FEBS Letters

FEBS PRESS science publishing by scientists

Aggregation of Mod5 is affected by tRNA binding with implications for tRNA gene-mediated silencing

David F. Read[1], Thomas J. Waller[1], Eric Tse[1,2], Daniel R. Southworth[1,2], David R. Engelke[1,3] and Philip J. Smaldino[1,4]

1 Biological Chemistry, University of Michigan, Ann Arbor, MI, USA
2 Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA
3 University of Colorado Denver/Anschutz Medical Campus, Aurora, CO, USA
4 Biology, Ball State University, Muncie, IN, USA

Correspondence
David R. Engelke, University of Colorado Denver/Anschutz Medical Campus, Mail Stop C296 Academic Office 1, L15-1503, 12631 E. 17th Avenue, Aurora, CO 80045, USA
Fax: +1 303 556 4776
Tel: +1 303 556 3259
E-mail: david.engelke@ucdenver.edu
and
Philip J. Smaldino, Ball State University, Cooper Life Sciences Building, CL073Muncie, IN 47306, USA
Fax: +1 765 285 8804
Tel: +1 765 285 3675
E-mail: pjsmaldino@bsu.edu

The first two authors of this manuscript (DFR and TJW) contributed equally.

(Received 22 February 2017, revised 9 March 2017, accepted 13 March 2017, available online 22 May 2017)

doi:10.1002/1873-3468.12627

Edited by Ned Mantei

Mod5 is a multifunctional protein that modifies a subset of tRNAs in the cytoplasm and is also required for an RNA-mediated form of transcriptional silencing. Previous *in vivo* studies have shown that the nuclear silencing function of Mod5 does not require that the causative tRNA gene encode a Mod5 substrate, although Mod5 is still required. However, previous data have not directly tested whether Mod5 can directly bind substrate and nonsubstrate RNAs. We herein demonstrate that Mod5 directly binds to both substrate and nonsubstrate RNAs, including a highly structured, non-tRNA sequence (5S-rRNA), consistent with previous *in vivo* data. Furthermore, we show that some RNAs drastically change the aggregation behavior of Mod5 with implications for tRNA gene-mediated silencing.

Keywords: amyloid fiber; isopentenyl transferase; Mod5; protein aggregation; RNA binding; tRNA modification

tRNA isopentenyl transferases are highly conserved enzymes that transfer an isopentenyl group from dimethylallylpyrophosphate (DMAPP) to the N6 position of A37, adjacent to the anticodon [1–9].The *Saccharomyces cerevisiae* enzyme, Mod5, resides primarily in the cytoplasm where it modifies mitochondrial and cytoplasmic tRNAs [10–12]. Mod5 modifies a subset of tRNAs with specific sequence requirements (AAA) surrounding the target nucleotide at A37 [13,14]. Substrates in yeast and humans [15] include Tyr−, Ser−, Trp−, and Cys-isoacceptor tRNAs. This modification on a tRNA increases tRNA stability [16] and the fidelity and efficiency of translation—an effect related to the tRNA's affinity for the ribosome [2,5,17,18].

A small fraction of the enzyme also resides in the nucleus [19,20], where it is required for a form of

Abbreviations
DMAPP, dimethylallylpyrophosphate; EM, electron microscopy; GMSA, gel mobility shift assay; RSC, remodel the structure of chromatin; tgm, tRNA gene-mediated.

transcriptional silencing, termed tRNA gene-mediated (tgm) silencing [11,21–23]. tgm silencing was originally observed in budding yeast as antagonizing pol II gene promoters adjacent to tRNA genes [24]. The silencing does not involve steric interference, was shown to occur upstream or downstream of the tRNA gene [24], and is dependent on histone modifications and chromatin remodeling [25]. This phenomenon also requires the clustering of the tRNA genes to the nucleolus, demonstrating a role for nuclear architecture and localization [23,26,27]. Deletion of the *MOD5* gene relieves silencing of a pol II promoter adjacent to a tRNA gene on a reporter plasmid [21,23]. Recently, a form of tgm silencing has been proposed to exist in human cells [28].

Mod5 involvement in tgm silencing mechanisms was further confirmed by the following observations [22]: (a) Mod5 protein is physically associated with tRNA gene loci, (b) Mod physically associates with pol III transcription complex proteins, (c) Mod5 copurifies with multiple proteins of a remodel the structure of chromatin (RSC) and histones that affect tgm silencing, and (d) Mod5 coimmunoprecipitates with precursor-tRNAs (pre−tRNA) that are thought to be found exclusively in the nucleus. Interestingly, Mod5 was found to coimmunoprecipitate with both substrate and nonsubstrate tRNAs for isopentenylation, but preferentially with corresponding nuclear pre−tRNAs in both types of RNA. This was consistent with the observation that tRNA genes encoding substrate or nonsubstrate pre-tRNAs are both capable of conferring tgm silencing [22], suggesting that the specificity of Mod5 for enzymatic modification of tRNA in the cytoplasm is distinct from that of its nuclear role in transcriptional silencing. Lastly, truncation of the pre-tRNA transcripts by early termination compromised tgm silencing, suggesting the mechanism required a tRNA-like transcript for efficiency.

Based on these data, we hypothesize that complexes between Mod5 and nascent pre−tRNAs at the site of transcription might recruit chromatin modification and remodeling proteins that affect local chromatin structure to impart silencing of nearby transcription by RNA polymerase II. This mechanism would require that Mod5 binds pre−tRNAs whether or not they are substrates for modification at A37, which has not previously been shown. Testing this was complicated by the prion-like tendency of Mod5 to aggregate in solution and *in vivo,* in agreement with recent studies showing that Mod5 forms heritable amyloid-like aggregates [29]. It is possible to select for this heritable aggregation *in vivo* by growth in certain fungicides that require loss of Mod5 modifications of tRNAs (e.g., fluconazole). However, this aggregation of Mod5 affects only cytoplasmic tRNA modification and not the nuclear silencing function [11,22].

In this study, we show for the first time that Mod5 directly binds to both substrate and non−substrate RNAs *in vitro*, as well as the corresponding primary transcript pre-tRNAs. Further, binding to tRNA-like molecules facilitates aggregation of Mod5. We propose a model for tgm silencing that includes binding of pre-tRNAs by Mod5.

