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Identification of the enzymes responsible for m^{2,2}G and acp^{3}U formation on cytosolic tRNA from insects and plants

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Abstract

Posttranscriptional modification of tRNA is critical for efficient protein translation and proper cell growth, and defects in tRNA modifications are often associated with human disease. Although most of the enzymes required for eukaryotic tRNA modifications are known, many of these enzymes have not been identified and characterized in several model multicellular eukaryotes. Here, we present two related approaches to identify the genes required for tRNA modifications in multicellular organisms using primer extension assays with fluorescent oligonucleotides. To demonstrate the utility of these approaches we first use expression of exogenous genes in yeast to experimentally identify two *TRM1* orthologs capable of forming *N2,N2*-dimethylguanosine (m^{2,2}G) on residue 26 of cytosolic tRNA in the model plant *Arabidopsis thaliana*. We also show that a predicted catalytic aspartate residue is required for function in each of the proteins. We next use RNA interference in cultured *Drosophila melanogaster* cells to identify the gene required for m^{2,2}G$_{26}$ formation on cytosolic tRNA. Additionally, using these approaches we experimentally identify *D. melanogaster* gene *CG10050* as the corresponding ortholog of human *DTWD2*, which encodes the protein required for formation of 3-amino-3-propylcarboxyuridine (acp^{3}U) on residue 20a of cytosolic tRNA. We further show that *A. thaliana* gene *AT2G41750* can form acp^{3}U$_{20b}$ on an *A. thaliana* tRNA expressed in yeast cells, and that the aspartate and tryptophan residues in the DXTW motif of this protein are required for modification activity. These results demonstrate that these approaches can be used to study tRNA modification enzymes.

Introduction

Posttranscriptional modification of tRNA is required for efficient and accurate protein translation, and tRNA from all organisms is extensively modified [1]. In the model yeast

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Saccharomyces cerevisiae, defects in certain tRNA modifications can lead to temperature sensitivity, impaired cell growth, or even lethality [2]. Mutations in genes encoding enzymes responsible for tRNA modifications in humans, with their corresponding loss of modifications, cause diseases including intellectual disability [3], mitochondrial disorders [4–8], and familial disautonomia [9–11]. Additionally, genes encoding tRNA modification enzymes have been associated with diseases such as cancer and metabolic disorders [12, 13]. tRNA modifications have also been shown to be involved in response to stress and other environmental stimuli [14–17]. The study of tRNA modifications in multicellular model organisms such as the plant *Arabidopsis thaliana* and the insect *Drosophila melanogaster* has also recently added insight into the roles of modifications in development and disease [18–23]. Due to the roles of tRNA modifications in varied processes, identifying and characterizing the enzymes that form them is of importance in our understanding of gene expression and human health.

The roles that many post-transcriptional modifications play in tRNA function and stability have been identified [24, 25]. In general, modifications in and around the anticodon loop are critical for translational fidelity by ensuring proper tRNA charging [26–28], reading frame maintenance [29–31], and decoding of the RNA [32]. Modifications in the body of the tRNA are generally important for proper folding of the tRNA and for tRNA stability [33–36]. For example, eukaryotic tRNAs lacking certain body modifications undergo degradation by the 5' to 3' exonucleases Rat1 and Xrn1 through a process known as rapid tRNA decay (RTD) [33, 37]. This process is exacerbated by high temperature, primarily due to instability of the acceptor and T-stems [38–40]. Some of these modifications in the body of the tRNA enhance stability by preventing unwanted intramolecular base-paring and/or by modulating base-pairing. Thus, the $N2,N2$-dimethylguanosine ($m^{2,2}G$) modification found at position 26 of most G26:A44 base-pair containing eukaryotic tRNAs likely modulates non-canonical pairing with A44, and blocks alternative tRNA conformations by blocking base-pairing of G_{26} with other residues [41–44]. The $m^{2,2}G_{26}$ modification is formed on both cytosolic and mitochondrial tRNA by Trm1 in yeast [45, 46] and by its homolog TRMT1 in humans [47, 48]. Yeast *TRM1* and human *TRMT1* are encoded in the nucleus, but once expressed, localize to both the nucleus and the mitochondria [47, 49]. Defects in $m^{2,2}G_{26}$ cause temperature sensitivity in yeast [50], and intellectual disability in humans [51–54]. Additionally, correct pre-tRNA folding in the yeast *Schizosaccharomyces pombe* was shown to require $m^{2,2}G_{26}$ modification by Trm1, and/or La RNA chaperone activity [55].

Most eukaryotic tRNA modifications and the enzymes that form them have been identified experimentally, and both appear to be conserved throughout eukaryotes [1]. For example, of the 25 modifications known to occur on *Saccharomyces cerevisiae* cytoplasmic tRNA, 19 have been found on human cytoplasmic tRNA [1, 56]. To date, genes encoding the majority of the proteins required for all 25 of these modifications in *S. cerevisiae* have been identified [2]. Most of these genes were identified using one of two general approaches. In the first approach, yeast homologs were predicted by sequence homology to known tRNA modification enzymes, often from *Escherichia coli*, followed by modification analysis of tRNA from yeast knockout strains. This method was used to identify most of the yeast pseudouridine synthases [57–61], which convert uridine to pseudouridine on yeast tRNA, as well as most of the tRNA methyltransferases [62–68]. In the second approach, several other tRNA modification enzymes were discovered using proteomic libraries to detect in vitro enzymatic activity towards a tRNA substrate [69]. Yeast members of the tRNA dihydrouridine synthase family [70, 71], and three other tRNA methyltransferases [72–74] were discovered using this approach. After their discovery in yeast, many human tRNA modification enzymes have been identified in humans by sequence homology and direct experimental analysis of cells deficient for candidate genes [75].

Humans and other multicellular eukaryotes also contain tRNA modifications that are not found in yeast. For example, some plant and animal tRNAs contain the 3-amino-3-propylcarboxyuridine (acp^{3}U) at residues 20, 20a, or 20b on cytoplasmic tRNAs [1, 76]. For a description of tRNA numbering, please see reference [77]. The human enzymes responsible for acp^{3}U$_{20}$ and acp^{3}U$_{20a}$ were recently identified as DTWD1 and DTWD2, respectively [76]. Lack of these modifications in tandem resulted in slow growth of cultured human cells, suggesting a role for these modifications in translation [76]. This modification is also found at residue 47 of bacterial, mitochondrial, and plastid tRNAs [1]. In bacteria, this modification increases thermal stability [76]. The *E. coli* protein YfiP (now named TuaA or TapT) was recently identified as the enzyme that forms acp^{3}U$_{47}$ on bacterial tRNA [76, 78], and lack of this modification results in a motility defect and genome instability in cells [76].

In this report, we describe two different approaches that we use to experimentally identify the plant and insect orthologs of the Trm1 family of enzymes required for the m^{2,2}G$_{26}$ modification of cytosolic tRNA. The first approach involves co-expression of an exogenous tRNA and a candidate gene in yeast, followed by detection of the modification by fluorescent primer extension (Fig 1A). Using this approach we identify two *A. thaliana* genes that encode enzymes capable of forming m^{2,2}G$_{26}$ on cytosolic plant tRNA. In the second approach, we use RNA interference (RNAi) and primer extension analysis to identify the m^{2,2}G$_{26}$ enzyme in the model insect *D. melanogaster* (Fig 1B). We then use these approaches to identify orthologs of the newly discovered DTWD2 family of proteins that are required for the acp^{3}U$_{20a}$ tRNA modification of cytosolic tRNA in *D. melanogaster*, and that can form the acp^{3}U$_{20b}$ modification on an *A. thaliana* tRNA.

Materials and methods

Yeast strains and plasmids

Yeast strains used in this study are listed in Table 1. The BY4742 *trm1Δ::kanMX* strain was purchased from Dharmacon. The BY4742 *trm1Δ::kanMX met22Δ::ble^R* strain (yMG2129-4) was constructed by standard methods [37]. Plasmids used in this study are listed in Table 2. The *A. thaliana TRM1a* (*AT3G02320*), *TRM1b* (*AT5G15810*), *TRM1c* (*AT3G56330*), *DTWD2A* (*AT2G41750*), and *DTWD2B* (*AT5G54880*) yeast expression plasmids were constructed by PCR amplification of genes from an *A. thaliana* cDNA library [79] with primers
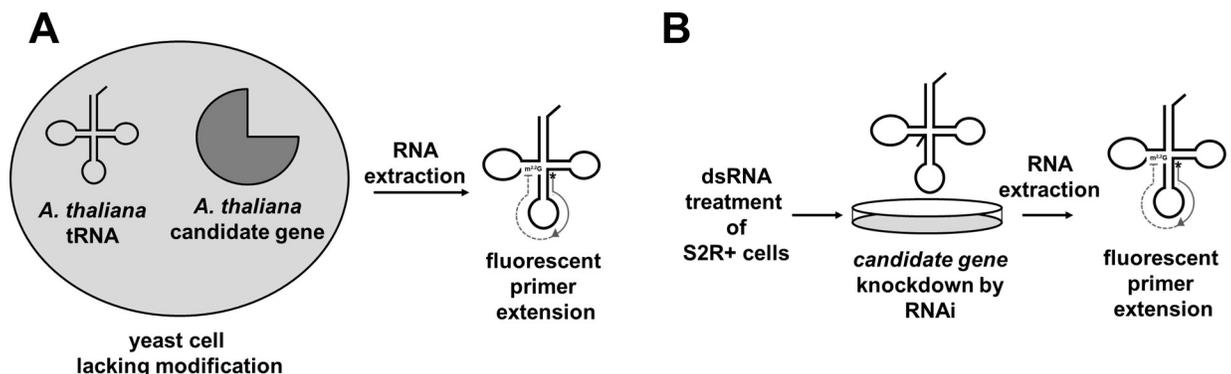
Fig 1. Schematic of approaches to identify tRNA modification enzymes. (A) Expression of tRNA and candidate genes in *S. cerevisiae*. An exogenous tRNA known or expected to receive the modification of interest, and a candidate modification gene are co-expressed in a yeast strain lacking the modification. RNA is extracted from the yeast and analyzed by primer extension with a fluorescent oligonucleotide specific for the tRNA to determine if the modification is present. **(B)** Analysis of *D. melanogaster* tRNA in dsRNA-treated S2R+ cells. S2R+ cells are treated with dsRNA complementary to the gene of interest, and tRNA is analyzed using fluorescent primer extension to detect the modification.

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Table 1. Yeast strains used in this study.

Strain	Genotype	Source
BY4742	*MATα his3-Δ1 leu2Δ0 met15-Δ0 ura3-Δ0*	Open Biosystems
BY4742 *trm1Δ*	BY4742, *trm1Δ::kanMX*	Dharmacon
yMG2129-4	BY4742, *trm1Δ::kanMX, mett22Δ::ble^R*	This study

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that added PacI and EagI cut sites, followed by digestion of the product with PacI and EagI and ligation into vector BG2596 [80]. Variants of *A. thaliana* genes were generated by performing site-directed mutagenesis on plasmids containing wild type genes using the Q5 site-directed mutagenesis kit (Promega) following manufacturer's instructions. The *A. thaliana* tRNA^Leu(AAG)-G37C plasmid was generated by PCR amplification of the tRNA gene from *A. thaliana* genomic DNA with primers that added XhoI and BglII sites, followed by digestion of the product with XhoI and BglII, and ligation into plasmid pMG24A [56] to replace the *S. cerevisiae* tRNA^Leu(UAA) gene, yielding plasmid pMG669B. A G37C mutation was then added by performing site-directed mutagenesis on plasmid pMG669B using Q5 site-directed mutagenesis to yield *A. thaliana* tRNA^Leu(AAG)-G37C (pMG671B).

RNA isolation

Yeast strains were grown in YPD or selective synthetic media to late log phase. For primer extension, qRT-PCR, and tRNA purification, bulk low molecular weight RNA was extracted using hot phenol as previously described [72]. RNA extraction from *D. melanogaster* S2R + cells was performed using TRIzol (Thermo Fisher) according to manufacturer's instructions.

Primer extension assays

For primer extension, 10–20 μmol 5' Tye665-labeled oligonucleotides (Integrated DNA Technologies) was annealed to 2–10 μg RNA, heated to 95°C and slow cooled to 37°C. Labeled oligonucleotides are listed in Table 3. The entire reaction was then incubated with 1 mM dNTPs and 1.89 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase. Reactions were incubated at 37°C overnight, and analyzed by 15% PAGE with 7M Urea. The gel was placed

Table 2. Plasmids used in this study.

