA modification writers: *SelU, CmoA, CmoB, Tgt,* and *MnmE* had a disrupted stress response while a different Keio strain lacking the tRNA modification writers: *queA, thiL, mnmC, ttcA* and *mnmE* also showed sensitivity to CAM. After this, RNA from mutant □*selU* was isolated in order to identify RNA modification levels using mass spectrometry analysis in the future.

In this work, I also analyzed RNA modification enzymes thorough computational analysis. One database, Modomics: [http://genesilico.pl/modomics/](http://genesilico.pl/modomics/) (Boccaletto et al., 2017), can be used to gather information about modifications, pathways, RNA sequences, proteins, guide RNAs and building blocks of numerous different organisms including *E. coli*. Specifically, for tRNA modification enzymes, information about writer protein sequences, enzymatic activities, position of catalyzed modification, sequence identifier number (GI number), open reading frame (Orf), cluster of orthologous groups for phylogenetic classification (COG), and summary of the activity pathway can be found. Using information from Modomics, such as the GI number, Basic Local Alignment Search Tool (BLAST) analysis can be performed. BLAST is a program as well as an algorithm tool that can be used to find similarities between biological sequences (Altschul et al., 1990). It forms local alignment for a given sequence by comparing/aligning to various sequence databases (Altschul et al., 1990). There are different versions for aligning different sequences: nucleotide to nucleotide, translated nucleotide to protein, translated protein to nucleotide and protein to protein (Altschul et al., 1990). For this project, BLAST analysis was used to determine if the writer enzyme is specific to just bacteria or just humans, or if the writer enzymes were conserved between species. In general, almost all of the tRNA modification enzymes mentioned
previously were found to be homologous in some disease-causing organisms such as *Mycobacterium tuberculosis, Vibrio cholera, Haemophilus influenza, Streptococcus pneumonia and Salmonella enterica*; however, homology was not found with human genes.

Lastly, human epitranscriptomic writer enzymes were analyzed for their roles in different cancer types through cBioPortal database as a computational analysis tool. In general, cells become cancerous through failures in multiple systems leading to uncontrollable cell division. Specifically, different types of amplifications, deletions and mutations arise in genes that can promote cell proliferation (Lodish et al., 2000). One type of mutation that may result in tumor growth is through amplification (Lodish et al., 2000). For example, if there is a mutation that leads to more than one copy of the same gene for positive cell division regulators, accumulation of extra proteins can promote cancer (Lodish et al., 2000). Similarly, damage to or deletion of negative cell regulator genes may lead to nonstop cell division, thus promoting tumor growth (Lodish et al., 2000). As mentioned earlier, prior to translation, tRNAs are modified by epitranscriptomic writers in order to ensure proper decoding and thus play an important role in gene expression. Therefore, it can be hypothesized that mutations such as amplification and deletions of epitranscriptomic writers could affect cancer development.

Thus, cBioPortal database was used to characterize mutations of human epitranscriptomic enzymes on different cancer types. The cBioPortal database contains tools for investigating cancer genomic data set from larger genomic databases like The Cancer Genome Atlas (TCGA) program (Cerami et al., 2012; Gao et al., 2013). One of the many ways cBioPortal database can be used is to analyze cancer types based on chromosomal amplification, mutation and deep deletion levels of different epitranscriptomic writers (Cerami et al., 2012; Gao et al., 2013). For this project, 27 epitranscriptomic modification enzymes were analyzed for amplification, mutation and deep
deletion levels. The epitranscriptomic modification enzymes of interest were: ALRYEF, ADAT3, ALKBH1, ALKBH2, ALKBH3, ALKBH4, CDKAL1, CTU2, ELP2, ELP3, ELP4, ELP5, ELP6, FTSJ1, GTPBP3, IKPKAB, METTL11B, METTL14, METTL15, METTL16, METTL17, METTL18, METTL21A, METTL21C, METTL22, METTL23, and METTL24. Similar to the *E. coli* cells that were sensitive when a specific writer was removed and could be used as a target for antibiotics, epitranscriptomes with high levels of mutations for specific cancer types could be used as potential target for cancer treatment. The human epitranscriptomic modification enzymes: ALRYEF, CDKAL1, FTSJ1, GTPBP3, METTL11B, METTL18, METTL23 and METTL24 were found to have high levels of alteration frequencies for specific cancer types.
Materials and Methods

LB Liquid Media

To make 1000 mL of LB media (Becton, Dickson and Company, Sparks), 25.0 g of LB powder was added into an autoclave bottle and the bottle was filled to 1000 mL with deionized water. Stirring plate with a stirring rod inside the autoclave bottle was used to mix the powder evenly with water. The cap of the autoclave bottle was put loosely on the bottle before placing the bottle in the autoclave on liquid cycle.

