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Key RNA-binding domains in the La protein establish tRNA modification levels in *Trypanosoma brucei*

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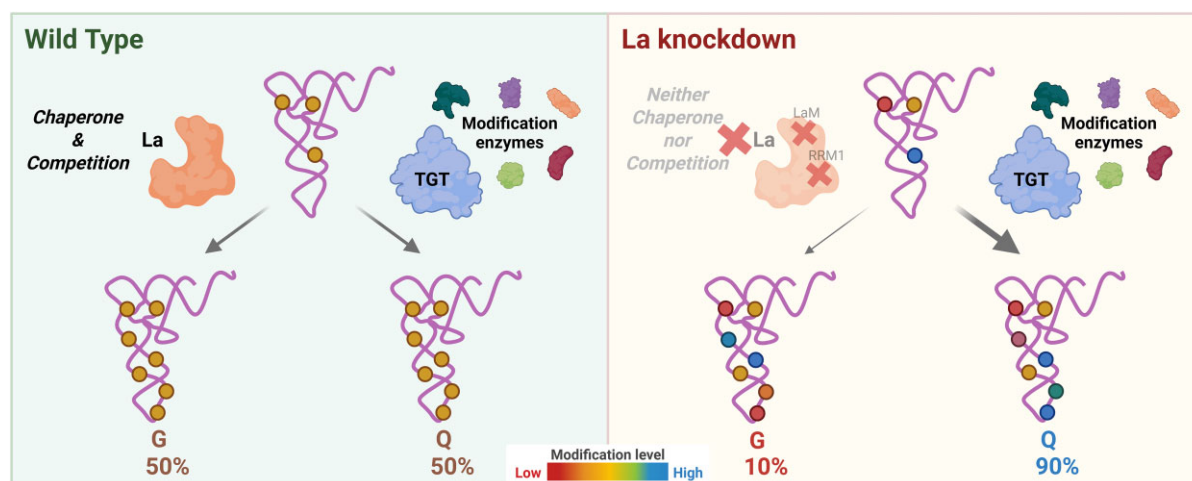
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Abstract

The RNA-binding protein La is found in most eukaryotes, and despite being essential in many organisms, its function is not completely clear. *Trypanosoma brucei*, the causative agent of human African trypanosomiasis, encodes a 'classical' La protein (TbLa) composed of a La-motif, two RNA recognition motifs (RRM1 and RRM2 α), a C-terminal short basic motif (SBM), and a nuclear localization signal (NLS). In *T. brucei*, like in most eukaryotes, position 34 of tRNA^{Tyr}, -Asp, -Asn and -His is modified with queuosine (Q₃₄). The steady-state levels of queuosine-modified tRNA in the insect form (procyclic) of *T. brucei* can fluctuate dynamically depending on growth conditions, but the mechanism(s) controlling Q₃₄ levels are not well understood. A well-established function of La is in precursor-tRNA 3'-end metabolism, but in this work, we demonstrate that La also controls Q₃₄-tRNA levels. Individual domain deletions showed that while deletion of La motif or RRM1 causes dysregulation of Q₃₄-tRNA levels, no other domain plays a similar role. We also show that La is important for the normal balance of several additional tRNA modifications. These findings are discussed in the context of substrate competition between La and modification enzymes, also highlighting subcellular localization as a key determinant of tRNA function.