Plasmid	Parent	Description	Source
pBG2596		2μ *URA3* P_GAL110 LIC	(Quartley et al. 2009)
pMG662B	pBG2596	2μ *URA3* P_GAL10 *A. thaliana TRM1a*	This study
pMG729E	pBG2596	2μ *URA3* P_GAL10 *A. thaliana TRM1a-D200A*	This study
pMG717A	pBG2596	2μ *URA3* P_GAL10 *A. thaliana TRM1b*	This study
pMG730E	pBG2596	2μ *URA3* P_GAL10 *A. thaliana TRM1b-D200A*	This study
pMG718A	pBG2596	2μ *URA3* P_GAL10 *A. thaliana TRM1c*	This study
pMG735B	pBG2596	2μ *URA3* P_GAL10 *A. thaliana TRM1c-D172A*	This study
pMG24a		2μ *LEU2 S. cerevisiae* tRNA^Leu(UAA)	(Guy et al. 2012)
pMG669B	pMG24a	2μ *LEU2 A. thaliana* tRNA^Leu(AAG)	This study
pMG671B	pMG669B	2μ *LEU2 A. thaliana* tRNA^Leu(AAG)-G37C	This study
pMG725A	pBG2596	2μ *URA3* P_GAL10 *A. thaliana DTWD2A*	This study
pMG726A	pBG2596	2μ *URA3* P_GAL10 *A. thaliana DTWD2B*	This study
pMG731C	pBG2596	2μ *URA3* P_GAL10 *A. thaliana DTWD2A-D133A*	This study
pMG732E	pBG2596	2μ *URA3* P_GAL10 *A. thaliana DTWD2A-W136A*	This study

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Table 3. Tye665-labeled oligonucleotides used for primer extension.

Primer	Target tRNA	Sequence	Target Nucleotides
MPG951	*S. cerevisiae* tRNA^{Tyr}	CGAACGCCCGATCTCAAGATT	55–37
MPG1012	*S. cerevisiae* tRNA^{Trp}	GTGAAACGGACAGGAATTGAACCTG	74–50
MPG1587	*A. thaliana* tRNA$^{Leu(AAG)}$-G37C	CTTTCGGACCAGAAGCTTAATCT	e5-29
MPG1772	*A. thaliana* tRNA$^{Leu(AAG)}$-G37C	CCTTTCGGACCAGAAGCTTAA	47–33
MPG1045	*D. melanogaster* tRNA^{Tyr}	GAACCAGCGACCTATGGATCTACAG	56–32
MPG1126	*D. melanogaster* tRNA$^{Val(CAC)}$	GAACCGGGGACCTTGTGCGTGTG	56–34

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between overhead projector sheets and visualized using a Typhoon 9200 scanner with a 620 BP30 Cy5 emission filter at high sensitivity and PMT between 600–800. Sequencing reactions were annealed similarly and extended overnight with the addition of 0.1mM ddNTP's. Primer MPG1772 was used for detection of m^{2,2}G$_{26}$ on *A. thaliana* tRNA$^{Leu(AAG)}$-G37C, and MPG1587 was used for detection of acp^{3}U$_{20b}$ on the same tRNA.

Northern blot analysis

Bulk RNA was analyzed by PAGE and transferred to a nitrocellulose membrane as previously described [33]. For detection by Northern blot, *S. cerevisiae* tRNA^{Trp} was detected with 5' Tye665-labeled probe MPG1012 and *A. thaliana* tRNA$^{Leu(AAG)}$-G37C was detected with MPG1587 (Table 3).

Quantitative Real Time PCR (qRT-PCR)

RNA was treated with RQ1 RNase-Free DNase (Promega) and then reverse transcribed using a Verso cDNA Kit (Thermo Scientific) using a 3:1 (v/v) mix of random hexamers and anchored oligo-dT primers. DNA was then PCR amplified using DyNAmo HS SYBR™ Green qPCR Kit (Thermo Scientific) master mix and primers specific to indicated genes. Primers used for qRT-PCR are listed in Table 4. In yeast, RNA was normalized to *ACT1*, and in *D. melanogaster*, RNA was normalized to *Act42A*.

tRNA purification

tRNA$^{Leu(AAG)}$-G37C was purified from yeast total RNA extracts using a 5'-biotinylated oligonucleotide with the sequence 5'-TGGTGTTGACAGTGGGATTTGAACCC-3', which is complementary to nucleotides 76–51 in the tRNA, as previously described (72). Briefly, biotinylated oligonucleotide bound to streptavidin magnetic particles was incubated with yeast RNA to capture tRNA$^{Leu(AAG)}$-G37C. Bound tRNA was then washed extensively to remove non-specific tRNA. Eluted tRNA was quantified using A$_{260}$ prior to LC-MS/MS analysis.

LC-MS/MS analysis of nucleosides

tRNA (2–4 μg) was digested to single nucleosides as previously described using nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase [81, 82]. The lyophilized samples were reconstituted in mobile phase A. Reversed phase chromatography was carried out with a high-strength silica column (Acquity UPLC HSS TS, 1.8 μm, 1.0 mm X 100 mm, Waters, Milford, MA) on an ultra-high-performance liquid chromatography (UHPLC) system (Vanquish Flex Quaternary, Thermo Fisher Scientific, San Jose, CA). Mobile phase A contained 5.3 mM ammonium acetate in water, pH 5.3, and mobile phase B contained a mixture of acetonitrile/water (40:60) with 5.3 mM ammonium acetate.

Table 4. Oligonucleotides used for qRT-PCR.

Primer	Organism	Target	Sequence
MPG1598	*S. cerevisiae*	*ACT1*	GAAATGCAAACCGCTGCTCA
MPG1599	*S. cerevisiae*	*ACT1*	TACCGGCAGATTCCAAACCC
MPG1756	*S. cerevisiae*	*TUB1*	CCAAGGGCTATTTACGTGGA
MPG1757	*S. cerevisiae*	*TUB1*	GGTGTAATGGCCTCTTGCAT
MPG1617	*A. thaliana*	*TRM1a*	GGGACTCATGTGAATCCGCT
MPG1618	*A. thaliana*	*TRM1a*	CGAACCAATGTGACGCGAAA
MPG1652	*A. thaliana*	*TRM1b*	CCACGTCGGTTCGCTTAGTA
MPG1653	*A. thaliana*	*TRM1b*	CGACCTGCCCTTATCTTGGG
MPG1654	*A. thaliana*	*TRM1c*	AATGAGATTGGGCTGCGGAT
MPG1655	*A. thaliana*	*TRM1c*	CATGTAGCTTCCCACGGTGA
MPG1266	*D. melanogaster*	*TRM1*	CGGAAGAAGGTCAAGGAACA
MPG1267	*D. melanogaster*	*TRM1*	GATCCGAAGTCCATCCTCATATC
MPG1635	*D. melanogaster*	*ACT42a*	CAAGAGTACGACGAGTCGGG
MPG1636	*D. melanogaster*	*ACT42a*	TTCGATGAGGAACGACCACG
MPG1706	*D. melanogaster*	*DTWD2A*	GTCCGTCATCAAACCGGACT
MPG1707	*D. melanogaster*	*DTWD2A*	GCATGGTAGGTGTGCTTTGC
MPG1708	*A. thaliana*	*DTWD2A*	TGGTCAAAGCTAGCGAGGTG
MPG1709	*A. thaliana*	*DTWD2A*	CTCCACTCACTGATGCGTCT
MPG1710	*A. thaliana*	*DTWD2B*	CAAAGAAGTACGACGGCAGC
MPG1711	*A. thaliana*	*DTWD2B*	TTCACCGATCTCCTTCATCGC

The gradient program consisted of: 0% B (from 0 to 7.6 min), 2% B at 15.7 min, 3% B at 19.2 min, 5% B at 25.7 min, 25% B at 29.5 min, 50% B at 32.3 min, 75% B at 36.4 min (hold for 0.2 min), 99% B at 39.6 min (hold for 7.2 min), then returning to 0% B at 46.9 min. After that, a re-equilibration step at 0% B for 18.1 min was employed prior to the next injection. A flow rate of 100 μL min^{-1} was used. The column temperature was set at 30˚C.

An Orbitrap Fusion^{TM} Lumos^{TM} Tribrid mass spectrometer (Thermo Fisher Scientific) interfaced with a heated electrospray (H-ESI) source was used for the identification and relative quantification of m^{2,2}G. All instrument parameters were as described previously [83], except that the collision-induced dissociation (CID) energy was set at 40%, and higher-energy collisional dissociation (HCD) energy was set at 80 arbitrary units.

Quantiva^{TM} triple quadrupole mass spectrometer interfaced with an H-ESI source (Thermo Fisher Scientific) was used for the identification and relative quantification of acp^{3}U. Instrument settings included detection in positive polarity with an H-ESI electrospray voltage of 3.8 kV, Ion Transfer Tube temperature at 290˚C, vaporizer temperature of 100˚C, sheath gas, auxiliary gas and sweep gas at 35, 10, 0 arbitrary units respectively. Collision gas pressure was 1.5 mTorr. Q1 and Q3 Resolution (FWHM) was set at 0.7 Da. Selected reaction monitoring (SRM) transitions for acp^{3}U were m/z 346–214, 346–197, 346–168 with collision energy (CE) of 20 V, RF lens was 58 V. SRM transition for U was m/z 245–113 with collision energy (CE) of 10 V and RF lens 30 V.

Data were analyzed using Qual browser of Xcalibur 3.0.

dsRNA generation

dsRNA for RNAi was generated by PCR amplification of the desired gene from *D. melanogaster* genomic DNA with primers containing 5' T7 promoter sites. Primers used for dsRNA generation are listed in Table 5. Regions for dsRNA were selected using the *Drosophila* RNAi

Table 5. Oligonucleotides used for D. *melanogaster* dsRNA generation.

Primer	Target	Sequence
MPG932	*TRM1*	GAATTAATACGACTCACTATAGGGAGATCTTTCCATGGTCCAAGAGG
MPG933	*TRM1*	GAATTAATACGACTCACTATAGGGAGAGATAATGCCGATTTGCGACT
MPG736	*TRM7*	GAATTAATACGACTCACTATAGGGAGAGAGTGCCTTCAAGTTGCTCC
MPG737	*TRM7*	GAATTAATACGACTCACTATAGGGAGAGCATCTGGGAGCTCAATAGC
MPG1698	*DTWD2A*	GAATTAATACGACTCACTATAGGGAGAACACCCTGCAGAGGAGAAGA
MPG1699	*DTWD2A*	GAATTAATACGACTCACTATAGGGAGAAGTCACTTATACCCACGGCG
MPG1700	*DTWD2A*	GAATTAATACGACTCACTATAGGGAGATCTACAAAGGCAAGCGGTTT
MPG1701	*DTWD2A*	GAATTAATACGACTCACTATAGGGAGAAGGCGGTGTTTCTAAGCAGA

Screening Center (DRSC) website to minimize off-target effects. The PCR product was analyzed by agarose gel, purified using a QIAquick gel extraction kit under low salt conditions (Qiagen), and 0.2 μg was used as a template for in vitro transcription using a T7 Megascript kit and purified according to manufacturer's instructions (Thermo Fisher).

D. *melanogaster* S2R+ cell culture and RNAi treatment

D. *melanogaster* S2R+ cells [84] were grown in Schneider's medium (Gibco) + 10% fetal bovine serum (FBS) (Gibco) at 27°C in culture flasks. RNAi was performed in 6-well plates as described on the DRSC website, essentially as previously described [85]. Briefly, 2×10^6 cells were plated to each well of a 6-well plate in 1 mL of serum free media, 10 μg of dsRNA was added to each well, and cells were incubated at room temperature for 30 minutes. After incubation, 3 mL of media + 10% FBS was added to each well. Cells were harvested after 3 days, or for multiple RNAi treatments, medium was removed, each well was resuspended in 1 mL of serum-free medium, and 250 μL of cells were plated in new wells already containing 750 μL serum-free medium. Cells were then treated with dsRNA as previously described and harvested or passaged again after 3 days.