Materials and methods

Recombinant Mod5 expression and purification

The endogenous Mod5 ORF was amplified by PCR from a genomic DNA preparation of yeast BY4741 cells using primers with *Nde*1 and *Xho*1 sequences. PCR products were digested with *Nde*1 and *Xho*1 and ligated into the pet15B vector, a bacterial expression plasmid with a 6× histidine C−terminal tag. The plasmid was transformed into BL21 *Escherichia coli* by electroporation using a Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA) Gene Pulser at 2.5 kV, 25 μF, 200 Ohms (http://springerlab.tch.harvard.edu/springer/uploads/Equipment/Genepulsermanual.pdf). Eight liters of *E. coli* was grown to OD (600 nm) 0.55 in LB + 100 $\mu g \cdot mL^{-1}$ ampicillin (37 °C) and then induced with 1.5 mM IPTG (5 h, 17 °C). Cells were pelleted at 2620 RCF, 15 min, 4 °C, and frozen at −80 °C. Pellets were resuspended in 120 mL buffer (50 mM Tris/HCl, 50 mM NaCl, pH 7.5) on ice. Cells were lysed using a Divitech Microfluidizer (model 110Y) using two passes with 200-μm orifice cartridge and then 8 passes with 100-μm cartridge. Lysate was centrifuged at 5470 RCF, 15 min, 4 °C, and the supernatant combined with 60-mL packed, pre-equilibrated BioRex 70 (Bio-Rad Laboratories, Inc.) cation exchange resin (50 mM Tris/HCL, 50 mM NaCl, pH 7.5). The lysate was allowed to bind for 1 h (4 °C) and spun for 5 min at 152 RCF, and the supernatant was discarded. Resin was washed twice with one volume buffer (200 mM NaCl, 50 mM Tris/HCl, pH 7.5). Protein was eluted with two resuspensions using 500 mM NaCl, 50 mM Tris/HCl, pH 7.5 using one packed resin volume. Elutions were loaded onto a 5 mL packed column of TALON nickel affinity resin at 4 °C. The column was washed with 50 mL buffer (300 mM NaCl, 20 mM $NaPO_4$, pH 7.0) and eluted with 5.5 mL 450 mM imidazole, 50 mM $NaPO_4$, 300 mM NaCl, pH 8.0. Eluent was run on an 80 mL Sephacryl S-100 column (2.7 cm diameter × 19.1 cm height) using 50 mM Tris/HCl, 50 mM NaCl, pH 7.5 buffer at a rate of 1 mL flow-through per minute at 4 °C. Seven hundred and fifty microliter fractions were collected, examined by SDS/PAGE, and then the 10 highest concentration fractions were combined, aliquoted, and stored at −80 °C.

DNA template synthesis

Template DNA fragments for transcription (Fig. S2) were ordered through Integrated DNA Technologies. Lyophilized DNA pellets were dissolved in 10 mM Tris/HCl containing 1 mM EDTA, to a concentration of 100 μM. Equal volumes of overlapping, complementary strand pairs were combined and hybridized at 37 °C overnight. Ten microliters of the 50 μM solutions of hybridized DNA was then added to a solution with a final concentration of 0.4 μM DNTPs, 10 mM Tris/HCl, pH 7.4, 50 mM KCl, 1.5 mM $MgCl_2$, and up to 5 units Taq DNA polymerase [30] in a final volume of 50 μL. The solutions were then incubated at 35 °C for 5 min, 45 °C for 5 min, 55 °C for 5 min, 65 °C for 30 min, and 37 °C for 15 min, ethanol-precipitated, resuspended in 15 μL of water, and stored at −20 °C.

In vitro transcription and RNA purification

For each transcription reaction, 200 μL solutions of 50 mM Tris/HCl pH 7.5, 15 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 2 mM ATP, UTP, GTP, CTP, 2/25 of volume template (see above), and empirically titrated His6−tagged T7 RNA polymerase [31] were incubated 12 h at 42 °C. Solutions were centrifuged at 15 400 RCF for 5 min to remove pyrophosphate precipitate, and the supernatants were ethanol-precipitated, gel−purified through a 12% polyacrylamide gel, visualized by shadowing with long-wave UV light, and passively eluted out of gel slices. RNA was ethanol-precipitated, resuspended in 1× TE (10 mM Tris/HCl pH 7.5, 0.1 mM EDTA), and stored at −80 °C. Radioactive transcripts were prepared as above, except 0.5 mM ATP containing 10 μCi radioactive α−ATP (Perkin−Elmer, Waltham, MA, USA) was substituted in the above reaction and $MgCl_2$ was reduced to 12 mM.

End-labeling of 5S-rRNA

5S-rRNA was prepared as described above. RNA was incubated with calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA, USA; 20 μL reaction, 1 unit CIP, 17 pmol 5S-rRNA, 1× final (New England Biolabs) cutSmart buffer) for 30 min at 37 °C. RNA was diluted to 50 μL using H_2O, then extracted twice, first with 50 μL of 25 : 24 : 1 phenol : chloroform : isoamyl alcohol, and then once with 50 μL of chloroform. RNA was ethanol-precipitated and then resuspended for radioactive end-labeling (50 μL volume, 1× (New England Biolabs) polynucleotide kinase buffer, 20 units PNK (New England Biolabs), 10 μCi radioactive gamma ^{32}P−ATP). The reaction was incubated for 30 min at 37 °C, extracted and ethanol-precipitated, and then resuspended in 50 μL of 200 mM NaCl.

Gel mobility shift assays

Sixteen micromolar Mod5 was serially diluted threefold into buffer (50 mM Tris/HCl pH 7.2, 50 mM NaCl), in 10 μL aliquots. Next, for all tRNA, 5 μL of ^{32}P−labeled RNA (~ 100 CPM·μL^{-1}) diluted in 3× binding buffer (24 mM Tris/HCl pH 7.2, 150 mM NaCl, 15 mM $MgCl_2$) was added to each tube and incubated at 37 °C for 30 min. For the 5S binding, ~ 36 000 CPM·μL^{-1} was used, approximately a sixfold higher RNA concentration than for tRNA binding. After incubation, 3 μL of 6× loading buffer (60% glycerol, 0.1% xylene cyanol) was added and 10 μL was run on an 8% native polyacrylamide gel in TBE buffer at 30 mA (1 mm thick gel, 7 cm length, Aquebogue Machine Shop Model 200). Gels were dried overnight, exposed on Phosphorimager screens overnight, and imaged on a Typhoon 9210 Imager (GE Healthcare, Chicago, IL, USA). Band intensities were analyzed using ImageQuant software, and statistical analysis was performed with GRAPHPAD Prism (GraphPad Software, Inc., La Jolla, CA, USA).