LB Agar Media

To make agar plates, ~100 mL of distilled water was first added to a 1000 mL sterile autoclave bottle. Then 12.5 g of LB media (Becton, Dickson and Company, Sparks) and 7.5 g of agar (Becton, Dickson and Company, Sparks) was added to the bottle. The bottle was filled up to 500 mL with distilled water, a stir rod was added, and it was autoclaved for two hours. It was then left to cool for another hour while stirring to prevent congealing. Afterwards, the media was poured into the plates and left to cool until it was solid. Finally, the plates were put, upside down and tilted, in the 55 °C incubator for 15 minutes to dry.

Sterile Streaking Technique

A loop was used to streak bacteria from a plate. First, the rod was sterilized using fire and a single colony of bacteria was picked. The bacteria containing rod was then streaked into a new plate by handling the rod at almost parallel level to the plate. The rod was sterilized before each streaking. Then the streaked plates were placed in the 37 °C for 24 hours.
RNA Isolation

The mutant strains were acquired from the Keio gene deletion library (Baba et al., 2006). Each strain was streaked on kanamycin (5 µg/mL) agar plates. One colony of each strain was inoculated for 18 hours in six separate, sterile tubes: three contained 10 mL LB broth with 3.4 µg/mL of CAM while three contained 10 mL of LB media. In order to ensure that good concentration of bacteria had grown prior to RNA isolation, optical density of the inoculated media was measured. These samples were then spun in a JA 25.5 rotor centrifuge for 20 minutes at 2,500 rpm and 20 °C. After discarding the supernatant without disturbing the pellet, 1 mL of TRIzol was added to each sample to break apart the pellet. Chloroform (200 µL) was then added to each sample which were then vortexed and incubated for 3 minutes. Samples were then centrifuged at 14,500 rpm for 15 minutes. 400 µL of the top layer of the sample was added to a new micro tube along with 500 µL of isopropanol and rotated around 5 to 10 times and incubated for 10 minutes in room temperature before centrifuging for 15 minutes at 14,500 rpm, 4 °C. Then the supernatant was removed, and 1 mL of 70 % ethanol was added to the micro tube before centrifuging for 10 minutes at 10,000 rpm, 4 °C. Finally, the ethanol was decanted, and the pellet was dried and solubilized with deionized water. RNA concentration and purity were determined using NanoDrop 1000.

Computational Analysis: Modomics and BLAST

Using the protein button on the main page of Modomics: http://genesilico.pl/modomics/, information about different tRNA modification proteins were found. After gathering information on the protein, “Send to NCBI Blast” button on the top right of the summary page was pressed, leading to the NCBI BLAST program that is already set with a GI number specific to the protein of choice. There, several settings were changed: under organism, E. coli was excluded and under “Algorithm Parameters”, the maximum targeted sequence was changed from 50 to 5000. The E-
value threshold was set to 0.05. Then BLAST was performed. Alignments for different organisms along with the e-value under the “Organism” section of “Taxonomy” was tabulated.

**Computational Analysis: cBioPortal Database**

In the query tab on the main screen of cBioPortal database website:

https://www.cbioportal.org, “query the curated set of non-redundant studies” was selected. Under the quick search tab, the gene of interest was selected. In the “Cancer Type Detailed” settings, the minimum number of total cases was changed to 50 and the minimum percent of altered cases was changed to 1%. The resulting graph along with the downloaded data from the top right corner of the graph were used to analyze the different mutation levels. Lastly, the downloaded data was compiled and clustered into heat maps using Morpheus:

https://software.broadinstitute.org/morpheus/.
Results

Growth Inhibition of *E. coli* Using CAM

In order to investigate the roles of tRNA modification enzymes during stress response, 10 different *E. coli* mutants lacking a specific modification enzyme, along with wild type (WT), were exposed to and grown in the presence (5 µg/mL CAM) or absence (0 µg/mL CAM) of chloramphenicol antibiotic (CAM). As seen in Figure 17.1-19.1, all of the mutants and the wild type cells grew on plates that did not contain CAM. Despite the 5-fold serial dilution that was performed seven times, all of the mutants and wild type grew densely in the regular agar plate lacking CAM. Some of the first strain of mutants showed growth defects in 5 µg/mL CAM plates relative to the wild type (WT), and they included *queA*, *thiL*, *mnmC*, *ttcA* and *mnmE*. However, when the second strain of the mutants were grown on 5 µg/mL CAM, only *queA*, *thiL*, *mnmC*, *ttcA* and *mnmE* showed sensitivity to CAM.
Three of the mutants lacking cmoA, queA, cmoB, modifications along with the controls were serially diluted, spotted on CAM agar plates and incubated overnight.

Three of the mutants, lacking tgt, selU, thiL, modifications along with the controls were serially diluted, spotted on 0 and 5 ug/mL CAM agar plates and incubated overnight.

Four of the mutants, lacking cmoM, mnmC, mnmE, ttcA, modifications along with the controls were serially diluted, spotted on 0 and 5 ug/mL CAM agar plates and incubated overnight.
RNA Isolation and Purity

RNA concentration and purity were determined spectroscopically. Contamination of the purified RNA with DNA, Trizol, ethanol, chloroform or isopropanol was also assessed using NanoDrop 1000. The A260/280 ratio represents the level of DNA contamination, with 2 being the ideal number. The A260/230 ratio represents the level of Trizol, ethanol, chloroform and isopropanol contamination, with 2 being the ideal value. Lastly, ng/µL represents the concentration of RNA in each sample. As seen in Table 1.1, compared to the rest of the samples, control 2 contained a lot of contamination. Similarly, CAM 1 sample had a lower concentration of RNA compared to the rest of the samples.
Table 1

Contamination of DNA (260/280), Trizol, ethanol, isopropanol and chloroform contamination (260/230) and concentration of RNA (ng/µL) from RNA isolation of ΔSelU mutants

<table>
<thead>
<tr>
<th></th>
<th>260/280</th>
<th>260/230</th>
<th>ng/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>1.93</td>
<td>2.01</td>
<td>2938.5</td>
</tr>
<tr>
<td>Control 2</td>
<td>1.39</td>
<td>1.47</td>
<td>3821.2</td>
</tr>
<tr>
<td>Control 3</td>
<td>1.79</td>
<td>1.84</td>
<td>3349.2</td>
</tr>
<tr>
<td>CAM 1</td>
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<td>2.02</td>
<td>2257.4</td>
</tr>
<tr>
<td>CAM 2</td>
<td>1.86</td>
<td>1.89</td>
<td>3258.1</td>
</tr>
<tr>
<td>CAM 3</td>
<td>1.84</td>
<td>1.84</td>
<td>3398.4</td>
</tr>
</tbody>
</table>

Homology of tRNA Modification Enzyme Genes

In order to analyze the homology of tRNA modification enzymes, Modomics and BLAST programs were used. Genes are homologous to each other if they are conserved between different organisms. As seen in Table 2.1, *E. coli* genes for the tRNA modification enzymes were found to be homologous to corresponding proteins in organisms such as *Mycobacterium tuberculosis*, *Vibrio cholera*, *Haemophilus influenza*, *Streptococcus pneumonia* and *Salmonella enterica*. However, *selU* homologs were not identified in *V. cholera*. Similarly, no homologs were seen for *cmoA* in *H. influenzae* and for *mnmc* in *M. tuberculosis*, *V. cholerae*, and *H. influenzae*. Yeast mitochondrial ortholog known as *Mss1* was also found for *mnme* in Modomics but nothing was found through BLAST alignment. Similarly, while a homolog for *tgt* was indicated for *Homo sapiens* as *TGT* in Modomics, nothing was found through BLAST alignment. Meanwhile, we can see the Expect-value (E-value) of tRNA modification genes in different organisms in Table 3.1. E-value indicates the statistical significance of the alignments. For each E-value, the number given is the number of hits that is expected to be random and found by chance. As we can see from Table
2.1, most of the E-value found is 0 or very close to 0. Hence, it can be concluded that these alignments are non-random.