Results and discussion

Primer extension assays using fluorescent oligonucleotides sensitively detect $m^{2,2}G_{26}$ on tRNA from yeast cells

We first determined whether fluorescently-labeled oligonucleotides could be used to sensitively detect tRNA modifications in yeast by primer extension [78, 86]. Thus, we used a Tye665 5'-end labeled oligonucleotide specific to *Saccharomyces cerevisiae* tRNA^{Tyr} (Fig 2A) to perform primer extension on RNA from wild type and *trm1Δ* cells, to detect the $m^{2,2}G_{26}$ modification. In a primer extension assay, the presence of a base-pair blocking modification such as $m^{2,2}G$ prohibits incorporation of a nucleotide complementary to the modified nucleotide by reverse transcriptase. Thus, presence of $m^{2,2}G_{26}$ results in a stop at residue 27 of the tRNA when analyzed by polyacrylamide gel electrophoresis (PAGE). We mixed wild type cells and *trm1Δ* cells in different ratios, extracted small molecular weight RNA, and then performed a primer extension assay specific to tRNA^{Tyr}. PAGE of the reactions showed a robust block at position 27 on tRNA^{Tyr} from wild type cells (Fig 2B), whereas a block at this position was not observed on tRNA^{Tyr} from *trm1Δ* cells. Instead, a larger band representing a primer extension block closer to the 5' end of tRNA^{Tyr} was observed, which corresponds to the string of dihydrouridines found in the D-loop of the tRNA (Fig 2A and 2B). Detection of $m^{2,2}G$ by this method appears to be sensitive, because a primer extension block corresponding to $m^{2,2}G_{26}$ could be easily detected in tRNA from wild-type cells mixed with *trm1Δ* cells at a ratio of 1/10,
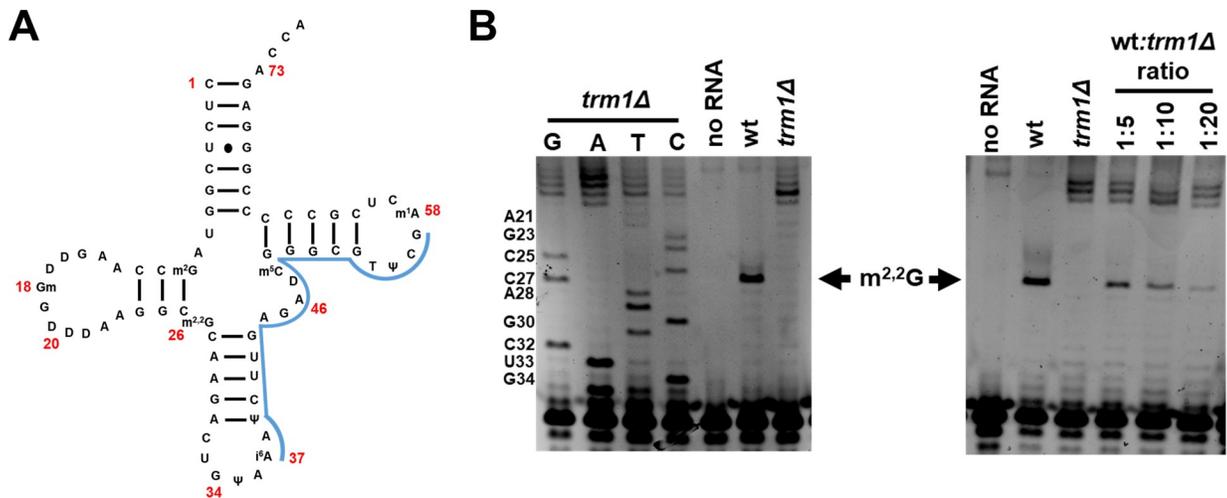
Fig 2. Detection of m^{2,2}G$_{26}$ by fluorescent primer extension in yeast cells. (A) Schematic of *S. cerevisiae* tRNA^{Tyr}. Location of primer binding is shown in blue. Selected nucleotides are numbered in red. **(B)** Detection of m^{2,2}G$_{26}$ by fluorescent primer extension in yeast cells. Left, bulk RNA was extracted from indicated strains and analyzed by primer extension to yeast tRNA^{Tyr}. Selected nucleotides from the sequencing reactions are provided on the left of the gel. Right, bulk RNA was extracted from culture of indicated individual strains or culture from mixtures of strains in indicated ratios, and then analyzed by primer extension to yeast tRNA^{Tyr}.

with detection at a ratio of 1/20 also possible (Fig 2B). Our ability to detect the m^{2,2}G$_{26}$ modification in wild-type cells from a background of *trm1Δ* cells indicates that this technique is sensitive enough to detect cells with a tRNA modification from a pool of cells lacking the modifications, which could be useful in the development of a screen to identify genes required for tRNA modifications. This sensitivity also further suggests that in many instances fluorescence could be a viable alternative to traditional primer extension [78, 86], which uses ^{32}P. ^{32}P has exquisite sensitivity, and is critical for the study of tRNA modifications. However, the use of radioactivity has several drawbacks, which include expense, safety concerns requiring extensive additional training of personnel, and a relatively short window in which the radioactivity can be used, due to the short half-life of ^{32}P [86].

Identification of *A. thaliana* TRM1 enzymes using yeast cells and primer extension

We next sought to identify tRNA modification genes from multicellular organisms by expressing the genes in yeast and using fluorescence-based primer extension to detect modification of the tRNA. This approach could provide some advantage over purely in vitro assays, because subcellular trafficking and localization of the protein in yeast could yield insights into the tRNA specificity of the enzyme. However, it is also possible that exogenously expressed proteins could be targeted incorrectly in yeast, thus confounding results. Nonetheless, this system provides a convenient method to rapidly determine if an enzyme has the ability to modify a given tRNA. To demonstrate the feasibility of this approach, we determined which predicted TRM1 enzymes can form the m^{2,2}G$_{26}$ modification on *Arabidopsis thaliana* tRNA. Interest in the study of tRNA modifications in *A. thaliana* has increased, with recent reports showing that tRNA modifications are involved in development [20, 87–89], disease resistance [18, 90], and stress response [23, 91, 92].

There are three known m^{2}G/m^{2,2}G$_{26}$ methyltransferases in eukaryotes, which include Trm1, Trm11, and Trm14 [45, 68, 93]. All three of these eukaryotic enzymes have a similar Rossman-fold methyltransferase domain, but unlike Trm11 and Trm14 proteins, Trm1

proteins lack a THUMP domain which is involved in tRNA binding [94]. Three predicted *TRM1* candidate genes have been identified in *A. thaliana* based on sequence homology [23] (S1 Fig), and the lack of a THUMP domain. To determine if these Trm1 proteins form $m^{2,2}G_{26}$, we co-expressed an *A. thaliana* tRNA with *A. thaliana TRM1* candidate genes in *S. cerevisiae*, and then detected $m^{2,2}G_{26}$ by fluorescent primer extension. To monitor $m^{2,2}G$ modification upon expression of *A. thaliana* candidate genes, we generated a high copy yeast expression plasmid encoding *A. thaliana* tRNA$^{Leu(AAG)}$ with a G37C mutation (tRNA$^{Leu(AAG)}$-G37C). The modification profile of cytosolic *A. thaliana* tRNA$^{Leu(AAG)}$ has not been determined, but cytosolic tRNA$^{Leu(AAG)}$ from the plant *Lupinus luteus* contains a $m^{2,2}G_{26}$ modification [95], and *A. thaliana* tRNA$^{Leu(AAG)}$ has a G at position 26 [1], suggesting that the *A. thaliana* tRNA should also receive the $m^{2,2}G_{26}$ modification in the presence of a TRM1 enzyme. Moreover, because *L. luteus* tRNA$^{Leu(AAG)}$ contains a 1-methylguanosine (m^1G) modification at residue 37, and *A. thaliana* tRNA$^{Leu(AAG)}$ contains a G37 [1], we added a G37C mutation to *A. thaliana* tRNA$^{Leu(AAG)}$ to ensure that G37 on the *A. thaliana* tRNA would not be modified to m^1G by yeast Trm5 [62] (Fig 3A). m^1G blocks base-pairing, and would therefore likely interfere with primer binding or primer extension in the assay.

We transformed yeast *trm1Δ* cells with the plasmid expressing cytosolic *A. thaliana* tRNA-$^{Leu(AAG)}$-G37C and with a high copy plasmid expressing *A. thaliana* gene *TRM1a* (*AT3G02320*) [23] under control of the P_{GAL} promoter, and then detected the $m^{2,2}G_{26}$ modification on tRNA$^{Leu(AAG)}$-G37C using a primer extension assay. Cells were grown in selective media containing galactose, and were grown at room temperature to decrease the likelihood that the tRNA$^{Leu(AAG)}$-G37C would be degraded by RTD [33, 40]. Northern blot analysis using a fluorescent oligonucleotide specific to *A. thaliana* tRNA$^{Leu(AAG)}$-G37C tRNA demonstrated that the tRNA was readily expressed from the plasmid (Fig 3B). Two bands of differing mobilities specific to tRNA$^{Leu(AAG)}$-G37C were detected. Expression of *TRM1a* mRNA in cells transformed with a *TRM1a* expression plasmid was confirmed by quantitative real-time PCR (qRT-PCR) (Table 6). Primer extension specific to *A. thaliana* tRNA$^{Leu(AAG)}$-G37C resulted in a primer extension block consistent with the m2,2G$_{26}$ modification in *trm1Δ* cells when *TRM1a* was expressed (Fig 3C), suggesting that TRM1a is a Trm1 ortholog in *A. thaliana*.

Because there are two other predicted *TRM1* genes in *A. thaliana* [23], we also tested the ability of *TRM1b* (*AT5G15810*) and *TRM1c* (*AT3G56330*) to form $m^{2,2}G$ on *A. thaliana* tRNA-$^{Leu(AAG)}$-G37C from high copy expression plasmids in yeast. Expression of *A. thaliana TRM1b* resulted in a primer extension block on *A. thaliana* tRNA$^{Leu(AAG)}$-G37C consistent with the presence of $m^{2,2}G_{26}$, whereas expression of *A. thaliana TRM1c* did not result in a block within the limits of our detection (Fig 3C). The lack of $m^{2,2}G$ formation on tRNA from cells expressing *TRM1c* was not due to lack of mRNA expression (Table 6). This data suggests that TRM1b is also a Trm1 ortholog. It is possible that TRM1c is responsible for the $m^{2,2}G_{26}$ modification found on several plastid tRNAs [1], because it is predicted to contain a chloroplast transit peptide and is predicted to localize to this cellular organelle [96, 97]. We cannot rule out the possibility that expression of *TRM1c* failed to generate a detectable primer extension block due to lack of protein expression and/or incorrect subcellular localization of the protein resulting from expression of this exogenous plant gene in yeast.

To further determine if the primer extension block observed upon the expression of TRM1b is due to Trm1 activity, we mutated a predicted active site aspartate residue [98] in Trm1a and in TRM1b (S1 Fig), expressed the variants in yeast, and measured modification activity by primer extension assay. Cells expressing a TRM1a or TRM1b variant containing an alanine substitution of the predicted active site aspartate (D200A) failed to generate a primer extension block (Fig 3C), even though the genes were expressed (Table 6). These data further support the conclusion that TRM1a and TRM1b are *A. thaliana* Trm1 orthologs.
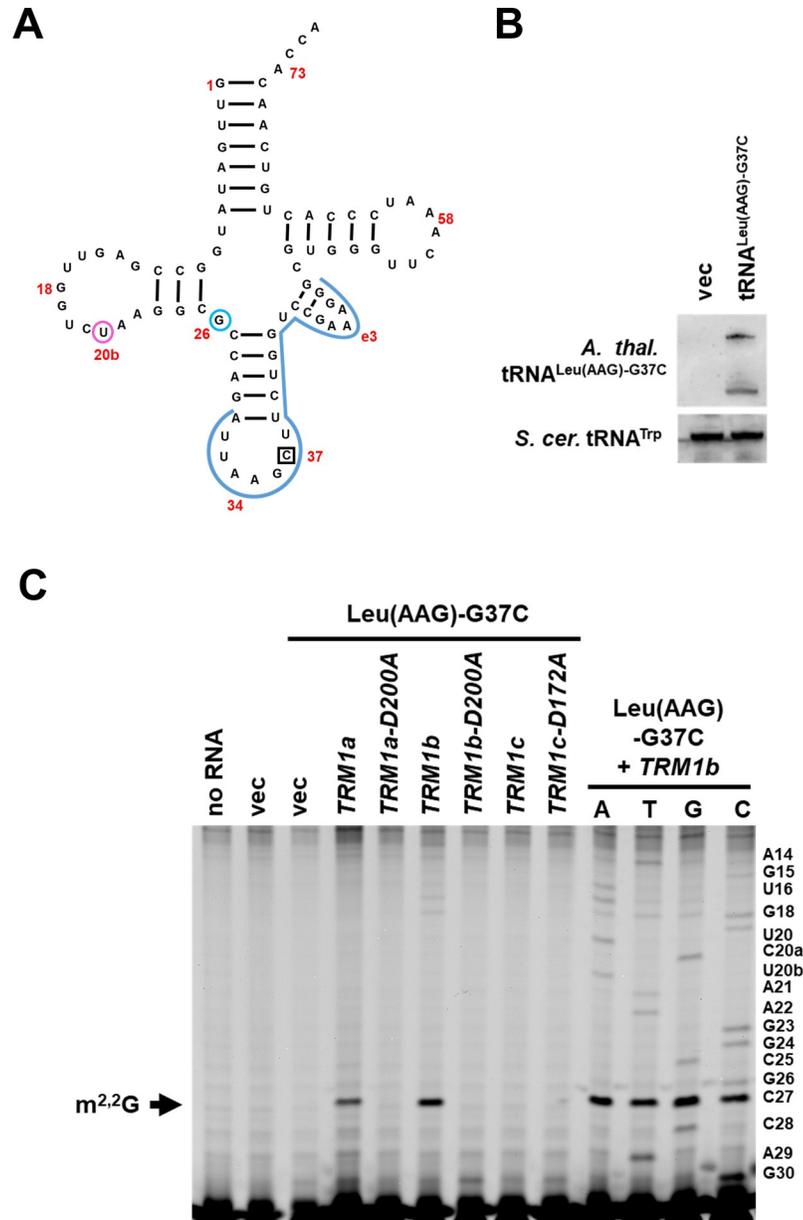
Fig 3. Identification of *TRM1* genes from *A. thaliana* in yeast cells. (A) Schematic of *A. thaliana* tRNA[Leu(AAG)]-G37C. Location of primer binding is shown in blue. G37C mutation is boxed. Predicted location of $m^{2,2}G_{26}$ (cyan) and acp^3U_{20b} (magenta) modifications are circled. Selected nucleotides are numbered in red. **(B)** *A. thaliana* tRNA[Leu(AAG)]-G37C is expressed in yeast cells. RNA was extracted from yeast expressing the indicated plasmid (vertical label) and analyzed by Northern blot to the indicated probe (horizontal label) as described in Materials and methods. **(C)** TRM1a or TRM1b expression results in a primer extension block consistent with $m^{2,2}G_{26}$ on *A. thaliana* tRNA. Bulk RNA was extracted from a *trm1Δ* yeast strain expressing the indicated plasmids and then analyzed by primer extension to *A. thaliana* tRNA[Leu(AAG)]-G37C.