RNA competition assays

Ten micromolar of 20 μM unlabeled competitor RNAs was titrated using twofold serial dilutions in 1× TE (10 mM Tris/HCl pH 7.5, 0.1 mM EDTA) to a final volume of 5 μL each. Five microliters of ^{32}P-labeled pre−Tyr RNA (~ 100 CPM·μL^{-1}) diluted in 3× binding buffer (24 mM Tris/HCl pH 7.2, 150 mM NaCl, 15 mM $MgCl_2$) was added to unlabeled competitor RNA tubes. Five microliters of 16 μM purified Mod5 was added to the above mixture for a final Mod5 concentration of 5.3 μM, 15 μL final reaction volume. Samples were incubated at 37 °C for 30 min; then, 3 μL of 6× loading buffer (60% glycerol, 0.1% xylene cyanol) was added, and samples were run on an 8% native polyacrylamide gel in TBE buffer at low power (30 mA, 1 mm thick gel, 7 cm length, Aquebogue Machine Shop Model 200). Gels were analyzed as above. IC_{50} values and confidence intervals were calculated using GRAPHPAD Prism. Data were fit to

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(x - \text{Log}_{10}(\text{IC}_{50}))}}$$

'Top' was given a constraint of ≤ 100% bound, while 'Bottom' was constrained to ≥ 0%.

Isopentenylation assay

Reactions were performed in 58 mM Tris/HCl pH 7.2, 1.2 mM ATP, 5.8 mM $MgCl_2$, ± 0.2 mM DMAPP, 10 U SuperRNase−In (Thermo Fisher Scientific, Waltham, MA, USA), 40 000 CPM of internally ^{32}P−labeled RNA, 5.3 μM Mod5, 1.2 mM β-mercaptoethanol in a 17 μL final reaction volume. Reactions were incubated at 37 °C for 1 h, and

RNAs were ethanol-precipitated. RNA pellets were washed one time with 70% ethanol, air-dried, and resuspended in 10 μL of 8 M urea, and 150 U of RNase T1 (Roche) was added to each. Samples were incubated overnight at 37 °C. Next, 2 μL of 6× loading buffer (60% glycerol, 0.1% xylene cyanol) was added to each and 10 μL was loaded onto a 20% polyacrylamide, 7 M urea denaturing gel (pre−run at 20 mA, for 2 h, 1 mm thick gel, 70 cm length, Aquebogue Machine Shop Model 200). Samples were run at 25 mA, 1 mm thick gel, 7 cm length, Aquebogue Machine Shop Model 200, 2 h. The gel was exposed on a phosphorscreen for 3 h and imaged on Typhoon 9210 (GE Healthcare).

Light scattering assay

Samples (7.5 μM Mod5, ± 7.5 μM RNA, in 125 mM NaCl, 5 mM potassium phosphate, 12.5 mM Tris/HCl, pH 7.4), 100 μL final volume, were loaded into a 96−well Costar plate. A_{405} was recorded using a Tecan Safire II at 37 °C. Readings were performed in duplicate and normalized to buffer-only A_{405}.

Fluorescent microscopy

Imaging used an Olympus IX70 inverted microscope with a CoolSNAP HQ2 monochrome camera, 100× oil immersion lens. Mod5 was incubated with a fluorescein-conjugated RNA samples (1 μM Fl−pre−Ser-tRNA, 7.5 μM Mod5, 125 mM NaCl, 5 mM potassium phosphate, 12.5 mM Tris/HCl, pH 7.4, 20 μL volume) for 1.5 h in humid conditions at 37 °C on No. 1.5 glass coverslips. Imaging used 492 nm excitation, 535 nm emission.

Electron microscopy

Eighteen micromolar Mod5, ± 7.5 μM pLeu/mLeu/pTyr/mTyr/5S-rRNA, 75 mM NaCl, 3 mM $NaPO_4$, 7.5 mM Tris/HCl (pH 7.4) was incubated for 24 h at 37 °C and sonicated briefly [32]. Samples were prepared using conventional methods for negative stain preparation [33]. Imaging was at room temperature using a Morgagni 268 at 100 kV. Images were acquired at 22 000× with an Orius 2000 CCD camera with a pixel size of 2.4 A.

Results

Mod5 directly binds to substrate and nonsubstrate RNAs

In our *in vitro* assays, we worked with three tRNAs that are predicted substrates for Mod5−modification: mat−Tyr−tRNA (mature tRNA with 5′ leader, intron and 3′ trailer removed), pre−Ser−tRNA (with 5′ leader and 3′ trailer), and mat−Ser−tRNA (with leader and trailer sequences removed). We also used nonsubstrate mature tRNAs (mat−Leu−tRNA), nonsubstrate pre−tRNAs (pre−Tyr−tRNA, pre−Leu−tRNA, and pre−Ala−tRNA), and nonsubstrate 5S rRNA (Table S1). Mat−Tyr, pre−Ser, and mat−Ser−tRNAs contain the $AAA_{36–38}$ sequence requirement for modification, while the intron in the pre−Tyr−tRNA disrupts the AAA and thus is not predicted to be modified by the enzyme at A37. The mat−Leu, pre−Leu, mat−Ala, and pre−Ala−tRNA sequences do not contain the required AAA at the anticodon loop, nor an A at position 37, and are therefore predicted to be nonsubstrates for isopentenylation (Table S1).

To confirm these predictions and the activity of the isolated Mod5 enzyme, we performed isopentenyl transferase assays in the presence or absence of DMAPP substrate and each RNA. The modification reaction was followed by the digestion of the products with RNase T1 (which cleaves on the 3′ side of guanosine residues leaving a 3′GMP) and analysis of oligonucleotide fragments on denaturing polyacrylamide gels. The transfer of a dimethylallyl/isopentenyl group from DMAPP to the RNA would result in a DMAPP-dependent shift of the indicated RNase T1−fragment containing the modified base (Fig. S1). As predicted, mat−Tyr, mat−Ser, and pre-Ser tRNAs are modified at the oligo containing the A37 position (Fig. 1A), while pre-Ala, pre−Tyr−tRNA, pre−Leu−tRNA, and Leu−tRNA are not modified (Fig. 1B; summarized in Table S1). These data confirm the Mod5 substrate specificity in our recombinant enzyme [1,3,5] and show that the enzyme is capable of recognizing the folded substrates used for *in vitro* binding assays. We note that we see an additional 7mer oligonucleotide that becomes modified in the substrate mat-Ser and pre-Ser-tRNAs in the presence of Mod5 and DMAPP (Fig. 1A). A possible explanation for this is that there is high sequence similarity in this 7 nt fragment compared to the 10 nt A37-containing fragment (GAAUCC compared to AAAUCC in the A37-containing fragment). Furthermore, although these two sequences reside in different arms of the tRNA, they are at similar relative positions within their respective arms (Fig. S3). The lack of a third A in the 7 nt fragment as well as differences in positioning within the tRNA might explain the differences in efficiency of modification between the two, in which only ~ 30% of the 7 nt fragment becomes modified under the conditions used compared to nearly 100% in the A37-containing 10 nt fragment.