Graphical abstract



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Introduction

La is an RNA binding protein originally identified as an autoantigen in patients with chronic inflammatory disorders, systemic Lupus erythematosus and Sjögren's syndrome [1, 2]. La is found in most eukaryotes and is essential in most [3–6]. Among the many functions that have been ascribed to La, its role in pre-tRNA 3'-end metabolism is well established [7, 8]. One or more La-related proteins (LARPs) are also found in eukaryotic genomes, defined by the conserved RNA-binding domain named La motif (LaM) and an adjacent LARP family-specific RNA-recognition motif (RRM), which together form the so-called La-module characteristic of LARPs [3, 4, 9]. The number of LARPs vary among eukaryotic lineages [4], for example, humans encode seven; La (aka LARP3), as well as LARPs 1, 1B, 4/4A, 4B, 6, and 7, while some single-cell eukaryotes, including *T. brucei*, only encode "genuine" La (LARP3). LARPs expanded as a polyphyletic family during eukaryotic evolution involving major domain variations beyond the La-module [3, 4, 9]. For example, the La modules of different LARPs are appended to various types of RNA-binding or other functional domains and motifs: DM15 in LARP1 [3, 10], PolyA-binding protein-associated motif-2 (PAM2) in LARP4A/B and LARP1/B [11–14], the La and S1 associated motif (LSA) in LARP6 [3], the conserved region-2 (CR2) in LARP4A/B [3, 15], and the conserved basic region (CBR) found in LARP7 preceding its RRM2 [16]. Indeed, the various domains serve to diversify La/LARP function, which ranges from binding mRNA, to binding rRNA, and/or small non-coding RNAs including tRNAs [9, 16]. Finally, LARPs contain subcellular localization elements, which in the case of human La includes a nuclear retention element (NRE), nuclear export sequence (NES), nucleolar localization (NoLS), and nuclear localization sequence (NLS) [16–22].

The well-characterized high-affinity binding target of La is $U_{(1-3)}U\text{-}3'\text{OH}$ [23–25], formed by transcription of the $T_{(4-7)}$ termination signal specific to RNA polymerase III (Pol III), itself a hallmark of eukaryotes. La proteins bind newly synthesized Pol III transcripts, the most numerous and diverse of which are the pre-tRNAs [26–28]. By binding 3'-terminal $U_{(1-3)}U\text{-}3'\text{OH}$, La shields Pol III transcripts from 3'-exonucleolytic digestion and untimely degradation or processing [29–31]. La's relationship with tRNA modifications is also notable, as some modifications contribute to tRNA folding. Human La-associated precursor-tRNAs show stoichiometric levels of modifications like m^1A_{58} [2, 32, 33], which is involved in tRNA structure and function [34, 35]. Yeast La (Lhp1) binding to tRNA reduces the levels of $m^{2,2}G_{26}$ modification by Trm1 and prevents misfolding in tRNAs [36–38]. However, no direct physical interaction between the two proteins has been observed, yet both can redundantly support structurally challenged tRNAs, highlighting their conserved roles as tRNA chaperones [36–42].

Beyond pre-tRNAs, La can associate with mature tRNAs, rRNAs and mRNAs species that bind by unknown mechanisms [43–46]. For example, human La binds fully end-matured tRNAs with high affinity via protein regions not involved in $U_{(1-3)}U\text{-}3'\text{OH}$ recognition [47]. Although the significance of La binding mature tRNAs is not fully clear, the reported association of the La protein with various tRNA species [45, 47] raises the question of whether La may also play a role in affecting tRNA modification levels.

In a previous report, we showed that in *Trypanosoma brucei* queuosine modification at the first position of the anticodon (Q_{34}) in $tRNA^{Tyr, -Asp, -His}$ and $-Asn$ changes dynamically in response to alterations in the levels of certain amino acids in the media, consequent to increased dwell time of tRNA in the nucleus [48]. Since *T. brucei* tRNA guanine transglycosylase (TbTGT), the enzyme responsible for Q_{34} formation in tRNA is nuclear, we concluded that there is a competition between the rate of tRNA nuclear export and the ability of TbTGT to target tRNAs for modification [49, 50]. Indeed, downregulation of the tRNA exporter (TbMex67) increased tRNA dwell time in the nucleus, with concomitant increase in Q_{34} [51], to phenocopy the effect seen when cells are grown in the presence of specific, low amino acid levels [48].