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To verify that the primer extension block observed on *A. thaliana* tRNA[Leu(AAG)]-G37C at position 27 was due to the $m^{2,2}G_{26}$ modification, we purified tRNA[Leu(AAG)]-G37C in the presence or absence of TRM1a or TRM1b, and analyzed nucleotide content by liquid chromatography followed by mass spectrometry (LC-MS). To this end, tRNA[Leu(AAG)]-G37C was expressed in the presence or absence of *A. thaliana TRM1* genes in *trm1Δ mett22Δ* yeast,

Table 6. Relative mRNA levels of *A. thaliana* TRM1 homologs expressed in yeast.

A. thaliana gene	target mRNA	relative levels[a]
TRM1a	*TRM1a*	1.51 ± 0.34
TRM1a-D200A	*TRM1a*	8.76 ± 2.41
TRM1b	*TRM1b*	68.6 ± 35.2
TRM1b-D200A	*TRM1b*	32.4 ± 9.98
TRM1c	*TRM1c*	12.86 ± 5.11

[a]Relative to *S. cerevisiae* tubulin (*TUB1*), after both genes were normalized to *S. cerevisiae* actin (*ACT1*). Values are from three independent growths.

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purified using a biotinylated nucleotide specific to the tRNA, and then digested to nucleosides. The *met22Δ* mutation was included in these cells to inhibit the rapid tRNA decay pathway [37], which would be expected to reduce degradation of tRNA$^{Leu(AAG)}$-G37C and increase tRNA yields. Nucleosides were separated by reversed phase HPLC and identified using a high-resolution, accurate-mass (HRAM) orbitrap mass spectrometer [83]. The m^{2,2}G modification on tRNA$^{Leu(AAG)}$-G37C purified from cells expressing TRM1a or TRM1b was readily detected using this method, whereas levels of m^{2,2}G on the same tRNA purified from cells expressing a vector were near background (S2 Fig). We therefore conclude that *A. thaliana* TRM1a and TRM1b form m^{2,2}G on *A. thaliana* tRNA$^{Leu(AAG)}$.

Our finding that *A. thaliana* has two enzymes capable of forming cytosolic m^{2,2}G$_{26}$ is different than what has been observed in yeast and humans, which each have been shown to only encode one enzyme responsible for formation of cytosolic m^{2,2}G$_{26}$ [45, 47]. Although humans encode the TRMT1L protein, which is related to TRMT1 by sequence, it does not form m^{2,2}G$_{26}$ on cytosolic tRNA [47]. Human TRMT1L and *A. thaliana* TRM1c share only 22% sequence identity, whereas human TRMTL1 shares 27% identity with *A. thaliana* TRM1a, and *A. thaliana* TRM1c shares 27% identity with human TRMT1. This sequence data strongly suggests that TRMTL and TRM1c do not have similar functions. Homologs to *A. thaliana* TRM1c are found in a wide array of plant species with amino acid sequence identities ranging from 37% in the green algae *Micromonas pusilla* to 68% in *Carica papaya* (S3 Fig). Furthermore, as mentioned previously, TRM1c contains a predicted chloroplast transit peptide. Thus, it is highly likely that TRM1c is responsible for m^{2,2}G$_{26}$ modification of plastid tRNA.

Identification of the *D. melanogaster* TRM1 enzyme in cultured cells using RNA interference and primer extension

In a separate approach to identify tRNA modification enzymes in a multicellular eukaryote, we used RNA interference (RNAi) coupled with fluorescent primer extension to identify the *D. melanogaster* TRM1 enzyme. Robust RNAi can be achieved by treating cultured S2R+ cells with double stranded RNA (dsRNA) specific to the gene of interest generated directly from a PCR product [85, 99–101]. We tested the effect of RNAi to the predicted *D. melanogaster* TRM1 gene (*CG6388*), followed by primer extension to detect m^{2,2}G on tRNA^{Tyr}, which receives the modification (Fig 4A) [102]. We treated S2R+ cells with dsRNA to *TRM1* twice over the course of 6 days, harvested cells, extracted RNA, and used fluorescent primer extension specific to tRNA^{Tyr} to detect the presence of m^{2,2}G. Quantification of *TRM1* gene expression at day 6 by qRT-PCR revealed that *TRM1* mRNA levels were significantly reduced compared to untreated cells (Fig 4B), verifying that dsRNA treatment resulted in a significant reduction in gene expression. Primer extension specific to tRNA^{Tyr} from cells treated with no dsRNA or with dsRNA to *D. melanogaster* TRM7/FTSJ1 [99, 103] resulted in the presence of
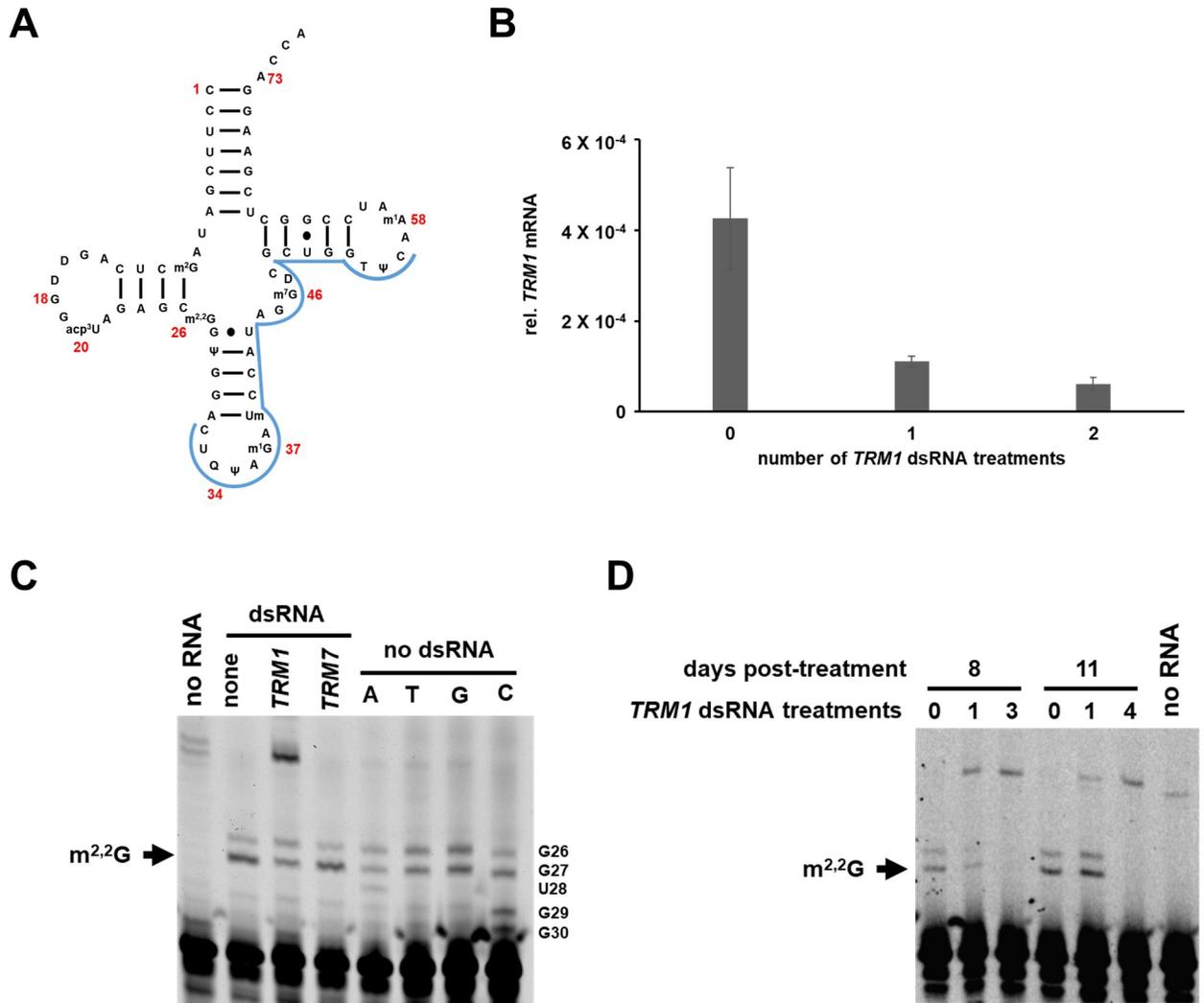
Fig 4. *CG6388* **encodes the TRM1 enzyme in *D. melanogaster*. (A)** Schematic of *D. melanogaster* tRNA^{Tyr}. Location of primer binding is shown in blue. Selected nucleotides are numbered in red. **(B)** *TRM1* mRNA levels significantly decrease in dsRNA-treated S2R+ cells. Cells were treated with dsRNA to *TRM1* as indicated and mRNA levels were measured by qRT-PCR. RNA levels are expressed relative to actin (*ACT42a*). **(C)** Knockdown of *CG6388* by RNAi results in loss of a primer extension block consistent with m^{2,2}G$_{26}$. S2R+ cells were treated twice over 6 days with dsRNA to indicated gene as described in Materials and methods. After harvest of cells, RNA was extracted and primer extension to tRNA^{Tyr} was performed. **(D)** Time course of *CG6388* knockdown by RNAi. S2R+ cells were treated as indicated, RNA was extracted, and primer extension to tRNA^{Tyr} was performed.

two primer extension blocks, the darker, bottom one of which was consistent with the size expected for an m^{2,2}G modification on residue 26 (Fig 4C). In contrast, primer extension analysis of tRNA^{Tyr} from cells treated with dsRNA to *TRM1* showed a marked decrease in the intensity of the bottom band of the doublet, with a corresponding appearance of a band of lesser mobility, consistent with the presence of the acp^{3}U modification found at position 20 of the tRNA [102] (Fig 4C, S4 Fig). No obvious difference in cell growth or morphology was observed between cells treated with either *TRM1* or *TRM7* dsRNA and untreated cells. The top band of the doublet is consistent with a pause at residue C$_{25}$, so although *D. melanogaster* tRNA^{Tyr} has G residues at positions 26 and 27, it does not appear that the doublet is due to m^{2,2}G modification of both residues 26 and 27, as has been observed for human tRNA^{Tyr} [104] and for tRNA^{Cys} from the eubacterium *Aquifex aeolicus* [105].

To further optimize RNAi for knockdown of tRNA modifications in S2R+ cells, we varied the number of dsRNA treatments over the course of 8 and 11 days, and harvested cells at different time points. We found that three dsRNA treatments of cells over the course of 8 days was sufficient to knock out nearly all detectable levels of the primer extension block corresponding to $m^{2,2}G$ (Fig 4D). Thus, our results demonstrate that *CG6388* encodes the TRM1 enzyme responsible for the $m^{2,2}G_{26}$ modification in *D. melanogaster*, because tRNA^{Tyr} in *D. melanogaster* has previously been shown to have an $m^{2,2}G_{26}$ modification [102], and because of the high sequence homology between this *D. melanogaster* protein and other bona fide Trm1 proteins. These results also suggest that this system could be used to rapidly screen candidate genes for novel tRNA modifications in *D. melanogaster*.