Table 2
Homology of tRNA Modification Genes

<table>
<thead>
<tr>
<th></th>
<th>H. sapiens</th>
<th>S. cerevisiae</th>
<th>M. Tuberculosis</th>
<th>V. cholerae</th>
<th>H. influenzae</th>
<th>S. pneumoniae</th>
<th>S. enterica</th>
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<tbody>
<tr>
<td>cmoA</td>
<td>-</td>
<td>-</td>
<td>Homologous</td>
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<td>-</td>
<td>Homologous</td>
<td>Homologous</td>
</tr>
<tr>
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<td>-</td>
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<td>Homologous</td>
<td>Homologous</td>
<td>Homologous</td>
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<tr>
<td>mnmC</td>
<td>-</td>
<td>-</td>
<td></td>
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<td>-</td>
<td>Homologous</td>
<td>Homologous</td>
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<tr>
<td>mnmE</td>
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<td>Homologous</td>
<td>Homologous</td>
<td>Homologous</td>
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<tr>
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<td>Homologous</td>
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<td>Homologous</td>
</tr>
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<td>tgt</td>
<td>?</td>
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<td>Homologous</td>
<td>Homologous</td>
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<td>Homologous</td>
<td>Homologous</td>
</tr>
</tbody>
</table>

Table 3
E-value of tRNA Modification Genes

<table>
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<th>M. Tuberculosis</th>
<th>V. cholerae</th>
<th>H. influenzae</th>
<th>S. pneumoniae</th>
<th>S. enterica</th>
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<tbody>
<tr>
<td>cmoA</td>
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<td>8.00E-130</td>
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<td>-</td>
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<td>3.00E-152</td>
<td>4.00E-156</td>
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<td>0</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>0</td>
</tr>
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<td>-</td>
<td>-</td>
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<td>0</td>
</tr>
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<td>-</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>-</td>
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<td>-</td>
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<td>0</td>
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<td>2.00E-171</td>
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</table>
Computational Analysis of Human tRNA Modification Enzymes

In order to analyze human tRNA modification enzymes for their links to different cancer types, cBioPortal database was used. Figures 20.1-46.1 show the alteration frequency in percentage (y-axis) of different cancer types (x-axis) for epitranscriptomic modifications obtained from the cBioPortal database. In the graph, the different alteration types are given in different colors: green represents mutations, red represents amplification, blue represents deep deletion, purple represents fusion and grey represents multiple alterations. As seen in Figure 20.1, there was 13.88% amplification level in Bladder/Urinary Tract for ALYREF modification. Amplification level of 23.61% was seen in Bladder/Urinary Tract cancer for CDKAL1 (Figure 26.1). Deep deletion level of 24.09% was seen in Stomach Adenocarcinoma for FTSJ1 (Figure 33.1). Amplification level of 14.68% was seen in Uterine Serous Carcinoma/Uterine Papillary Serous Carcinoma for GTPBP3 (Figure 34.1). For METTL11B, amplification levels of 25.31%, 14.96% and 13.95% were seen in Breast Mixed Ductal and Lobular Carcinoma, Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma respectively (Figure 36.1). Similarly, for METTL18, amplification levels of 25.73%, 15.2% and 14.05% were seen in Breast Mixed Ductal and Lobular Carcinoma, Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma respectively (Figure 41.1). Amplification level of 13.89% were seen in Bladder/Urinary Tract cancer for METTL23 (Figure 45.1). Lastly, mutation level of 13.46% was seen in Prostate Neuroendocrine Carcinoma for METTL24 (Figure 46.1).
Figure 20

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic reader ALYREF obtained from the cBioPortal database.

Figure 21

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ADAT3 obtained from the cBioPortal database.
Figure 22

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ALKBH1 obtained from the cBioPortal database.

Figure 23

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ALKBH2 obtained from the cBioPortal database.
Figure 24

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ALKBH3 obtained from the cBioPortal database.

Figure 25

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ALKBH4 obtained from the cBioPortal database.
Figure 26

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer CDKAL1 obtained from the cBioPortal database.

Figure 27

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer CTU2 obtained from the cBioPortal database.
Figure 28

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ELP2 obtained from the cBioPortal database.

Figure 29

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ELP3 obtained from the cBioPortal database.
Figure 30

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ELP4 obtained from the cBioPortal database.