Since *T. brucei* La (TbLa) and TbTGT are nuclear enzymes [49, 50, 52] and given the previous observations described above, we hypothesized that there may be competition for the tRNA substrates between TbLa and other tRNA-interacting factors, including modification enzymes. In this study, we show that downregulation of TbLa expression by RNA interference leads to an increase in the Q_{34} content of all four tRNAs that are targets of TbTGT without significant changes in transcript levels. By using a combination of genetic and biochemical approaches we dissected the contribution of each La domain to tRNA Q_{34} content, leading to the conclusion that the La module (LaM and RRM1) and, implicitly, tRNA binding are essential for regulating the levels of Q_{34} . We also provide OTTR-seq and LC-MS/MS data showing that the effect of TbLa extends to additional tRNA modifications with varied effects; some modifications increase in the absence of TbLa, others decrease. This indicates that TbLa influences global tRNA modification levels by different mechanisms, but in the specific case of queuosine it helps maintain ~50% steady state levels presumably needed based on translational decoding arguments [48, 50]. Conceptually, substrate competition may offer a rapid response to modification changes that goes beyond transcription and can easily be adapted to changes in environmental cues. Given the near-universal occurrence of La in eukaryotes, our findings likely extend beyond the *T. brucei* system.

Materials and methods

T. brucei cell culturing and generation of RNAi cell lines expressing TbLa mutants

Procyclic form of *Trypanosoma brucei* 29–13 cells were grown in SDM-79 media supplemented with 10% FBS and Hemin at 27°C. TbLa was knocked down using a p2T7-177 vector through expression of a double stranded RNA (dsRNA) targeting TbLa mRNA (Tb927.10.2370) via head-to-head oriented T7 promoters [53]. The dsRNA expression was induced by treating cells with 1 µg/ml of tetracycline [54]. The growth of the cultures was assessed through counting cells using a Neubauer hemocytometer. The region that was targeted by the La RNAi induction in the TbLa gene was recoded with synonymous codons to generate the TbLaRec gene (supplementary Fig. S3). pABPuro vector with Puromycin selection was used to express myc-TbLaRec and the myc-TbLaRec domain mutants in 29–13 cells expressing La RNAi. The Thermo Scientific Phusion site directed mutagenesis kit (Catalog: F541) was used to generate the TbLaRec domain mutants. All the vectors were transfected

into *T. brucei* 29–13 cells via electroporation followed by selection using appropriate drugs [55]. The selected clones were analyzed via polymerase chain reaction (PCR) with genomic DNA, western blotting, and Reverse Transcription (RT)-PCR. Following oligos were used to generate the myc-TbLaRec mutants. LaMΔ: FP- GGAGGAAAGTGGGAG-GTTCAAGTC, RP- TCCATCCAAACCGATCACCAAACC, RRM1Δ: FP- AGCTCAAAGACGAAAAATGGAGGAGC, RP- GTGATCGGTTTGGATGGATTCCGG, RRM2αΔ: FP- TCAGCTAACACAGGAGCGGAAGG, RP- TG-GCATAGGCGGAGTCTTTTTTTCAG, SBMΔ: FP- GGGCATAAACGCAGCAGAGAGTAAG, RP- GTTGT-TAGCTGATTGCATAGCCCTGT).

Reverse transcription—PCR

T. brucei cultures at log phase ($2\text{--}8 \times 10^6$ cells/ml) were used to extract total RNA using guanidinium thiocyanate-phenol-chloroform protocol as previously described [56]. RNA samples were treated with DNase I to remove genomic DNA. Around 2 µg of each RNA sample was subjected to reverse transcription and PCR using oligos specific to endogenous TbLa mRNA (FP1: CCGCTTG-GAAGTAGCTATCGTGTAAGC, RP1: TTCACGTGAC-CGCTTGTGTCCT) and Recoded TbLa mRNA (For myc-TbLa Rec, myc-TbLa LaMΔRec and myc-TbLa RRM1ΔRec; FP2: GTTAAAAACCTCTGGCCAGTTGAA-GAACA, RP2: GCTCCTGTTGTTAGCTGATTGCATAGC, For myc-TbLa RRM2αΔRec and myc-TbLa SBMΔRec; FP3: CCTGAAGGGGAAGATGGCAGAAAATG, RP3: TG-GCATAGGCGGAGTCTTTTTTTCAG).