DTWD2 homologs are responsible for the acp^3U_{20a} modification in flies and plants

DTWD proteins were recently identified as the enzymes responsible for acp^3U modifications on eukaryotic tRNA [76]. DTWD2 was shown to be responsible for the acp^3U_{20a} tRNA modification in human cells, and homologs of these genes in other organisms were also identified by sequence [76]. We used our fluorescent primer extension approach to determine if DTWD2 genes are responsible for the acp^3U_{20a} modification found on tRNA from *D. melanogaster* and for the acp^3U_{20b} modification found on *A. thaliana* [1]. To this end, we silenced *CG10050*, which is the predicted DTWD2 homolog in *D. melanogaster* [76] (Fig 5A), and determined acp^3U_{20a} modification levels on tRNA$^{Val(CAC)}$ from these cells by primer extension (Fig 5B). A robust primer extension block on tRNA$^{Val(CAC)}$ of a size consistent with that expected for acp^3U_{20a} was observed on tRNA from untreated cells or cells treated with *TRM1* dsRNA (Fig 5C). In contrast, treatment of S2R+ cells with either of two different dsRNA constructs directed to *D. melanogaster DTWD2* resulted in a significant loss of the primer extension block (Fig 5C). No obvious difference in cell growth or morphology was observed between S2R+ cells treated with either *DTWD2* dsRNA construct and untreated cells. These results strongly suggest that *D. melanogaster DTWD2* forms acp^3U_{20a} in *D. melanogaster* cells.

We next sought to identify the *A. thaliana* enzyme responsible for acp^3U_{20b} formation on tRNA. In *A. thaliana*, DTWD2A (AT2G41750) and DTWD2B (AT5G54880) are the putative homologs of human DTWD2 [76] (Fig 5A). We therefore expressed each gene in *trm1Δ met22Δ* yeast cells with *A. thaliana* tRNA$^{Leu(AAG)}$-G37C, which is expected to receive the acp^3U_{20b} modification, because tRNA$^{Leu(AAG)}$ from *L. luteus* contains acp^3U_{20b} [95]. We used yeast cells containing the *trm1Δ* mutation to ensure that yeast Trm1 would not form the base-pair blocking $m^{2,2}G_{26}$ modification, and cells containing the *met22Δ* mutation to inhibit the rapid tRNA decay pathway [37]. We extracted RNA from the yeast cells and detected the formation of acp^3U_{20b} by fluorescent primer extension. We found that expression of DTWD2A resulted in a primer extension block on tRNA$^{Leu(AAG)}$-G37C when both the gene and the tRNA were expressed in yeast, consistent with the size expected for the acp^3U_{20b} modification (Fig 6). This primer extension block was absent in yeast cells expressing only the tRNA. The block was also not present within the limits of our detection in cells expressing DTWD2B and the tRNA (Fig 6, Table 7).

To verify that the primer extension block at position 21 on tRNA$^{Leu(AAG)}$-G37C was due to the acp^3U_{20b} modification, we performed LC-MS/MS on nucleosides from purified tRNA. tRNA nucleosides from *trm1Δ mett22Δ* cells expressing tRNA$^{Leu(AAG)}$-G37C with or without *DTWD2A* were analyzed by reverse phased LC coupled with a triple quadrupole mass spectrometer. Selected reaction monitoring (SRM) was used to monitor the mass transitions for acp^3U and U. We readily detected acp^3U on tRNA$^{Leu(AAG)}$-G37C purified from cells
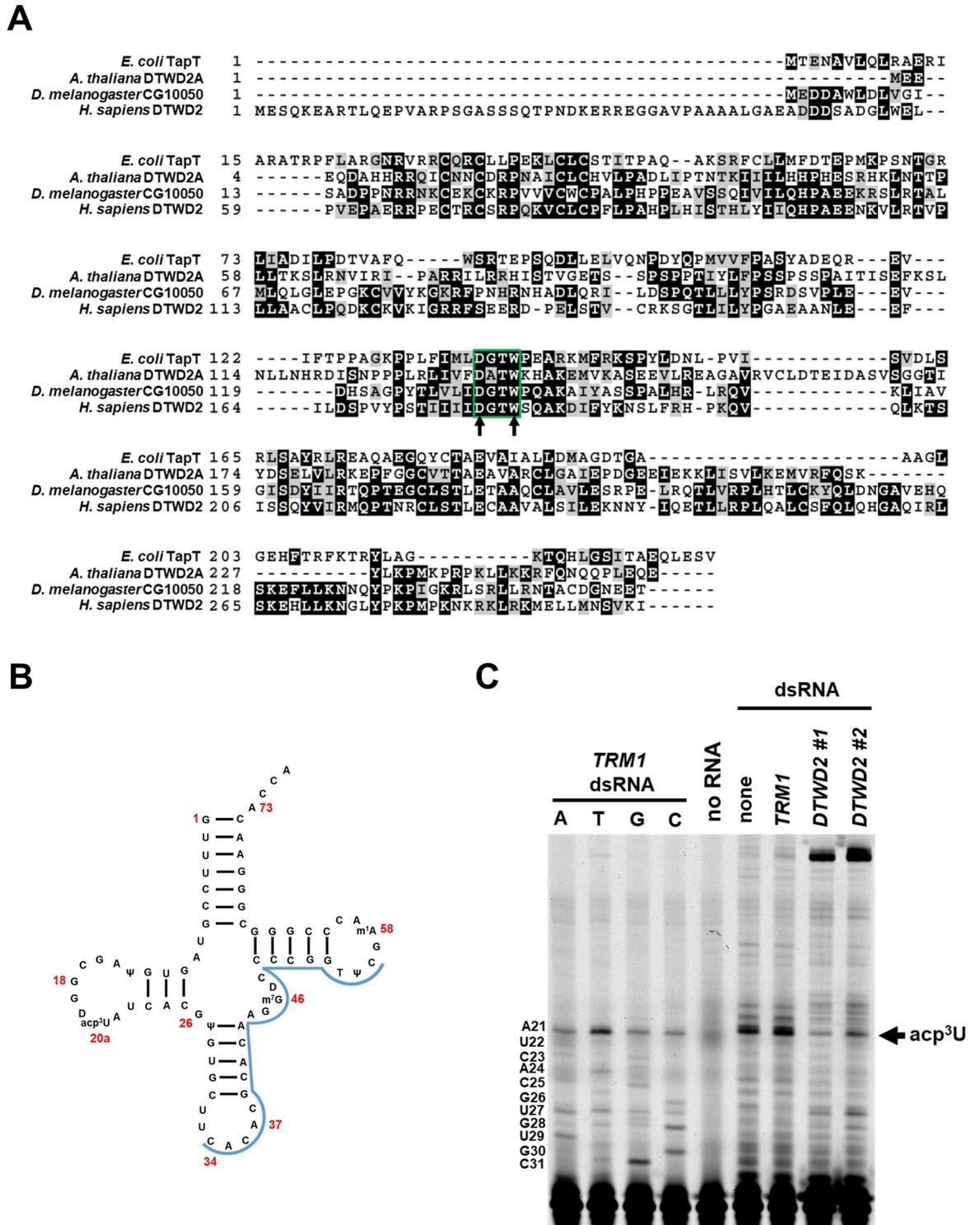
Fig 5. *CG10050* encodes the DTWD2A enzyme in *D. melanogaster*. (A) Sequence alignment of DTWD2 proteins. Green box represents the DTW domain, and arrows denote predicted active site residues. **(B)** Schematic of *D. melanogaster* tRNA$^{Val(CAC)}$. Location of primer binding is shown in blue. Selected nucleotides are numbered in red. **(C)** Knockdown of *CG10050* by RNAi results in loss of a primer extension block consistent with acp^{3}U$_{20b}$ on tRNA. S2R+ cells were treated three times over 8 days with dsRNA to indicated genes. After harvest of cells, RNA was extracted and primer extension to tRNA$^{Val(CAC)}$ was performed.

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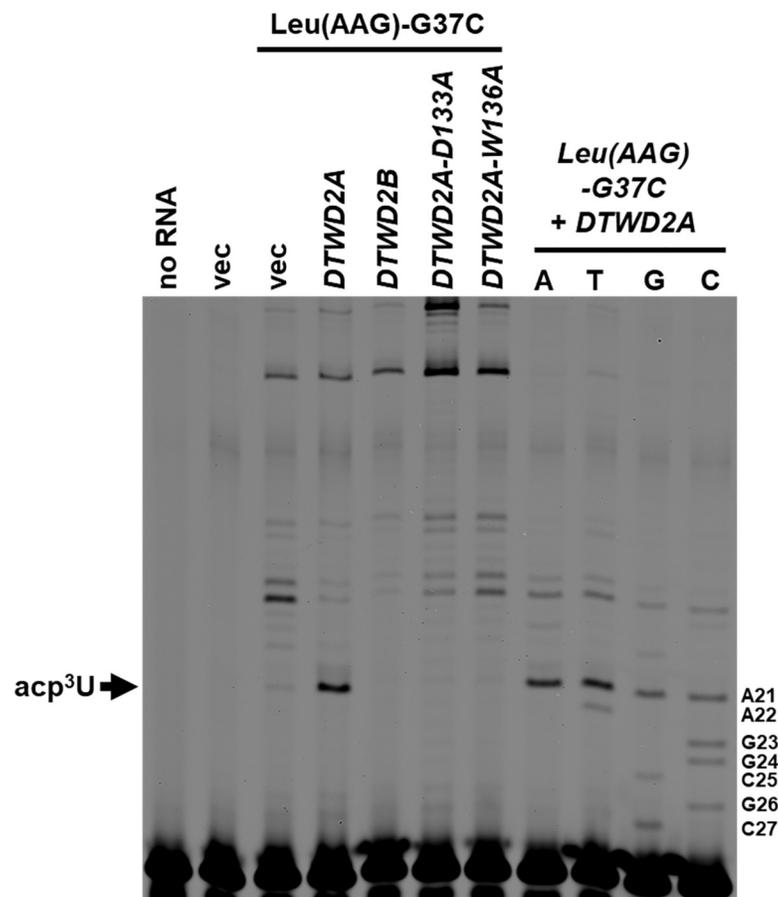
Fig 6. *A. thaliana* DTW2A expression results in a primer extension block consistent with acp^{3}U$_{20b}$ on *A. thaliana* tRNA. Bulk RNA was extracted from a *trm1Δ met22Δ* yeast strain expressing the indicated plasmids and then analyzed by primer extension to *A. thaliana* tRNA$^{Leu(AAG)}$-G37C.

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expressing DTWD2A, whereas levels of the modification on tRNA from cells expressing the vector control were near background levels (S5 Fig). To further verify that the observed LC peak was indeed acp^{3}U, the nucleoside digest from the DTWD2A expressing cells was compared to a total *E. coli* tRNA nucleoside digest. *E. coli* contains several tRNAs with the acp^{3}U modification [1]. The chromatogram and MS/MS spectra of acp^{3}U from purified tRNA^{Leu}$^{(AAG)}$-G37C match the retention time and fragment ratio of acp^{3}U from total *E. coli* tRNA (S6 and S7 Figs). Thus, we conclude that the primer extension block formed on tRNA$^{Leu(AAG)}$-G37C in the presence of DTWD2A is due to acp^{3}U.

Table 7. Relative mRNA levels of *A. thaliana* DTWD2 genes expressed in yeast.

A. thaliana gene	target mRNA	relative levels[a]
DTW2A	*DTW2A*	25.38 ± 2.05
DTWD2A-D133A	*DTWD2A*	12.12 ± 5.19
DTWD2A-W136A	*DTWD2A*	17.23 ± 7.35
DTW2B	*DTW2B*	39.23 ± 7.35

[a]Relative to *S. cerevisiae* tubulin (*TUB1*), after both genes were normalized to *S. cerevisiae* actin (*ACT1*). Values are from three independent growths.

https://doi.org/10.1371/journal.pone.0242737.t007

These data suggest that DTWD2A forms acp^3U_{20b} on plant tRNA, and are the first demonstration of an enzyme capable of forming an acp^3U modification at position 20b on a tRNA. This result is somewhat surprising, because *A. thaliana* DTWD2A is more closely related to human DTWD2 than is *A. thaliana* DTWD2B [76]. Whether DTWD2A forms the modification in plant cells, and whether it can also form acp^3U_{20a} is not clear. Additionally, we note that it is possible that DTWD2B is also capable of forming acp^3U_{20b}, but that the protein is not expressed well in yeast, or that it is incorrectly targeted in yeast cells.