Figure 31

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ELP5 obtained from the cBioPortal database.
Figure 32

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer ELP6 obtained from the cBioPortal database.

Figure 33

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer FTSJ1 obtained from the cBioPortal database.
Figure 34

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer GTPBP3 obtained from the cBioPortal database.

Figure 35

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer IKPKAB obtained from the cBioPortal database.
Figure 36

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL11B obtained from the cBioPortal database.

Figure 37

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL14 obtained from the cBioPortal database.
Figure 38

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL15 obtained from the cBioPortal database.

Figure 39

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL16 obtained from the cBioPortal database.
Figure 40

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL17 obtained from the cBioPortal database.

Figure 41

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL18 obtained from the cBioPortal database.
Figure 42

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL21A obtained from the cBioPortal database.

Figure 43

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL21C obtained from the cBioPortal database.
Figure 44

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL22 obtained from the cBioPortal database.

Figure 45

Alternation frequency in percentage (y-axis) for different cancer types (x-axis) for the epitranscriptomic writer METTL23 obtained from the cBioPortal database.
Several trends were seen when the different alteration types were clustered on a heat map in which darker color corresponds to higher alteration frequency. As seen in Figure 47.1, the cancer type that is most affected by amplification of the different epitranscriptome was found to be Bladder/ Urinary Tract cancer. Although at very low frequencies for the most part, amplification can also be seen in most of the modification enzymes in several cancer types: Serous Ovarian Cancer, Uterine Serous Carcinoma, Prostate Adenocarcinoma, Adrenocortical Carcinoma, and Dedifferentiated Liposarcoma. AlKBH4, CDKAL1, METTL11B and METTL18 were shown to have the highest levels of amplifications compared to other epitranscriptomes. Similarly, Figure 48.1 shows Uterine Serous Carcinoma to be one of the cancer types most affected by deep deletion. Compared to amplification levels, the lack of deep deletion of modification enzymes in different cancer types is very noticeable. Lastly, as seen in Figure 49.1, cancer types with the highest levels
of mutation frequency was found to be Uterine Endometrioid Carcinoma, Mucinous Adenocarcinoma of the Colon and Rectum and Skin Cancer, Non-Melanoma. Similar to deep deletion, many of the cancer types can be seen to lack in mutation of modification enzymes.

Figure 47

Compilation of amplification levels for 69 writers, erasers and readers in 65 different cancer types.
Figure 48

Compilation of deep deletion levels for 69 writers, erasers and readers in 65 different cancer types.

Figure 49

Compilation of mutation levels for 69 writers, erasers and readers in 65 different cancer types.
Discussion

Relative to the wild type, the mutants CmoA, CmoB, Tgt, and MnmE of the first strain and the mutants QueA, ThiL, MnmC, TtcA, and MnmE of second strain were sensitive to CAM and displayed perturbation. CmoA and CmoB are required for the formation of 5-oxyacetyl uridine modification through the transfer of carboxymethyl group. Tgt is involved in the modified base queuine in tRNAs Asp, Asn, His, and Tyr. MnmE is necessary for 5-methylaminomethyl-2-thioridine modification in tRNAs Lys, Leu, Glu, Gln, Gly and Arg. QueA is required for the addition of the 2,3-epoxy-4,5-dihydroxycyclopentane ring to epoxyqueuosine in tRNAs (Asp, Asn, His and Tyr) for the formation of the anticodon loop. ThiL catalyzes the phosphorylation of thiamine monophosphate to produce thiamine diphosphate. MnmC catalyzes the formation of 5-methylaminomethyl-2-thiouridine from 5-carboxymethylaminomethyl-2-thiouridine for tRNAs (Glu, Lys and Gly). TtcA catalyzes the ATP-dependent 2-thiolation of cytidine in position 32 of tRNA (Arg and Ser) to produce 2-thiocytidine. Meanwhile, MnmE adds a carboxymethylaminomethyl (cmnm) group at the wobble position U34 for tRNAs (Lys, Leu, Glu, Gln, Gly and Arg) and is necessary for the biosynthesis of 5-methylaminomethyl-2-thiouridine modification. Hence, we can speculate that these tRNA modifications and tRNAs play an important role in inhibiting the effects of CAM in protein synthesis.