Gel mobility shift assay (GMSA) analyses demonstrate that Mod5 binds to both substrate and nonsubstrate tRNAs and pre−tRNAs, as well as 5S RNA,

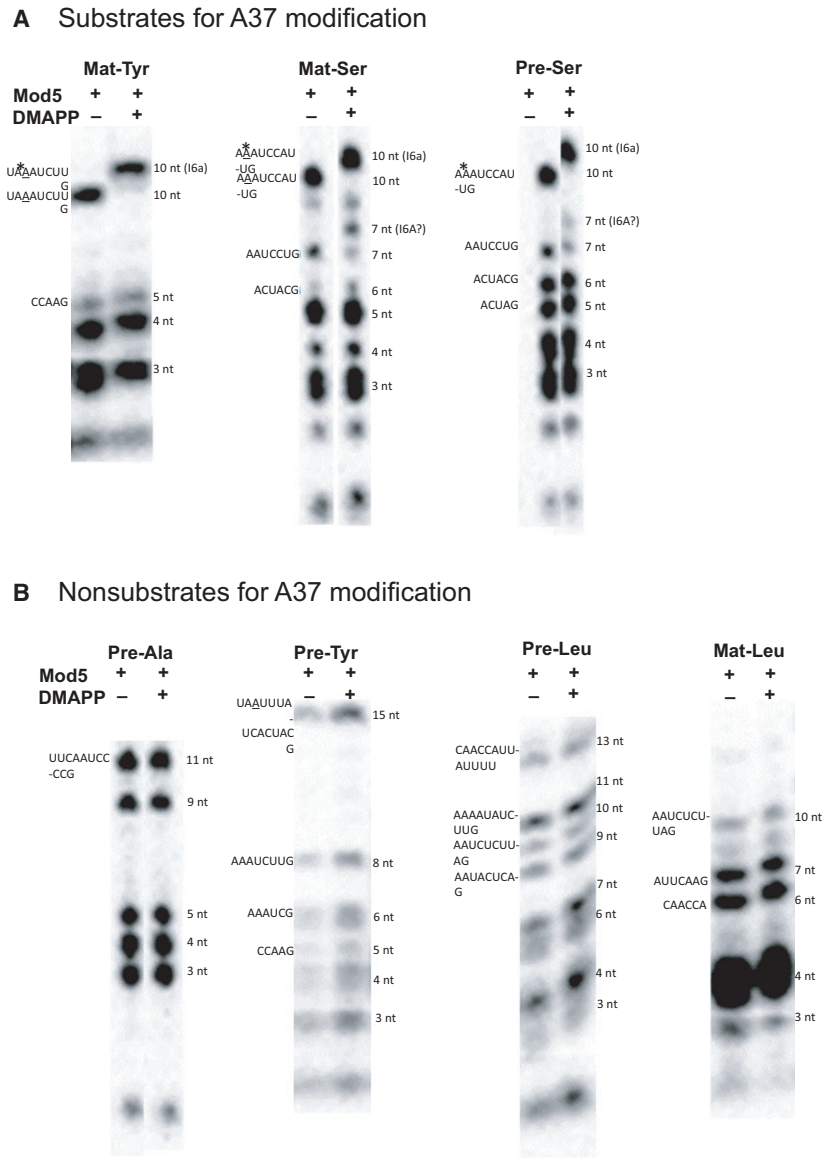


Fig. 1. Mod5 isopentenylation assays. (A) Substrate or (B) nonsubstrate tRNA genes were transcribed in the presence of ^{32}P-adenosine creating internally labeled RNAs. RNAs were then incubated with Mod5 in the presence or absence of DMAPP. The samples were subsequently digested with RNase T1 and fragments separated with 20% denaturing PAGE. Fragments containing the modifiable adenosine in the absence of DMAPP are indicated with an asterisk*. A shifted band in the presence of DMAPP indicates the presence of a modified adenosine containing an isopentenyl group (See Fig. S1).

within a similar range of protein concentrations (Fig. 2A–H). At low concentrations of Mod5, in some cases, there are discrete shifted RNA bands that likely represent monomer, dimer, or higher complexes of RNAs with Mod5, although there is some smearing of signal in lanes consistent with either semistable complexes or variably structured complexes under these conditions. At higher concentrations of protein, these give way for all RNAs to supershift at the gel interface, suggesting a much larger or insoluble complex (Fig. 2A–H, discussed below). We will address the implications of these slow-migrating complexes below, but at a minimum, these data suggest that Mod5 directly binds to RNAs regardless of whether the RNA is a substrate for modification.

To investigate the relative binding strengths of these RNAs with Mod5, we used a competition assay in which Mod5 was bound to a constant concentration of ^{32}P–labeled pre–Tyr–tRNA, in the presence of increasing amounts of unlabeled competitor RNAs. We determined apparent IC_{50} values for the nonsubstrate pre-Leu–tRNA, pre–Ala–tRNA, and pre–Tyr–tRNA to be similar (0.4, 1.0, and 0.8 μM, respectively, Fig. 3A, D, and E.). We further determined the competitive strength of two other nonsubstrates including a highly structured non–tRNA (5S rRNA, Fig. 3F) and Leu–tRNA (Fig. 3C), and one other substrate RNA, Tyr–tRNA (Fig. 3B). Leu–tRNA, Tyr–tRNA, and 5S-rRNA sequences (IC_{50} values 3.5, 1.5, and 1.6 μM, respectively) competed with similar strengths to other RNAs tested.