DTWD2 belongs to the TDD superfamily of proteins, and is localized to the nucleus and the cytoplasm [78, 106, 107]. This protein superfamily contains *E. coli* TuaA/TapT, as well as Tsr3, which is required to add the acp group to the 1-methyl-3-(3-amino-3-carboxypropyl)-pseudouridine modification ($m^1acp^3\Psi$) found at residue U_{1191} on the yeast 18s rRNA and residue U_{1248} on human 18s rRNA. [78, 107]. TDD superfamily proteins contain a DTW domain with an E/DXS/TW motif [78, 106] (Fig 5A). In previously studied TDD proteins, the Asp/Glu and Trp residues of the motif are found in the active site and are required for catalysis. Indeed, Asp137 of this motif in *E. coli* TuaA/TapT is required for activity, and is likely the catalytic base, while Trp140 is required for activity and S-adenosyl methionine (SAM) cofactor binding [78, 107]. To determine if the corresponding Asp and Trp residues of the motif are required for the activity of a eukaryotic DTWD2 protein (Fig 5A), we generated D133A and W136A variants of *A. thaliana* DTW2a and tested their modification activity. We found that coexpression of either the DTWD2A-D133A or DTWD2A-W136A variants in yeast with tRNA^{Leu}$^{(AAG)}$-G37C did not result in the formation of a primer extension block consistent with acp^3U_{20b} (Fig 6, Table 7), whereas expression of wild type DTWD2A resulted in formation of the primer extension block. These results further suggest that DTWD2A is responsible for the acp^3U_{20b} modification found on *A. thaliana* tRNA. Because an in vitro assay for eukaryotic DTWD2 has not been reported, these results are the first experimental evidence that the Asp and Trp residues of the E/DXS/TW motif of a eukaryotic acp^3U tRNA modification enzyme are required for activity. Whether *A. thaliana* DTWD2B and/or DTWD2A are required for the acp^3U_{20a} modification of cytosolic tRNA, and whether the *A. thaliana* TapT-like enzyme is required for acp_3U_{47} modification on plastid tRNA remains to be determined [76].

Supporting information

S1 Fig. Sequence alignment of TRM1 proteins. Green arrow denotes location of predicted active site aspartate residue.
(PDF)

S2 Fig. *A. thaliana* TRM1a and TRM1b form $m^{2,2}G$ on *A. thaliana* tRNA$^{Leu(AAG)}$. Overlay of extracted ion chromatograms showing the abundance of $m^{2,2}G$ (*m/z* 312.131 ± 5 ppm) on *A. thaliana* tRNA$^{Leu(AAG)}$-G37C expressed in *trm1Δ* mutant yeast expressing TRM1a (green), TRM1b (red), or a vector control (blue).
(PDF)

S3 Fig. Sequence alignment of TRM1c homologs in various plants species.
(PDF)

S4 Fig. Knockdown of *CG6388* by RNAi results in loss of a primer extension block consistent with $m^{2,2}G_{26}$ and the appearance of a block consistent with acp^3U_{20}. S2R+ cells were treated twice over 6 days with dsRNA to indicated gene as described in Materials and methods. After harvest of cells, RNA was extracted and primer extension to tRNA^{Tyr} was performed. Sequencing was performed on cells treated with dsRNA to *CG6388* to determine the location

of the new primer extension block which appeared upon loss of the $m^{2,2}G_{26}$ block.
(PDF)

S5 Fig. *A. thaliana* DTWD2A is necessary for acp^{3}U modification on *A. thaliana* tRNA^{Leu} $^{(AAG)}$. Chromatogram overlays of acp^{3}U (top) and uridine (bottom) from *A. thaliana* tRNA^{Leu} $^{(AAG)}$-G37C expressed in yeast cells expressing DTWD2A (red) or a vector control (blue). Equal amounts of tRNA from indicated samples were analyzed by nucleoside LC-MS/MS. The abundance of acp^{3}U (top; mass transition 346 → 214, 346 → 197, 346 → 168) and uridine (bottom; mass transition 245 → 113) are shown.
(PDF)

S6 Fig. Comparison of acp^{3}U chromatograms from *E. coli* tRNA nucleosides and *A. thaliana* tRNA$^{Leu(AAG)}$ nucleosides. Chromatograms of acp^{3}U (mass transition 346 → 214, 346 → 197, 346 → 168) from *E. coli* tRNA nucleosides (black) and *A. thaliana* tRNA$^{Leu(AAG)}$-G37C nucleosides from cells expressing DTWD2A (red) are shown.
(PDF)

S7 Fig. Comparison of MS/MS of acp^{3}U in *E. coli* tRNA nucleosides and *A. thaliana* tRNA$^{Leu(AAG)}$ nucleosides. MS/MS of acp^{3}U (mass transition 346 → 214, 346 → 197, 346 → 168) from *E. coli* tRNA nucleosides (top) and *A. thaliana* tRNA$^{Leu(AAG)}$-G37C nucleosides from cells expressing DTWD2A (bottom) are shown.
(PDF)

S1 Raw images.
(PDF)

Acknowledgments

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References

1. Boccaletto P, Machnicka MA, Purta E, Piatkowski P, Baginski B, Wirecki TK, et al. MODOMICS: a database of RNA modification pathways. 2017 update. Nucleic Acids Res. 2018 Jan 4; 46(D1):D303–7. https://doi.org/10.1093/nar/gkx1030 PMID: 29106616

2. Hopper AK. Transfer RNA post-transcriptional processing, turnover, and subcellular dynamics in the yeast Saccharomyces cerevisiae. Genetics. 2013 May; 194(1):43–67. https://doi.org/10.1534/genetics.112.147470 PMID: 23633143

3. Angelova MT, Dimitrova DG, Dinges N, Lence T, Worpenberg L, Carré C, et al. The Emerging Field of Epitranscriptomics in Neurodevelopmental and Neuronal Disorders. Front Bioeng Biotechnol. 2018; 6:46. https://doi.org/10.3389/fbioe.2018.00046 PMID: 29707539

4. Suzuki T, Suzuki T, Wada T, Saigo K, Watanabe K. Taurine as a constituent of mitochondrial tRNAs: new insights into the functions of taurine and human mitochondrial diseases. EMBO J. 2002 Dec 2; 21 (23):6581–9. https://doi.org/10.1093/emboj/cdf656 PMID: 12456664

5. Yarham JW, Lamichhane TN, Pyle A, Mattijssen S, Baruffini E, Bruni F, et al. Defective i6A37 modification of mitochondrial and cytosolic tRNAs results from pathogenic mutations in TRIT1 and its substrate tRNA. PLoS Genet. 2014 Jun; 10(6):e1004424. https://doi.org/10.1371/journal.pgen.1004424 PMID: 24901367

6. Yasukawa T, Suzuki T, Ishii N, Ueda T, Ohta S, Watanabe K. Defect in modification at the anticodon wobble nucleotide of mitochondrial tRNA(Lys) with the MERRF encephalomyopathy pathogenic mutation. FEBS Lett. 2000 Feb 11; 467(2–3):175–8. https://doi.org/10.1016/s0014-5793(00)01145-5 PMID: 10675533

7. Yasukawa T, Suzuki T, Ueda T, Ohta S, Watanabe K. Modification defect at anticodon wobble nucleotide of mitochondrial tRNAs(Leu)(UUR) with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. J Biol Chem. 2000 Feb 11; 275(6):4251–7. https://doi.org/10.1074/jbc.275.6.4251 PMID: 10660592

8. Zeharia A, Shaag A, Pappo O, Mager-Heckel A-M, Saada A, Beinat M, et al. Acute infantile liver failure due to mutations in the TRMU gene. Am J Hum Genet. 2009 Sep; 85(3):401–7. https://doi.org/10.1016/j.ajhg.2009.08.004 PMID: 19732863

9. Anderson SL, Coli R, Daly IW, Kichula EA, Rork MJ, Volpi SA, et al. Familial dysautonomia is caused by mutations of the IKAP gene. Am J Hum Genet. 2001 Mar; 68(3):753–8. https://doi.org/10.1086/318808 PMID: 11179021

10. Cuajungco MP, Leyne M, Mull J, Gill SP, Lu W, Zagzag D, et al. Tissue-specific reduction in splicing efficiency of IKBKAP due to the major mutation associated with familial dysautonomia. Am J Hum Genet. 2003 Mar; 72(3):749–58. https://doi.org/10.1086/368263 PMID: 12577200

11. Karlsborn T, Tükenmez H, Chen C, Byström AS. Familial dysautonomia (FD) patients have reduced levels of the modified wobble nucleoside mcm(5)s(2)U in tRNA. Biochem Biophys Res Commun. 2014 21; 454(3):441–5. https://doi.org/10.1016/j.bbrc.2014.10.116 PMID: 25450681

12. Pereira M, Francisco S, Varanda AS, Santos M, Santos MAS, Soares AR. Impact of tRNA Modifications and tRNA-Modifying Enzymes on Proteostasis and Human Disease. Int J Mol Sci. 2018 Nov 24; 19(12). https://doi.org/10.3390/ijms19123738 PMID: 30477220

13. Torres AG, Batlle E, Ribas de Pouplana L. Role of tRNA modifications in human diseases. Trends Mol Med. 2014 Jun; 20(6):306–14. https://doi.org/10.1016/j.molmed.2014.01.008 PMID: 24581449

14. Blanco S, Bandiera R, Popis M, Hussain S, Lombard P, Aleksic J, et al. Stem cell function and stress response are controlled by protein synthesis. Nature. 2016 16; 534(7607):335–40. https://doi.org/10.1038/nature18282 PMID: 27306184

15. Chan CTY, Dyavaiah M, DeMott MS, Taghizadeh K, Dedon PC, Begley TJ. A quantitative systems approach reveals dynamic control of tRNA modifications during cellular stress. PLoS Genet. 2010 Dec 16; 6(12):e1001247.

16. Endres L, Dedon PC, Begley TJ. Codon-biased translation can be regulated by wobble-base tRNA modification systems during cellular stress responses. RNA Biol. 2015; 12(6):603–14. https://doi.org/10.1080/15476286.2015.1031947 PMID: 25892531

17. Wang Y, Li D, Gao J, Li X, Zhang R, Jin X, et al. The 2'-O-methyladenosine nucleoside modification gene OsTRM13 positively regulates salt stress tolerance in rice. J Exp Bot. 2017 01; 68(7):1479–91. https://doi.org/10.1093/jxb/erx061 PMID: 28369540

18. An C, Ding Y, Zhang X, Wang C, Mou Z. Elongator Plays a Positive Role in Exogenous NAD-Induced Defense Responses in Arabidopsis. Mol Plant Microbe Interact. 2016 May; 29(5):396–404. https://doi.org/10.1094/MPMI-01-16-0005-R PMID: 26926998

19. de Brouwer APM, Abou Jamra R, Körtel N, Soyris C, Polla DL, Safra M, et al. Variants in PUS7 Cause Intellectual Disability with Speech Delay, Microcephaly, Short Stature, and Aggressive Behavior. Am J Hum Genet. 2018 Dec 6; 103(6):1045–52. https://doi.org/10.1016/j.ajhg.2018.10.026 PMID: 30526862

20. Nakai Y, Horiguchi G, Iwabuchi K, Harada A, Nakai M, Hara-Nishimura I, et al. tRNA wobble modification affects leaf cell development in Arabidopsis thaliana. Plant Cell Physiol. 2019 May 10; https://doi.org/10.1093/pcp/pcz064 PMID: 31076779

21. Rojas-Benítez D, Eggers C, Glavic A. Modulation of the Proteostasis Machinery to Overcome Stress Caused by Diminished Levels of t6A-Modified tRNAs in Drosophila. Biomolecules. 2017 06; 7(1). https://doi.org/10.3390/biom7010025 PMID: 28272317

22. Tang J, Jia P, Xin P, Chu J, Shi D-Q, Yang W-C. AtTRM61/AtTRM6 complex is a bona fide tRNA N1-methyladenosine methyltransferase in Arabidopsis. J Exp Bot. 2020 Feb 25;

23. Wang Y, Pang C, Li X, Hu Z, Lv Z, Zheng B, et al. Identification of tRNA nucleoside modification genes critical for stress response and development in rice and Arabidopsis. BMC Plant Biol. 2017 21; 17 (1):261. https://doi.org/10.1186/s12870-017-1206-0 PMID: 29268705

24. El Yacoubi B, Bailly M, de Crécy-Lagard V. Biosynthesis and function of posttranscriptional modifications of transfer RNAs. Annu Rev Genet. 2012; 46:69–95. https://doi.org/10.1146/annurev-genet-110711-155641 PMID: 22905870

25. Väre VYP, Eruysal ER, Narendran A, Sarachan KL, Agris PF. Chemical and Conformational Diversity of Modified Nucleosides Affects tRNA Structure and Function. Biomolecules. 2017 16; 7(1). https://doi.org/10.3390/biom7010029 PMID: 28300792

26. Han L, Guy MP, Kon Y, Phizicky EM. Lack of 2'-O-methylation in the tRNA anticodon loop of two phylogenetically distant yeast species activates the general amino acid control pathway. PLoS Genet. 2018; 14(3):e1007288. https://doi.org/10.1371/journal.pgen.1007288 PMID: 29596413