APB gel electrophoresis and Northern blot analysis

Total RNA of *T. brucei* was isolated as previously described [56]. RNA was deacetylated in 0.1 M Tris-HCl at pH 9.0 for 30 min at 37°C prior to gel electrophoresis. Samples of 5 µg of RNA were denatured at 70°C and separated on 8% polyacrylamide gels containing 8M Urea, 0.5 % 3-aminophenyl boronic acid (APB) and 1x TAE [57]. Electrophoresis was carried out at 75 V for 5.5 h at 4°C. The RNA in gels were visualized using ethidium bromide staining for 10 min and then, electroblotted into Zeta probe nylon membranes for 2 h at 200 mA at 4°C according to the manufacturer's protocol (Bio-Rad). RNA was crosslinked to the membranes by UV irradiation for 1 min before hybridization. Northern blot hybridization was done according to Bio-Rad manufacturer's protocol. The blots that were hybridized with radiolabeled probes were exposed to a phosphorimager screen overnight and imaged using a Typhoon FLA 9000 scanner. The band intensities were calculated using ImageLab software (Bio-Rad). The blots were stripped and re-probed according to the manufacturers' protocol. (Bio-Rad) Following oligonucleotides end-labeled with ^{32}P were used to probe for the tRNA species of interest. (tRNA^{Tyr}: CCTTCCGGC-CGGAATCGAACCAGCGAC, tRNA^{Asp}: CGGGTCACC-CGCGTGACAGG, tRNA^{His}: GGGAAGACCGGGAATC-GAAC, tRNA^{Asn}: GATTCGAACCAACGACCTGTAGGT, tRNA^{Glu}: TTCCGGTACCGGGAATCGAAC).

Fluorescence *in situ* hybridization (FISH)

T. brucei 29–13 cells were harvested on days 4, 5, and 6 post-La RNAi induction and washed twice with 1x Phosphate saline buffer (PBS). 600 000 cells resuspended in 4%

paraformaldehyde/PBS solution were smeared onto poly-L-lysine coated microscope slides and fixed overnight. Slides were washed with 1x PBS and then permeabilized by dehydration in a series of solutions with increasing ethanol concentrations (50, 80, and 100%) for 3 min each. Next, the slides were prehybridized for 2 h in hybridization buffer (2% BSA, 5 × Denhardt's solution, 4 × SSC, 5% dextran sulfate, 35% deionized formamide, 10 U/ml RNase inhibitor). The hybridization was carried out in the hybridization buffer containing 10 ng/µl Cy3-labeled oligonucleotide probe (TbtRNA^{Tyr-mature}: Cy3-AACCAGCGACCCTGTGATCTAC, TbtRNA^{Asp}: Cy3-CGGGTCACCCGCGTGACAGG), in a humid chamber overnight at room temperature. Then the slides were washed with 4 × SSC with 35% deionized formamide, 2x SSC, and 1x SSC (for 10 min in each). The mounting medium supplemented with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) was added to the slides at the end. A confocal microscope Olympus FluoView™ FV1000 was used for imaging and Fluoview and ImageJ (NIH) software were used for processing the images.

Immunofluorescence microscopy

T. brucei cultures were supplemented with 200 mM Mito-tracker Red dye for 30 mins at 27°C to stain mitochondria in cells. Cells were harvested by spinning at 2300g for 5 min, washed twice with 1x PBS and resuspended in 1x PBS solution containing the fixative agent, 4% paraformaldehyde. Around 6×10^5 cells were smeared onto a glass slide, air-dried, and washed with 1x PBS. Cells were permeabilized with methanol for 10 min at −20°C and rinsed with 1x PBS three times. Slides were blocked for 1 h using 5.5% fetal bovine serum (FBS) in 1x PBST (1x PBS with 0.1% Tween-20) followed by two washes with 1x PBS. Cells were incubated with anti-Myc Mouse primary antibody at 1:300 dilution in 3% FBS in 1x PBST for 1 h followed by washing thrice with 1x PBST and twice with 1x PBS. Cells were incubated with Alexa-488 conjugated to goat derived anti-Mouse secondary antibody at 1:1000 dilution in 3% FBS in 1x PBST for 1 h followed by washing thrice with 1x PBST and twice with 1x PBS. Cellular DNA was stained with 1x PBS containing 300 nM DAPI for 2 min followed by two washes with 1x PBS followed by air-dry. The smear was mounted with Vectashield, covered with a coverslip and the edges were sealed with nail polish. The cells were imaged using a Nikon Ti microscope and images were processed using ImageJ (NIH) and Hyugens Essentials 3.4 software.