After the RNA isolation was completed and measured for ΔselU, compared to the rest of the samples, we can see that control 2 contained a lot of DNA and other contaminations. Similarly, CAM 1 sample had a lower concentration of RNA compared to the rest of the samples. Therefore, our next step is to clean these samples to ensure that contamination does not interfere with future experiments. In general, RNA samples isolated using TRIzol protocol can be cleaned with a spin column kit such as RNA Clean and Concentrator kits (Norgen Biotek Corporation, 2016). In the
future I would also purify RNA from other mutant cells, including ΔmnmC, ΔmnmE, ΔTrmO, Wild type (as the positive control) and Alkb (as the negative control). Then, we will identify RNA modification levels using mass spectrometry analysis in order to test if their absence alters RNA modification levels and response to stress (CAM) (Basanta-Sanchez et al., 2006). Mass Spectrometry is an analytical technique that can be used to identify proteins based on the mass to charge ratio. The sample is digested with trypsin or other enzymes to make small peptides. Then the mass to charge ratio is measured. This ratio is compared with a database to identify the proteins (Brown et al., 2004).

Meanwhile, there are also other possible antibiotics that could be used for this experiment due to their similarity to chloramphenicol. As mentioned earlier, amphenicol class antibiotics includes antibiotics such as chloramphenicol thiamphenicol, florfenicol, etc., and works by binding to the 50 S subunit. Similar to chloramphenicol, thiamphenicol works by inhibiting protein synthesis but is more potent than chloramphenicol (Francis et al., n.d.). It is typically used as an antibiotic for treating infectious diseases in cattle, poultry, pigs etc. (Francis et al.). While thiamphenicol has similar side effects to that of chloramphenicol, it does not cause aplastic anemia (Francis et al., n.d.). Florfenicol is another amphenicol class antibiotic that also inhibits protein synthesis by binding to the ribosome (Florfenicol, 2007). It is also often used for veterinary medicine and is not associated with aplastic anemia, unlike chloramphenicol (Florfenicol, 2007). With these, we can compare and contrast the effects of CAM vs. other antibiotics in E. coli mutants to speculate about what types of antibiotics affect which tRNA modifications enzymes found in E. coli. Our next steps proceeding these results are also to replicate this experiment using the second strains two more times, test them in different concentrations of CAM and attempt to rescue these mutants back by adding back the deleted genes in E. coli.
E. coli is useful in experiments due to its simplicity as compared to other organisms such as yeasts and human cells. E. coli genome consists of around 4.6 million base pairs encoding around 4,000 different proteins (Cells as Experimental Models). Meanwhile, Saccharomyces cerevisiae (a type of yeast) has 12 million base pairs encoding around 6,000 genes and human genome in general consists of about 3 billion base pairs, encoding around 100,000 different proteins (Cells as Experimental Models). Similar to E. coli, yeasts and human cells have many different stress response proteins and systems to manage different stressors. Research in S. cerevisiae stress response include environmental stressors such as oxidative stress, metalloid and metal stress, osmotic stress, heat shock etc. (Rodrigues-Pousasa et al., 2005). Much of the work focuses on transcriptional regulation, particularly the Yap protein family that is involved in various environmental stressors (Rodrigues-Pousasa et al., 2005). Similarly, many different types of stress response proteins and thus systems exist for different types of cellular stress in human cells, such as cytotoxic agents, heavy metals, genotoxic agents, calorie restriction, oxidative stress, and heat (Nunes et al., 2019). These environmental stressors may trigger and/or contribute to different diseases. Thus, stress responses such as tRNA methyltransferase Alkbh8 enzyme, required for response against reactive oxygen species (ROS), are important for “restoring” the normal physiology of cells (Endres et al., 2015; Nunes et al., 2019). The lack of these stress responses can therefore lead to various diseases in humans. The tRNA methyltransferase Alkbh8 is homologous in S. cerevisiae as tRNA methyltransferase 9 (Trm9) (Endres et al., 2015). Alkbh8 in humans and Trm9 in S. cerevisiae catalyzes the methylation of 5-carboxymethyl uridine to 5-methylcarboxymethyl uridine (Boccaletto et al., 2017). Similarly, mnme, mentioned earlier as important for the biosynthesis of 5-methylaminomethyl-2-thiouridine modification in E. coli, is homologous in humans and S. cerevisiae as MSS1 (Gao et al., 2019).
However, when *mmnE* was run through BLAST, no homology was observed in humans. Similarly, while a homolog for *tgt* was seen for *Homo sapiens* as *TGT* in Modomics, nothing was found through BLAST alignment. This could be because these genes are orthologous (when genes diverge after speciation, but the main function is conserved) or paralogous (genes are duplicated but the function and sequence composition may have changed) in humans (Haszprunar et al., 1992). Therefore, a next step would be to use BLAST to compare different species with these genes and compare which ones are most similar to each other in order to determine which types of homology are found in humans. In order to determine the homology, studying the taxonomy for these genes are also important. Meanwhile, *E. coli* genes for the tRNA modification enzymes were found to be homologous to some disease-causing organisms such as *Mycobacterium tuberculosis*, *Vibrio cholera*, *Haemophilus influenza*, *Streptococcus pneumonia* and *Salmonella enterica*. This is important since the specific writer enzymes that are also found in these other bacteria can also be explored as potential antibiotic targets. Similarly, amphenicol antibiotics may also be used as potential antibiotics to treat illnesses caused by these bacteria.