27. Muramatsu T, Nishikawa K, Nemoto F, Kuchino Y, Nishimura S, Miyazawa T, et al. Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. Nature. 1988 Nov 10; 336(6195):179–81. https://doi.org/10.1038/336179a0 PMID: 3054566

28. Pütz J, Florentz C, Benseler F, Giegé R. A single methyl group prevents the mischarging of a tRNA. Nat Struct Biol. 1994 Sep; 1(9):580–2. https://doi.org/10.1038/nsb0994-580 PMID: 7634096

29. Bekaert M, Rousset J-P. An extended signal involved in eukaryotic -1 frameshifting operates through modification of the E site tRNA. Mol Cell. 2005 Jan 7; 17(1):61–8. https://doi.org/10.1016/j.molcel.2004.12.009 PMID: 15629717

30. Urbonavicius J, Qian Q, Durand JM, Hagervall TG, Björk GR. Improvement of reading frame maintenance is a common function for several tRNA modifications. EMBO J. 2001 Sep 3; 20(17):4863–73. https://doi.org/10.1093/emboj/20.17.4863 PMID: 11532950

31. Waas WF, Druzina Z, Hanan M, Schimmel P. Role of a tRNA base modification and its precursors in frameshifting in eukaryotes. J Biol Chem. 2007 Sep 7; 282(36):26026–34. https://doi.org/10.1074/jbc.M703391200 PMID: 17623669

32. Agris PF, Eruysal ER, Narendran A, Väre VYP, Vangaveti S, Ranganathan SV. Celebrating wobble decoding: Half a century and still much is new. RNA Biol. 2018; 15(4–5):537–53. https://doi.org/10.1080/15476286.2017.1356562 PMID: 28812932

33. Alexandrov A, Chernyakov I, Gu W, Hiley SL, Hughes TR, Grayhack EJ, et al. Rapid tRNA decay can result from lack of nonessential modifications. Mol Cell. 2006 Jan 6; 21(1):87–96. https://doi.org/10.1016/j.molcel.2005.10.036 PMID: 16387656

34. Hall KB, Sampson JR, Uhlenbeck OC, Redfield AG. Structure of an unmodified tRNA molecule. Biochemistry. 1989 Jul 11; 28(14):5794–801. https://doi.org/10.1021/bi00440a014 PMID: 2775736

35. Helm M, Giegé R, Florentz C. A Watson-Crick base-pair-disrupting methyl group (m1A9) is sufficient for cloverleaf folding of human mitochondrial tRNALys. Biochemistry. 1999 Oct 5; 38(40):13338–46. https://doi.org/10.1021/bi991061g PMID: 10529209

36. Kadaba S, Krueger A, Trice T, Krecic AM, Hinnebusch AG, Anderson J. Nuclear surveillance and degradation of hypomodified initiator tRNAMet in S. cerevisiae. Genes Dev. 2004 Jun 1; 18(11):1227–40. https://doi.org/10.1101/gad.1183804 PMID: 15145828

37. Chernyakov I, Whipple JM, Kotelawala L, Grayhack EJ, Phizicky EM. 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 May 15; 22(10):1369–80. https://doi.org/10.1101/gad.1654308 PMID: 18443146

38. Guy MP, Young DL, Payea MJ, Zhang X, Kon Y, Dean KM, et al. Identification of the determinants of tRNA function and susceptibility to rapid tRNA decay by high-throughput in vivo analysis. Genes Dev. 2014 Aug 1; 28(15):1721–32. https://doi.org/10.1101/gad.245936.114 PMID: 25085423

39. Payea MJ, Sloma MF, Kon Y, Young DL, Guy MP, Zhang X, et al. Widespread temperature sensitivity and tRNA decay due to mutations in a yeast tRNA. RNA. 2018; 24(3):410–22. https://doi.org/10.1261/rna.064642.117 PMID: 29259051

40. Whipple JM, Lane EA, Chernyakov I, D'Silva S, Phizicky EM. The yeast rapid tRNA decay pathway primarily monitors the structural integrity of the acceptor and T-stems of mature tRNA. Genes Dev. 2011 Jun 1; 25(11):1173–84. https://doi.org/10.1101/gad.2050711 PMID: 21632824

41. Bavi RS, Kamble AD, Kumbhar NM, Kumbhar BV, Sonawane KD. Conformational preferences of modified nucleoside N(2)-methylguanosine (m(2)G) and its derivative N(2), N(2)-dimethylguanosine (m(2)(2)G) occur at 26th position (hinge region) in tRNA. Cell Biochem Biophys. 2011 Dec; 61(3):507–21. https://doi.org/10.1007/s12013-011-9233-1 PMID: 21735129

42. Pallan PS, Kreutz C, Bosio S, Micura R, Egli M. Effects of N2,N2-dimethylguanosine on RNA structure and stability: crystal structure of an RNA duplex with tandem m2 2G:A pairs. RNA. 2008 Oct; 14 (10):2125–35. https://doi.org/10.1261/rna.1078508 PMID: 18772248

43. Steinberg S, Cedergren R. A correlation between N2-dimethylguanosine presence and alternate tRNA conformers. RNA. 1995 Nov; 1(9):886–91. PMID: 8548653

44. Urbonavicius J, Armengaud J, Grosjean H. Identity elements required for enzymatic formation of N2, N2-dimethylguanosine from N2-monomethylated derivative and its possible role in avoiding alternative conformations in archaeal tRNA. J Mol Biol. 2006 Mar 24; 357(2):387–99. https://doi.org/10.1016/j.jmb.2005.12.087 PMID: 16434050

45. Ellis SR, Morales MJ, Li JM, Hopper AK, Martin NC. Isolation and characterization of the TRM1 locus, a gene essential for the N2,N2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in Saccharomyces cerevisiae. J Biol Chem. 1986 Jul 25; 261(21):9703–9. PMID: 2426253

46. Hopper AK, Furukawa AH, Pham HD, Martin NC. Defects in modification of cytoplasmic and mitochondrial transfer RNAs are caused by single nuclear mutations. Cell. 1982 Mar; 28(3):543–50. https://doi.org/10.1016/0092-8674(82)90209-4 PMID: 7074684

47. Dewe JM, Fuller BL, Lentini JM, Kellner SM, Fu D. TRMT1-Catalyzed tRNA Modifications Are Required for Redox Homeostasis To Ensure Proper Cellular Proliferation and Oxidative Stress Survival. Mol Cell Biol. 2017 Nov 1; 37(21). https://doi.org/10.1128/MCB.00214-17 PMID: 28784718

48. Liu J, Strâby KB. The human tRNA(m(2)(2)G(26))dimethyltransferase: functional expression and characterization of a cloned hTRM1 gene. Nucleic Acids Res. 2000 Sep 15; 28(18):3445–51. https://doi.org/10.1093/nar/28.18.3445 PMID: 10982862

49. Li JM, Hopper AK, Martin NC. N2,N2-dimethylguanosine-specific tRNA methyltransferase contains both nuclear and mitochondrial targeting signals in Saccharomyces cerevisiae. J Cell Biol. 1989 Oct; 109(4 Pt 1):1411–9. https://doi.org/10.1083/jcb.109.4.1411 PMID: 2677019

50. Gustavsson M, Ronne H. Evidence that tRNA modifying enzymes are important in vivo targets for 5-fluorouracil in yeast. RNA. 2008 Apr; 14(4):666–74. https://doi.org/10.1261/rna.966208 PMID: 18314501

51. Blaesius K, Abbasi AA, Tahir TH, Tietze A, Picker-Minh S, Ali G, et al. Mutations in the tRNA methyltransferase 1 gene TRMT1 cause congenital microcephaly, isolated inferior vermian hypoplasia and cystic leukomalacia in addition to intellectual disability. Am J Med Genet A. 2018; 176(11):2517–21. https://doi.org/10.1002/ajmg.a.38631 PMID: 30289604

52. Davarniya B, Hu H, Kahrizi K, Musante L, Fattahi Z, Hosseini M, et al. The Role of a Novel TRMT1 Gene Mutation and Rare GRM1 Gene Defect in Intellectual Disability in Two Azeri Families. PLoS ONE. 2015; 10(8):e0129631. https://doi.org/10.1371/journal.pone.0129631 PMID: 26308914

53. Najmabadi H, Hu H, Garshasbi M, Zemojtel T, Abedini SS, Chen W, et al. Deep sequencing reveals 50 novel genes for recessive cognitive disorders. Nature. 2011 Sep 21; 478(7367):57–63. https://doi.org/10.1038/nature10423 PMID: 21937992

54. Zhang K, Lentini JM, Prevost CT, Hashem MO, Alkuraya FS, Fu D. An intellectual disability-associated missense variant in TRMT1 impairs tRNA modification and reconstitution of enzymatic activity. Hum Mutat. 2020 Mar; 41(3):600–7. https://doi.org/10.1002/humu.23976 PMID: 31898845

55. Vakiloroayaei A, Shah NS, Oeffinger M, Bayfield MA. The RNA chaperone La promotes pre-tRNA maturation via indiscriminate binding of both native and misfolded targets. Nucleic Acids Res. 2017 Nov 2; 45(19):11341–55. https://doi.org/10.1093/nar/gkx764 PMID: 28977649

56. Guy MP, Podyma BM, Preston MA, Shaheen HH, Krivos KL, Limbach PA, et al. Yeast Trm7 interacts with distinct proteins for critical modifications of the tRNAPhe anticodon loop. RNA. 2012 Oct; 18 (10):1921–33. https://doi.org/10.1261/rna.035287.112 PMID: 22912484

57. Ansmant I, Motorin Y, Massenet S, Grosjean H, Branlant C. Identification and characterization of the tRNA:Psi 31-synthase (Pus6p) of Saccharomyces cerevisiae. J Biol Chem. 2001 Sep 14; 276 (37):34934–40. https://doi.org/10.1074/jbc.M103131200 PMID: 11406626

58. Becker HF, Motorin Y, Planta RJ, Grosjean H. The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of psi55 in both mitochondrial and cytoplasmic tRNAs. Nucleic Acids Res. 1997 Nov 15; 25(22):4493–9. https://doi.org/10.1093/nar/25.22.4493 PMID: 9358157

59. Behm-Ansmant I, Urban A, Ma X, Yu Y-T, Motorin Y, Branlant C. The Saccharomyces cerevisiae U2 snRNA:pseudouridine-synthase Pus7p is a novel multisite-multisubstrate RNA:Psi-synthase also acting on tRNAs. RNA. 2003 Nov; 9(11):1371–82. https://doi.org/10.1261/rna.5520403 PMID: 14561887

60. Behm-Ansmant I, Grosjean H, Massenet S, Motorin Y, Branlant C. Pseudouridylation at position 32 of mitochondrial and cytoplasmic tRNAs requires two distinct enzymes in Saccharomyces cerevisiae. J Biol Chem. 2004 Dec 17; 279(51):52998–3006. https://doi.org/10.1074/jbc.M409581200 PMID: 15466869

61. Lecointe F, Simos G, Sauer A, Hurt EC, Motorin Y, Grosjean H. Characterization of yeast protein Deg1 as pseudouridine synthase (Pus3) catalyzing the formation of psi 38 and psi 39 in tRNA anticodon loop. J Biol Chem. 1998 Jan 16; 273(3):1316–23. https://doi.org/10.1074/jbc.273.3.1316 PMID: 9430663

62. Björk GR, Jacobsson K, Nilsson K, Johansson MJ, Byström AS, Persson OP. A primordial tRNA modification required for the evolution of life? EMBO J. 2001 Jan 15; 20(1–2):231–9. https://doi.org/10.1093/emboj/20.1.231 PMID: 11226173

63. Cavaillé J, Chetouani F, Bachellerie JP. The yeast Saccharomyces cerevisiae YDL112w ORF encodes the putative 2'-O-ribose methyltransferase catalyzing the formation of Gm18 in tRNAs. RNA. 1999 Jan; 5(1):66–81. https://doi.org/10.1017/s1355838299981475 PMID: 9917067

64. Kalhor HR, Clarke S. Novel methyltransferase for modified uridine residues at the wobble position of tRNA. Mol Cell Biol. 2003 Dec; 23(24):9283–92. https://doi.org/10.1128/mcb.23.24.9283-9292.2003 PMID: 14645538

65. Motorin Y, Grosjean H. Multisite-specific tRNA:m5C-methyltransferase (Trm4) in yeast Saccharomyces cerevisiae: identification of the gene and substrate specificity of the enzyme. RNA. 1999 Aug; 5 (8):1105–18. https://doi.org/10.1017/s1355838299982201 PMID: 10445884

66. Nordlund ME, Johansson JO, von Pawel-Rammingen U, Byström AS. Identification of the TRM2 gene encoding the tRNA(m5U54)methyltransferase of Saccharomyces cerevisiae. RNA. 2000 Jun; 6 (6):844–60. https://doi.org/10.1017/s1355838200992422 PMID: 10864043