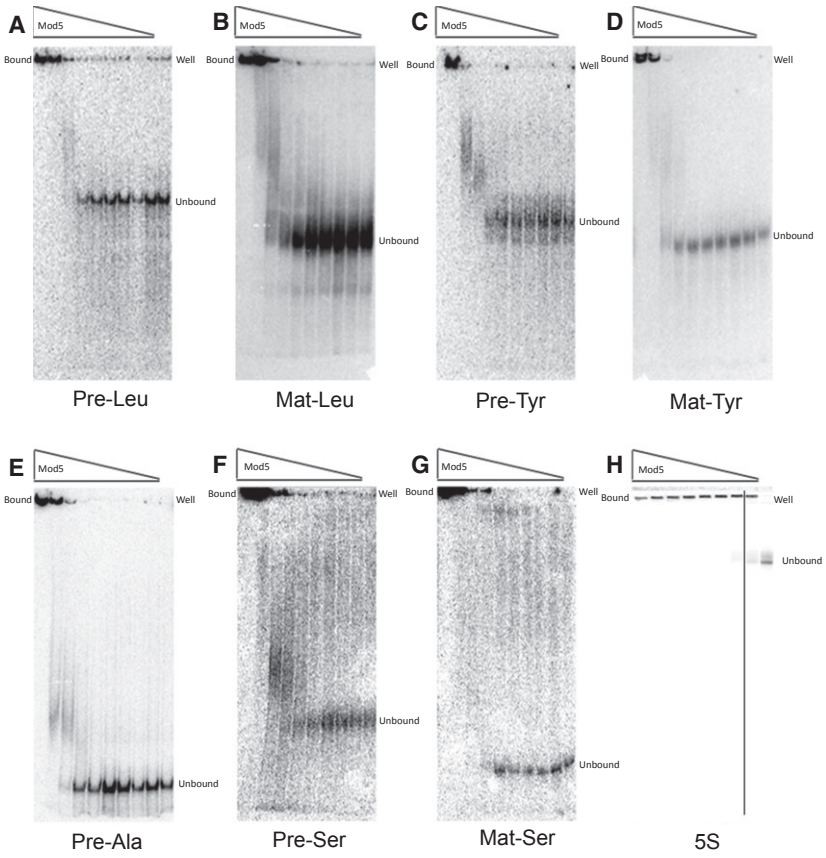


Fig. 2. Mod5 binds to substrate and nonsubstrate RNAs. Phosphorimage of representative nondenaturing gel electrophoretograms of purified recombinant Mod5 and ^{32}P-labeled RNAs (A) pre-Leu-tRNA, (B) mat-Leu-tRNA, (C) pre-Tyr-tRNA, (D) mat-Tyr-tRNA, (E) pre-Ala-tRNA, (F) h-pre-Ser-tRNA, and (G)mat-Ser-tRNA (H) 5S-rRNA with decreasing Mod5 concentrations (5.33, 1.78, 0.593, 0.198, 0.0658, 0.0219, 0.00732, 0.00244, 0.000813, and 0.00 μM. In (H), the lane with 0.00244 μM Mod5 was omitted).

In general, these data are consistent with our previously published *in vivo* data showing that Mod5 coimmunoprecipitates with both substrate and nonsubstrate tRNAs [22] and suggests that the capacity to serve as a substrate for modification does not predict which RNAs Mod5 can bind.

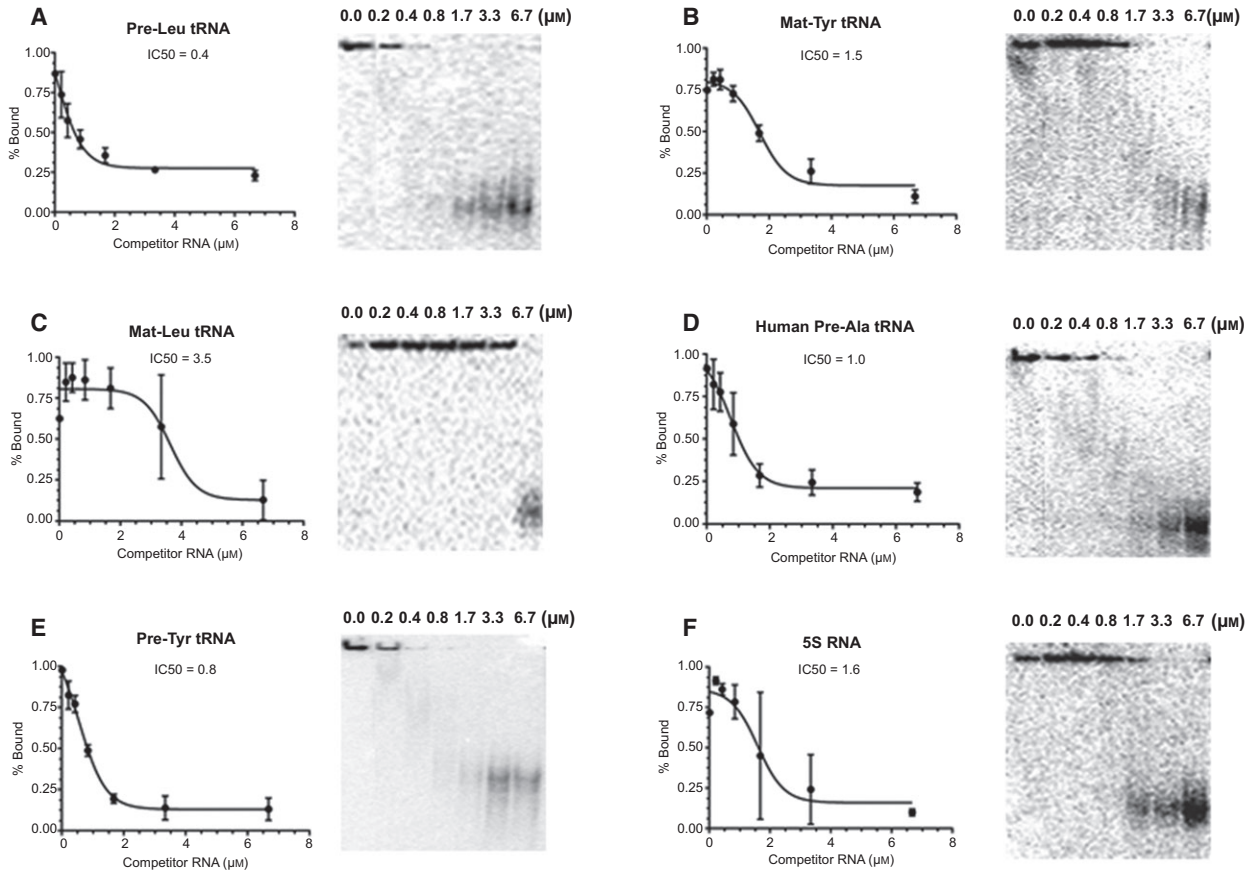
tRNA accelerates aggregation of Mod5

As noted above, at the higher Mod5:RNA ratios, the majority of the RNA is bound in a complex with proteins that remains near the well interface of the gel, suggesting that the RNA is bound to a multimerized or aggregated form of Mod5. This suggested that aggregation of Mod5 is not prevented by its interaction with RNA and possibly takes the form of amyloid fibers as observed by others [29]. We thus examined the physical form of these interactions further.