Recombinant his-TbLa purification

The coding sequence of TbLa was cloned into the expression vector pET15b and the TbLa domain mutants (LaMΔ and RRM1Δ) in pET15b were constructed as described above. Recombinant His(8x)-TbLa protein and mutants were expressed in *Escherichia coli* BL21 cells. For protein purification, 1.5 L cultures were grown at 37°C while shaking at 200 rpm, until OD₆₀₀ reached 0.6–0.8. Then protein expression was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and cultures were grown at room temperature (~25°C) overnight while shaking. Harvested cell pellets were resuspended in lysis buffer (20 mM Tris pH 8.0, 1 M NaCl, 10% glycerol, 20 mM Imidazole, 1x protease inhibitor cocktail, 1x PMSF and 0.01% NP40) and lysed by sonication with a Sonifier 450. Lysate was cen-

trifuged at 40 000 $\times$ g for 30 min, followed by 50 000 $\times$ g for 30 min to remove cellular debris. Resultant supernatant was injected through a 5 mL HisTrap HP column (Cytiva, Ni²⁺-nitrilotriacetic acid (NTA) column) via AktaPure FPLC. Bound material was washed with lysis buffer and a wash buffer (20 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol, 50 mM Imidazole, 1x protease inhibitor cocktail, and 1x PMSF), 20 column volumes in each. Bound protein was eluted (20 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol, 600 mM Imidazole, 1x protease inhibitor cocktail, and 1x PMSF). Peak fractions were pooled, and buffer was exchanged using the size exclusion column (HiLoadTM 16/600 SuperdexTM 75 pg) to a storage buffer (50 mM Tris pH 8.0, 50 mM NaCl, 14% glycerol, 1x protease inhibitor cocktail, and 1x PMSF). Final isolated protein was aliquoted and stored at -80°C .

Electrophoretic mobility shift assay (EMSA)

Native tRNA^{Tyr} lacking the Q modification was extracted from total RNA preps of TbTGT2Δ cells using biotinylated oligos following the protocol published by Spears *et al.* in 2011 [58]. The tRNA was end-labeled with γ -(³²P) ATP using T4 polynucleotide kinase (PNK) (NEB: M0201L) and subsequent radiolabeled mature tRNA^{Tyr} was gel-purified. The radiolabeled tRNA was denatured at 70°C for 3 min, then folded by slowly cooling down at room temperature, and incubated at 37°C for 15 min. Approximately 4000 CPM of folded tRNA (~ 0.6 nM) was incubated on ice for 30 min with His-TbLa solutions of varying concentrations (0–1.6 μM) in 1x HKM buffer (50 mM HEPES pH 7.5, 1 mM MgCl₂, 5 mM KCl) in a total volume of 10 μL . Next, 5 μL of 50% glycerol was mixed with each solution and the products were separated on a 6% nondenaturing polyacrylamide gel at 90 V for 2.5 h. Gels were vacuum dried and exposed to a phosphorimager screen overnight. A Typhoon FLA 9000 phosphorimager was used to visualize the screen and band intensities were measured using ImageLab software. The fraction of bound tRNA was calculated using the equation $\text{tRNA}_{\text{complex}} / (\text{tRNA}_{\text{free}} + \text{tRNA}_{\text{complex}})$ and plotted against the concentration of TbLa in each reaction. The data points were fit to a Hill plot and the apparent dissociation constants (K_{Dapp}) were estimated by nonlinear regression using Graphpad Prism software.