67. Pintard L, Lecointe F, Bujnicki JM, Bonnerot C, Grosjean H, Lapeyre B. Trm7p catalyses the formation of two 2'-O-methylriboses in yeast tRNA anticodon loop. EMBO J. 2002 Apr 2; 21(7):1811–20. https://doi.org/10.1093/emboj/21.7.1811 PMID: 11927565

68. Purushothaman SK, Bujnicki JM, Grosjean H, Lapeyre B. Trm11p and Trm112p are both required for the formation of 2-methylguanosine at position 10 in yeast tRNA. Mol Cell Biol. 2005 Jun; 25 (11):4359–70. https://doi.org/10.1128/MCB.25.11.4359-4370.2005 PMID: 15899842

69. Jackman JE, Grayhack EJ, Phizicky EM. The use of Saccharomyces cerevisiae proteomic libraries to identify RNA-modifying proteins. Methods Mol Biol. 2008; 488:383–93. https://doi.org/10.1007/978-1-60327-475-3_25 PMID: 18982304

70. Xing F, Martzen MR, Phizicky EM. A conserved family of Saccharomyces cerevisiae synthases effects dihydrouridine modification of tRNA. RNA. 2002 Mar; 8(3):370–81. https://doi.org/10.1017/s1355838202029825 PMID: 12003496

71. Xing F, Hiley SL, Hughes TR, Phizicky EM. The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. J Biol Chem. 2004 Apr 23; 279(17):17850–60. https://doi.org/10.1074/jbc.M401221200 PMID: 14970222

72. Jackman JE, Montange RK, Malik HS, Phizicky EM. Identification of the yeast gene encoding the tRNA m1G methyltransferase responsible for modification at position 9. RNA. 2003 May; 9(5):574–85. https://doi.org/10.1261/rna.5070303 PMID: 12702816

73. Kotelawala L, Grayhack EJ, Phizicky EM. 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 Jan; 14(1):158–69. https://doi.org/10.1261/rna.811008 PMID: 18025252

74. Wilkinson ML, Crary SM, Jackman JE, Grayhack EJ, Phizicky EM. The 2'-O-methyltransferase responsible for modification of yeast tRNA at position 4. RNA. 2007 Mar; 13(3):404–13. https://doi.org/10.1261/rna.399607 PMID: 17242307

75. de Crécy-Lagard V, Boccaletto P, Mangleburg CG, Sharma P, Lowe TM, Leidel SA, et al. Matching tRNA modifications in humans to their known and predicted enzymes. Nucleic Acids Res. 2019 Mar 18; 47(5):2143–59. https://doi.org/10.1093/nar/gkz011 PMID: 30698754

76. Takakura M, Ishiguro K, Akichika S, Miyauchi K, Suzuki T. Biogenesis and functions of aminocarboxy-propyluridine in tRNA. Nat Commun. 2019 05; 10(1):5542. https://doi.org/10.1038/s41467-019-13525-3 PMID: 31804502

77. Sprinzl M, Horn C, Brown M, Ioudovitch A, Steinberg S. Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. 1998 Jan 1; 26(1):148–53. https://doi.org/10.1093/nar/26.1.148 PMID: 9399820

78. Meyer B, Immer C, Kaiser S, Sharma S, Yang J, Watzinger P, et al. Identification of the 3-amino-3-carboxypropyl (acp) transferase enzyme responsible for acp3U formation at position 47 in Escherichia coli tRNAs. Nucleic Acids Res. 2019 Dec 21.

79. Elledge SJ, Mulligan JT, Ramer SW, Spottswood M, Davis RW. Lambda YES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and Escherichia coli mutations. Proc Natl Acad Sci USA. 1991 Mar 1; 88(5):1731–5. https://doi.org/10.1073/pnas.88.5.1731 PMID: 1848010

80. Quartley E, Alexandrov A, Mikucki M, Buckner FS, Hol WG, DeTitta GT, et al. Heterologous expression of L. major proteins in S. cerevisiae: a test of solubility, purity, and gene recoding. J Struct Funct Genomics. 2009 Sep; 10(3):233–47. https://doi.org/10.1007/s10969-009-9068-9 PMID: 19701618

81. Ross R, Cao X, Yu N, Limbach PA. Sequence mapping of transfer RNA chemical modifications by liquid chromatography tandem mass spectrometry. Methods. 2016 01; 107:73–8. https://doi.org/10.1016/j.ymeth.2016.03.016 PMID: 27033178

82. Crain PF. Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry. Meth Enzymol. 1990; 193:782–90. https://doi.org/10.1016/0076-6879(90)93450-y PMID: 1706062

83. Jora M, Burns AP, Ross RL, Lobue PA, Zhao R, Palumbo CM, et al. Differentiating Positional Isomers of Nucleoside Modifications by Higher-Energy Collisional Dissociation Mass Spectrometry (HCD MS). J Am Soc Mass Spectrom. 2018; 29(8):1745–56. https://doi.org/10.1007/s13361-018-1999-6 PMID: 29949056

84. Yanagawa S, Lee JS, Ishimoto A. Identification and characterization of a novel line of Drosophila Schneider S2 cells that respond to wingless signaling. J Biol Chem. 1998 Nov 27; 273(48):32353–9. https://doi.org/10.1074/jbc.273.48.32353 PMID: 9822716

85. Zhang L, Ten Hagen KG. Dissecting the biological role of mucin-type O-glycosylation using RNA interference in Drosophila cell culture. J Biol Chem. 2010 Nov 5; 285(45):34477–84. https://doi.org/10.1074/jbc.M110.133561 PMID: 20807760

86. Ying B-W, Fourmy D, Yoshizawa S. Substitution of the use of radioactivity by fluorescence for biochemical studies of RNA. RNA. 2007 Nov; 13(11):2042–50. https://doi.org/10.1261/rna.637907 PMID: 17848413

87. Cui X, Liang Z, Shen L, Zhang Q, Bao S, Geng Y, et al. 5-Methylcytosine RNA Methylation in Arabidopsis Thaliana. Mol Plant. 2017 06; 10(11):1387–99. https://doi.org/10.1016/j.molp.2017.09.013 PMID: 28965832

88. John F, Philipp M, Leiber R-M, Errafi S, Ringli C. Ubiquitin-related modifiers of Arabidopsis thaliana influence root development. PLoS ONE. 2014; 9(1):e86862. https://doi.org/10.1371/journal.pone.0086862 PMID: 24466270

89. Philipp M, John F, Ringli C. The cytosolic thiouridylase CTU2 of Arabidopsis thaliana is essential for posttranscriptional thiolation of tRNAs and influences root development. BMC Plant Biol. 2014 Apr 28; 14:109. https://doi.org/10.1186/1471-2229-14-109 PMID: 24774365

90. Ramírez V, González B, López A, Castelló MJ, Gil MJ, Zheng B, et al. A 2'-O-Methyltransferase Responsible for Transfer RNA Anticodon Modification Is Pivotal for Resistance to Pseudomonas syringae DC3000 in Arabidopsis. Mol Plant Microbe Interact. 2018 Dec; 31(12):1323–36. https://doi.org/10.1094/MPMI-06-18-0148-R PMID: 29975160

91. Chen Z, Zhang H, Jablonowski D, Zhou X, Ren X, Hong X, et al. Mutations in ABO1/ELO2, a subunit of holo-Elongator, increase abscisic acid sensitivity and drought tolerance in Arabidopsis thaliana. Mol Cell Biol. 2006 Sep; 26(18):6902–12. https://doi.org/10.1128/MCB.00433-06 PMID: 16943431

92. Zhou W, Karcher D, Bock R. Importance of adenosine-to-inosine editing adjacent to the anticodon in an Arabidopsis alanine tRNA under environmental stress. Nucleic Acids Res. 2013 Mar 1; 41(5):3362–72. https://doi.org/10.1093/nar/gkt013 PMID: 23355609

93. Menezes S, Gaston KW, Krivos KL, Apolinario EE, Reich NO, Sowers KR, et al. Formation of m2G6 in Methanocaldococcus jannaschii tRNA catalyzed by the novel methyltransferase Trm14. Nucleic Acids Res. 2011 Sep 1; 39(17):7641–55. https://doi.org/10.1093/nar/gkr475 PMID: 21693558

94. Fislage M, Roovers M, Tuszynska I, Bujnicki JM, Droogmans L, Versées W. Crystal structures of the tRNA:m2G6 methyltransferase Trm14/TrmN from two domains of life. Nucleic Acids Res. 2012 Jun; 40(11):5149–61. https://doi.org/10.1093/nar/gks163 PMID: 22362751

95. Barciszewska M, Keith G, Dirheimer G, Mashkova T, Kubli E, Barciszewski J. The primary structure of six leucine isoacceptor tRNAs of yellow lupin seeds. The structural requirements for amber tRNA suppressor activity. Biochim Biophys Acta. 1990 Jan 30; 1048(1):78–84. https://doi.org/10.1016/0167-4781(90)90025-w PMID: 2297534

96. Berardini TZ, Reiser L, Li D, Mezheritsky Y, Muller R, Strait E, et al. The Arabidopsis information resource: Making and mining the "gold standard" annotated reference plant genome. Genesis. 2015 Aug; 53(8):474–85. https://doi.org/10.1002/dvg.22877 PMID: 26201819

97. Emanuelsson O, Nielsen H, von Heijne G. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 1999 May; 8(5):978–84. https://doi.org/10.1110/ps.8.5.978 PMID: 10338008

98. Awai T, Ochi A, Ihsanawati null, Sengoku T, Hirata A, Bessho Y, et al. Substrate tRNA recognition mechanism of a multisite-specific tRNA methyltransferase, Aquifex aeolicus Trm1, based on the X-ray crystal structure. J Biol Chem. 2011 Oct 7; 286(40):35236–46. https://doi.org/10.1074/jbc.M111.253641 PMID: 21844194

99. Angelova MT, Dimitrova DG, Da Silva B, Marchand V, Jacquier C, Achour C, et al. tRNA 2'-O-methylation by a duo of TRM7/FTSJ1 proteins modulates small RNA silencing in Drosophila. Nucleic Acids Res. 2020 28; 48(4):2050–72. https://doi.org/10.1093/nar/gkaa002 PMID: 31943105

100. Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, et al. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc Natl Acad Sci USA. 2000 Jun 6; 97(12):6499–503. https://doi.org/10.1073/pnas.110149597 PMID: 10823906

101. Kulkarni MM, Booker M, Silver SJ, Friedman A, Hong P, Perrimon N, et al. Evidence of off-target effects associated with long dsRNAs in Drosophila melanogaster cell-based assays. Nat Methods. 2006 Oct; 3(10):833–8. https://doi.org/10.1038/nmeth935 PMID: 16964256

102. Suter B, Altwegg M, Choffat Y, Kubli E. The nucleotide sequence of two homogeneic Drosophila melanogaster tRNATyr isoacceptors: application of a rapid tRNA anticodon sequencing method using S-1 nuclease. Arch Biochem Biophys. 1986 May 15; 247(1):233–7. https://doi.org/10.1016/0003-9861(86)90552-7 PMID: 3010877

103. Guy MP, Phizicky EM. Conservation of an intricate circuit for crucial modifications of the tRNAPhe anticodon loop in eukaryotes. RNA. 2015 Jan; 21(1):61–74. https://doi.org/10.1261/rna.047639.114 PMID: 25404562

104. van Tol H, Stange N, Gross HJ, Beier H. A human and a plant intron-containing tRNATyr gene are both transcribed in a HeLa cell extract but spliced along different pathways. EMBO J. 1987 Jan; 6 (1):35–41. PMID: 3502708

105. Awai T, Kimura S, Tomikawa C, Ochi A, Ihsanawati null, Bessho Y, et al. Aquifex aeolicus tRNA (N2, N2-guanine)-dimethyltransferase (Trm1) catalyzes transfer of methyl groups not only to guanine 26 but also to guanine 27 in tRNA. J Biol Chem. 2009 Jul 31; 284(31):20467–78. https://doi.org/10.1074/jbc.M109.020024 PMID: 19491098

106. Burroughs AM, Aravind L. Analysis of two domains with novel RNA-processing activities throws light on the complex evolution of ribosomal RNA biogenesis. Front Genet. 2014; 5:424. https://doi.org/10.3389/fgene.2014.00424 PMID: 25566315

107. Meyer B, Wurm JP, Sharma S, Immer C, Pogoryelov D, Kötter P, et al. Ribosome biogenesis factor Tsr3 is the aminocarboxypropyl transferase responsible for 18S rRNA hypermodification in yeast and humans. Nucleic Acids Res. 2016 19; 44(9):4304–16. https://doi.org/10.1093/nar/gkw244 PMID: 27084949