To compare the rates of aggregation, we measured solution light scattering using absorbance at 405 nm as a measure of Mod5 precipitation/aggregation over time (Fig. 4A). The sample containing Mod5 without RNA demonstrated a modest increase in A_{405}, while the addition of substrates mat−Tyr−tRNA, pre−Ser−tRNA, or mat−Ser-tRNA led to a threefold to eightfold increase in A_{405} by 10 h compared to the Mod5−only sample. Similarly, addition of nonsubstrate pre−Leu−tRNA or pre−Tyr−tRNA led to sixfold to sevenfold increase in A_{405}. In contrast, combining Mod5 with 5S-rRNA did not lead to a significant change, notable given the GMSA and competition results (Figs 2 and 3) showing Mod5 binding to 5S rRNA. These results suggest that tRNA-like binding accelerates the aggregation of Mod5, but that the nature of the bound RNA is important.

Mod5 forms both punctate aggregates and extended fibers in the presence or absence of RNA

Mod5 was previously shown to form amyloid fibers *in vitro* and aggregates *in vivo* under selective pressure [11,29]. We tested whether RNA affects the ability of Mod5 to form amyloid fibers by examining protein samples +/− RNA using fluorescence and electron microscopy (EM). We also imaged fibers formed in the presence of fluorescently labeled pre−Ser−tRNA to test whether the RNA itself was associated with the protein aggregates. Consistent with our GMSAs (Fig. 2A–G), we observe Mod5 fibers and punctate aggregates bound to the fluorescent RNA (Fig. 4B). For both protein-



G

Relative IC50s of competitor RNAs		
Competitor RNA	IC50	95% CI
Pre-Leu	0.4	[0.3, 1.3]
Mat-Leu	3.5	[1.5, 6.0]
Pre-Tyr	0.8	[0.6, 1.1]
Mat-Tyr	1.5	[1.2, 1.8]
Pre-Ala	1.0	[0.8, 1.8]
5S	1.6	[0.9, 2.9]

Fig. 3. Effect of Mod5 binding to ^{32}P-labeled pre-Tyr RNA in the presence of substrate and nonsubstrate RNAs. Phosphorimages of representative (of three repetitions) nondenaturing gel electrophoretograms with indicated concentrations of unlabeled (A) pre-Leu-tRNA, (B) mat-Tyr-tRNA, (C) mat-Leu-tRNA, (D) h-pre-Ala-tRNA, (E) pre-Tyr-tRNA, and (F) 5S-rRNA, with a graphic representation (to the left of each phosphorimage) of concentration-dependent effect of Mod5 binding to ^{32}P-labeled pre-Tyr-tRNA by unlabeled competitor RNAs. (G) A table of IC_{50} values and 95% confidence intervals by RNA.

only samples and those incubated with various RNAs, we observed both punctate particulates and fibers using EM (Fig. 4C–H) confirming previous observations [29]. The size of the punctate particulates as well as the presence of fibers suggested protein multimers. Individual fibers had an approximate diameter of 5–10 nm and were often seen in bundles with diameters as large as 50 nm. The resolution of this analysis precluded detection of quantitative differences in the dimensions of individual fibers, while spectroscopic assays for changes in the rate of fiber versus particle formation were inconclusive (Fig. S4): thus, more detailed analysis of differences with or without RNA awaits further investigation.

Discussion

We herein demonstrated that Mod5 directly binds to both substrate and non−substrate RNAs *in vitro*. This is in agreement with our hypothesis that Mod5-tRNA interactions play a role in the mechanism of tgm -silencing, where Mod5 binds the nascent pre−tRNA

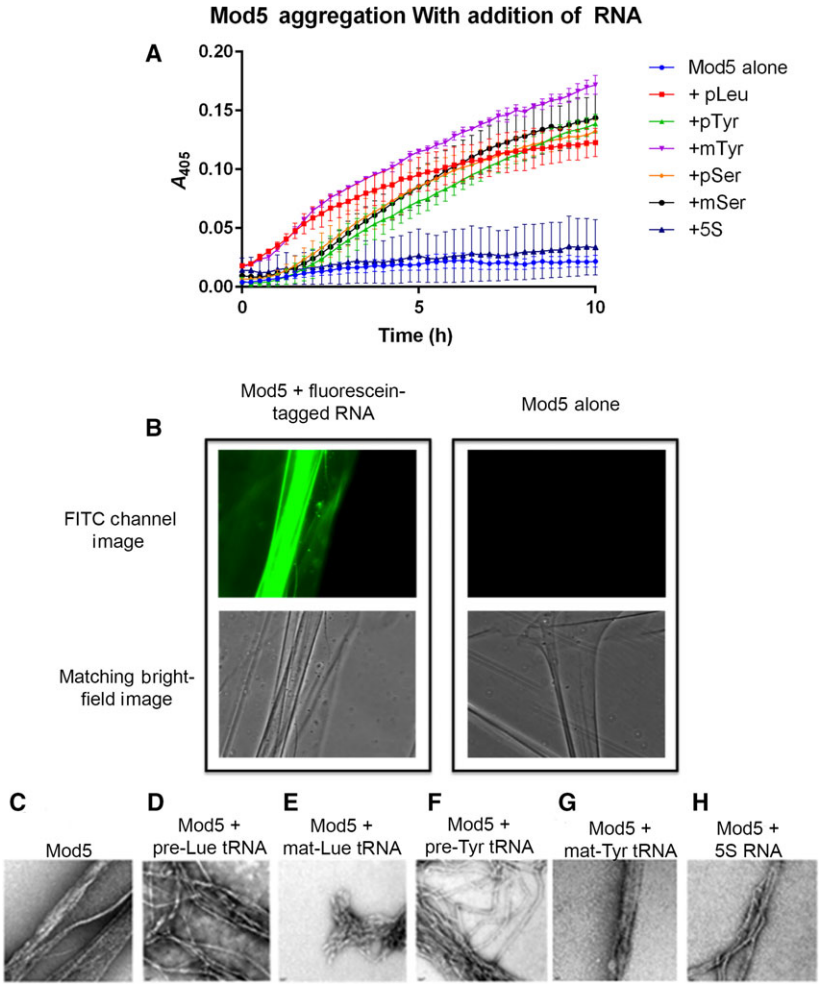


Fig. 4. tRNAs increase Mod5-aggregation, Mod5 forms amyloid fibers in the presence of RNA, and the fibers bind RNA. (A) Protein incubated with substrate tRNAs (mat-Tyr-tRNA, pre-Ser-tRNA, mat-Ser-tRNA) or nonsubstrate tRNAs (pre-Tyr-tRNA, pre-Leu-tRNA) demonstrated a ~ fivefold to eightfold increase in absorbance after 10 h compared to the Mod5-only sample. Protein incubated with 5S-rRNA did not increase overall absorbance. (B) Mod5 amyloid fibers bound to a fluorescein-labeled Ser-tRNA. (C–H) Electron micrographs of Mod5 amyloid fibers formed (from left to right) in the (C) absence of RNA, or the presence of (D) pre-Leu-tRNA, (E) mat-Leu-tRNA, (F) pre-Tyr-tRNA, (G) mat-Tyr-tRNA, or (H) 5S-rRNA. 22 000× magnification.