Lastly, when investigating the roles of human epitranscriptomic writers on cancer types using cBioPortal database, it was found that ALRYEF, CDKAL1, GTPBP3, METTL11B, METTL18 and METTL23 had high levels of amplification frequency; FTSJ1 had a high level of deep deletion frequency and METTL24 had high level of mutation frequency. ALYREF (*m^5^C*) is a methyltransferase reader enzyme that modifies position 48, 49 and 50 of tRNA (Yang et al., 2017, Boccaletto et al., 2017). CDKAL1 (*ms^2^t^6^A*) is a methylthiotransferase writer enzyme that modifies position 37 of tRNA (Wei et al., 2011). FTSJ1 (2’-O methyl ribose) is a methyltransferase writer enzyme that modifies position 32 and 34 of tRNA (Dimitrova et al., 2019). METTL11B, a methyltransferase-like 11B writer enzyme and METTLL18, a methyltransferase-like 18 writer
enzyme, had almost same levels of amplification frequency in Breast Mixed Ductal and Lobular Carcinoma, Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma among many other cancer types. This raises a question on whether these two writers are related or function with similar roles in the development of cancer cells. While the deep deletion frequency was low in different cancer types for ELP3 (mcm5's2U), an acetyltransferase enzyme that modifies position 34 of tRNA (Bjork et al., 2019), the highest type of alteration seen in each cancer type was deep deletion. Therefore, it can be assumed that ELP3 plays a big role in deep deletion for many different types of cancers.

Two common cancers that had high alteration frequency for many human epitranscriptomic writers were Bladder/ Urinary Tract cancer and Uterine Serous Carcinoma/ Uterine Papillary Serous Carcinoma. Bladder cancer is a specific Urinary Tract cancer and is one of the most common cancers in the world (Yaxley, 2016). Unlike most cancer types, the risk of having bladder cancer is not associated with family history (Yaxley, 2016). Rather, the most probable etiology of bladder cancer is found to be a mix between environmental and genetic factors (Yaxley, 2016). The biggest known risk factors for developing bladder cancer is smoking cigarette and everyday exposure for those working in “chemical and textile industries” (Yaxley, 2016). This disease is seen less frequently in women than men (Yaxley, 2016). Meanwhile, Uterine Serous Carcinoma (USC) is a rare variant (Type II) of endometrial cancer that includes less than 10% of endometrial cancers (Zhang et al., 2020). Despite this, USC causes almost 80% of all endometrial cancer deaths and is known to be a very aggressive variant (Zhang et al., 2020). It is most endemic in women 70 years and over in age (Zhang et al., 2020). Due to its poor prognosis, extremely aggressive effects and lack of proper treatment compared to other endometrial cancer, there has been a rapid upward trend in the number of deaths due to USC each year (Black et al., 2014; Zhang et al., 2020).
In general, it can be seen that human epitranscriptomic writers: ALRYEF, CDKAL1, FTSJ1, GTPBP3, METTL11B, METTL18, METTL23 and METTL24 have high levels of alteration frequencies for specific cancer types. Therefore, we can conclude that these writers could play an important role for those specific cancer developments, and hence can be used as a potential cancer treatment target, especially for cancers like USC where a typical cancer treatment such as chemotherapy and radiotherapy is not a great option.
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