transcripts as a prerequisite for subsequent interactions that modify and remodel local chromatin structure. This is consistent with our previous *in vivo* data showing that Mod5 copurifies with the precursor and mature forms of both substrate and nonsubstrate tRNAs [22]. The ability of the enzyme to bind with similar affinities to both substrate and nonsubstrate pre-tRNAs suggests that although the presence of an AAA at the A36–A38 position of the active site is predictive of modification activity [14], it is not a major determinant of RNA-binding behavior of Mod5. The observed differences in binding among tRNAs, pre-tRNAs, and 5S-rRNA are relatively small, suggesting substantial tolerance for variations in RNA structure, as well as sequence, for binding to Mod5.

Interestingly, upon the addition of RNA to purified Mod5, we observed a marked increase in light scattering with all tRNAs and pre-tRNAs tested, but not 5S-rRNA (Fig. 4A). These observations suggest that tRNA-like molecules can stimulate Mod5 aggregation whether or not they are substrates for isopentenylation (Fig. 4A). The reason for the markedly different aggregation behavior of 5S-rRNA, even though it binds to Mod5 and can compete with tRNA binding, is currently unclear, but could reflect a fundamentally different structure in the ribonucleoprotein. This would not be surprising, because the enzyme evolved specifically to fit a tRNA structure into the active site [14] and the overall effect on the structure of the complex could be quite distinct with a differently shaped RNA.

A second observation of interest comes from the effect of RNAs on aggregation by Mod5. It was previously shown that Mod5 is able to form amyloid-like aggregates and that this aggregation can be selected *in vivo* by selecting against functional tRNA-modifying activity in the cytoplasm with an antifungal agent, fluconazole [29]. However, we previously showed that the nuclear silencing function of Mod5 is not ablated when aggregation is selected by this stressor [11], suggesting that the nuclear pool of Mod5 that is associated with RNA polymerase III transcription complexes and pre−tRNAs was not subject to the aggregation behavior of the cytoplasmic pool.

As aggregation of the purified enzyme is not prevented by RNA binding, and if anything is enhanced by it, we infer that the immunity of the nuclear function to fluconazole-selected aggregation might be due to other interactions of Mod5 in the nucleus. We propose an alternative model in which the RNA-bound form of Mod5 could be the 'active' conformation of the nuclear enzyme, with the enhanced aggregation a necessary part of its nuclear function. Under this model, tgm silencing is facilitated by the multimerization of Mod5 which might extend the Mod5-particle to neighboring gene promoters to either attract chromatin modifiers or block access of the transcription machinery to the DNA.

Suzuki and colleagues originally reported that aggregated Mod5 in the cytoplasm conveys resistance to certain fungicides [29], which might be consistent with the property having evolved in fungi to provide a reserve population of yeast that are resistant to antifungal attack. Interestingly, the human orthologue, TRIT1, has retained both the cytoplasmic and nuclear functions of Mod5 [22], and we recently demonstrated that it retains the amyloid fibril formation potential of Mod5 [34]. It is not clear why fibril formation would have been retained in an organism as distant from fungi as humans if cytoplasmic aggregation were the selective pressure, although it is possible that this method of removing Mod5 might regulate cholesterol biosynthesis or protein prenylation by modulating the availability of substrates. We suggest an alternative hypothesis, that the fibril formation is a positive attribute in tgm silencing that has been conserved for that reason. The observation that apparent RNA-mediated silencing has been observed near pol III transcription units in human cells [28] would be consistent with such a conservation of function.

Acknowledgements

We thank Dr. Alice Telesnitsky for her generous gift of time, laboratory space, reagents, and guidance. We thank May Tsoi for her technical assistance. We thank Dr. David Barton for helpful discussion. This work was supported by NIH Grant R01 GM082875 to DRE and support from Michigan Undergraduate Research Opportunity Program (UROP) for DFR.

Author contributions

DRE and PJS conceived and supervised the study; DRE, PJS, DRS, and DFR designed experiments; PJS, ET, TJW, and DFR performed experiments; PJS, ET, TJW, and DFR analyzed data; PJS, DFR, and DRE wrote the manuscript; PJS, DRE, TJW, and DFR made manuscript revisions.

References

1 Dihanich ME, Najarian D, Clark R, Gillman EC, Martin NC and Hopper AK (1987) Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of Saccharomyces cerevisiae. *Mol Cell Biol* **7**, 177–184.

2 Gefter ML and Bikoff E (1971) Studies on synthesis and modification of transfer RNA. *Can Res* **31**, 667–670.

3 Gillman EC, Slusher LB, Martin NC and Hopper AK (1991) MOD5 translation initiation sites determine N6-isopentenyladenosine modification of mitochondrial and cytoplasmic tRNA. *Mol Cell Biol* **11**, 2382–2390.

4 Golovko A, Hjalm G, Sitbon F and Nicander B (2000) Cloning of a human tRNA isopentenyl transferase. *Gene* **258**, 85–93.

5 Laten H, Gorman J and Bock RM (1978) Isopentenyladenosine deficient tRNA from an antisuppressor mutant of Saccharomyces cerevisiae. *Nucleic Acids Res* **5**, 4329–4342.

6 Lemieux J, Lakowski B, Webb A, Meng Y, Ubach A, Bussiere F, Barnes T and Hekimi S (2001) Regulation of physiological rates in Caenorhabditis elegans by a tRNA-modifying enzyme in the mitochondria. *Genetics* **159**, 147–157.

7 Seif E and Hallberg BM (2009) RNA-protein mutually induced fit: structure of *Escherichia coli* isopentenyl-tRNA transferase in complex with tRNA(Phe). *J Biol Chem* **284**, 6600–6604.

8 Soderberg T and Poulter CD (2000) Escherichia coli dimethylallyl diphosphate: tRNA dimethylallyltransferase: essential elements for recognition of tRNA substrates within the anticodon stem-loop. *Biochemistry* **39**, 6546–6553.

9 Warner GJ, Rusconi CP, White IE and Faust JR (1998) Identification and sequencing of two isopentenyladenosine-modified transfer RNAs from Chinese hamster ovary cells. *Nucleic Acids Res* **26**, 5533–5535.

10 Slusher LB, Gillman EC, Martin NC and Hopper AK (1991) mRNA leader length and initiation codon context determine alternative AUG selection for the yeast gene MOD5. *Proc Natl Acad Sci USA* **88**, 9789–9793.

11 Smaldino PJ, Read DF, Pratt-Hyatt M, Hopper AK and Engelke DR (2015) The cytoplasmic and nuclear populations of the eukaryote tRNA-isopentenyl transferase have distinct functions with implications in human cancer. *Gene* **556**, 13–18.

12 Zoladek T, Vaduva G, Hunter LA, Boguta M, Go BD, Martin NC and Hopper AK (1995) Mutations altering the mitochondrial-cytoplasmic distribution of Mod5p implicate the actin cytoskeleton and mRNA 3' ends and/or protein synthesis in mitochondrial delivery. *Mol Cell Biol* **15**, 6884–6894.

13 Benko AL, Vaduva G, Martin NC and Hopper AK (2000) Competition between a sterol biosynthetic enzyme and tRNA modification in addition to changes in the protein synthesis machinery causes altered nonsense suppression. *Proc Natl Acad Sci USA* **97**, 61–66.
14 Zhou C and Huang RH (2008) Crystallographic snapshots of eukaryotic dimethylallyltransferase acting on tRNA: insight into tRNA recognition and reaction mechanism. *Proc Natl Acad Sci USA* **105**, 16142–16147.
15 Lamichhane TN, Mattijssen S and Maraia RJ (2013) Human cells have a limited set of tRNA anticodon loop substrates of the tRNA isopentenyltransferase TRIT1 tumor suppressor. *Mol Cell Biol* **33**, 4900–4908.
16 Yarham JW, Lamichhane TN, Pyle A, Mattijssen S, Baruffini E, Bruni F, Donnini C, Vassilev A, He L, Blakely EL *et al*. (2014) Defective i6A37 modification of mitochondrial and cytosolic tRNAs results from pathogenic mutations in TRIT1 and its substrate tRNA. *PLoS Genet* **10**, e1004424.
17 Phizicky EM and Hopper AK (2010) tRNA biology charges to the front. *Genes Dev* **24**, 1832–1860.
18 Urbonavicius J, Qian Q, Durand JM, Hagervall TG and Bjork GR (2001) Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J* **20**, 4863–4873.
19 Boguta M, Hunter LA, Shen WC, Gillman EC, Martin NC and Hopper AK (1994) Subcellular locations of MOD5 proteins: mapping of sequences sufficient for targeting to mitochondria and demonstration that mitochondrial and nuclear isoforms commingle in the cytosol. *Mol Cell Biol* **14**, 2298–2306.
20 Tolerico LH, Benko AL, Aris JP, Stanford DR, Martin NC and Hopper AK (1999) Saccharomyces cerevisiae Mod5p-II contains sequences antagonistic for nuclear and cytosolic locations. *Genetics* **151**, 57–75.
21 Kendall A, Hull MW, Bertrand E, Good PD, Singer RH and Engelke DR (2000) A CBF5 mutation that disrupts nucleolar localization of early tRNA biosynthesis in yeast also suppresses tRNA gene-mediated transcriptional silencing. *Proc Natl Acad Sci USA* **97**, 13108–13113.
22 Pratt-Hyatt M, Pai DA, Haeusler RA, Wozniak GG, Good PD, Miller EL, McLeod IX, Yates JR 3rd, Hopper AK and Engelke DR (2013) Mod5 protein binds to tRNA gene complexes and affects local transcriptional silencing. *Proc Natl Acad Sci USA* **110**, E3081–E3089.
23 Wang L, Haeusler RA, Good PD, Thompson M, Nagar S and Engelke DR (2005) Silencing near tRNA genes requires nucleolar localization. *J Biol Chem* **280**, 8637–8639.
24 Hull MW, Erickson J, Johnston M and Engelke DR (1994) tRNA genes as transcriptional repressor elements. *Mol Cell Biol* **14**, 1266–1277.
25 Good PD, Kendall A, Ignatz-Hoover J, Miller EL, Pai DA, Rivera SR, Carrick B and Engelke DR (2013) Silencing near tRNA genes is nucleosome-mediated and distinct from boundary element function. *Gene* **526**, 7–15.
26 Haeusler RA, Pratt-Hyatt M, Good PD, Gipson TA and Engelke DR (2008) Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. *Genes Dev* **22**, 2204–2214.
27 Thompson M, Haeusler RA, Good PD and Engelke DR (2003) Nucleolar clustering of dispersed tRNA genes. *Science (New York, NY)* **302**, 1399–1401.
28 Woolnough JL, Atwood BL and Giles KE (2015) Argonaute 2 binds directly to tRNA genes and promotes gene repression in cis. *Mol Cell Biol* **35**, 2278–2294.
29 Suzuki G, Shimazu N and Tanaka M (2012) A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress. *Science (New York, NY)* **336**, 355–359.
30 Engelke DR, Krikos A, Bruck ME and Ginsburg D (1990) Purification of thermus aquaticus DNA polymerase expressed in Escherichia coli. *Anal Biochem* **191**, 396–400.
31 He B, Rong M, Lyakhov D, Gartenstein H, Diaz G, Castagna R, McAllister WT and Durbin RK (1997) Rapid mutagenesis and purification of phage RNA polymerases. *Protein Expr Purif* **9**, 142–151.
32 Serio TR, Cashikar AG, Kowal AS, Sawicki GJ, Moslehi JJ, Serpell L, Arnsdorf MF and Lindquist SL (2000) Nucleated conformational conversion and the replication of conformational information by a prion determinant. *Science (New York, NY)* **289**, 1317–1321.
33 Ohi M, Li Y, Cheng Y and Walz T (2004) Negative staining and image classification - powerful tools in modern electron microscopy. *Biol Proced Online* **6**, 23–34.
34 Waller TJ, Read DF, Engelke DR and Smaldino PJ (2016) The human tRNA-modifying protein, TRIT1, forms amyloid fibers *in vitro*. *Gene*, doi: 10.1016/j.gene.2016.10.041.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. RNAs used in this study.
Fig. S1. RNase T1 digest maps of RNAs used for modification assays (Fig. 1).
Fig. S2. DNA oligonucleotides used to construct templates for *in vitro* transcription of tRNAs.
Fig. S3. (*Homo sapiens* chr11.trna10) tRNA prediction of human mat-SerAGA with similarity in modified sequences (Fig. 1A) indicated within the ovals.
Fig. S4. The effect of RNA on Mod5 fiber formation is unclear.