Total nucleoside analysis by LC-MS/MS

Sample preparation

Total RNA extracted from La RNAi induced and uninduced *T. brucei* cells were separated on 8% urea-polyacrylamide gels and visualized by ethidium bromide staining under ultraviolet light. The band at the size of tRNA (~ 75 bp) was excised from the gel and RNA was eluted by incubating it in a solution of 0.3 M Sodium Acetate (pH 5.2) overnight. The eluted tRNA was concentrated by ethanol precipitation and was digested enzymatically to monomers as previously reported [59]. Briefly, the purified tRNA was denatured by heating at 100°C then quickly chilled in an ice water bath. 1/10 volume of 0.1 M ammonium acetate, 1U (units) of nuclease P1 (Sigma Aldrich, St. Louis MO.) was added to the tRNA and incubated at 45°C. After 2 h, 1/10 volume of 1 M ammonium carbonate was added with 1.2×10^{-4} U of snake venom phosphodiesterase (Worthington Biochemicals, Lakewood, NJ.) and 0.003 U FastAP (Thermo Fisher Scientific, San Jose, CA.), and incubated at 37°C for 2 h. The digested nucle-

osides were then dried in speed vac and resuspended in 10 μL of mobile phase A for analysis.

Liquid chromatography (LC) method

Around 1 μL of each sample was injected onto a microbore C18 UHPLC column, 0.5 $\times$ 100 mm, 1.7 μm (Mac-Mod Analytical, Chadds Ford, PA.) with the Vanquish Neo UHPLC system (Thermo Fisher Scientific, San Jose, CA.) at a flow rate of 30 $\mu\text{L min}^{-1}$ using an ammonium acetate/acetonitrile buffer system. Mobile Phase A was 5 mM Ammonium acetate pH 5, Mobile Phase B was 60% MPA and 40% ACN. Gradient starts at 0% B held for 0.1 min then increasing to 1.5% at 7.2 min, 3% at 9.7 min, 5% at 15 min, 25% at 18 min, 50% at 20 min, 75% at 23 min, and held for 1.5 min before increasing to 90% at 30 min, 99% at 35 min then returning to 0% for 10 column volume re-equilibration.

Mass spectrometry (MS) method

Acquisition was performed on an Orbitrap Ascend BioPharma Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA.) running Xcalibur 4.6. For all experiments, data were collected in small molecule application mode. Full scan at a 60 000 orbitrap resolution with a scan range of 240–600 m/z was used. AGC target was set to standard, microscans was 1, the RF lens was 35% and maximum injection time was auto. MS2 was performed with decision tree method involving CID (collision induced dissociation) and HCD (higher energy collisional dissociation) in orbitrap with a Resolution of 30000. For CID collision energy of 40%, activation time of 10 ms and activation q of 0.25 was used. For HCD, 60 V of energy, activation time of 100 ms and microscans of 1 was used.

Data analysis

Data were processed using Compound Discoverer 3.1 (Thermo Fisher Scientific, San Jose, CA.) as previously described [60]. The area under the peaks for each modification was added up and internally normalized to the Cytosine level in each sample. The value of each modification in different samples was divided by the average values of RNAi uninduced control to calculate the fold difference between RNAi induced and uninduced samples. Unpaired two-tailed t-test with Welch correction was used to determine the statistical significance.

Ordered two-template relay tRNA sequencing (OTTR-seq)

Total RNA extracted from La RNAi-induced and uninduced *T. brucei* cultures was incubated with T4 PNK (NEB, M0201L) to resolve 2'-3' cyclic phosphate ends as described previously [61], and then deacylated by bringing the reaction to pH 9.0 for 30 min at 45°C using 100 mM Na₂B₄O₇ [62]. Treated RNA was recovered using the RNA clean and concentrate kit (Zymo research, Irvine, CA, USA, R1013). OTTR-seq libraries were prepared according to previously described methods [63] with modifications. Briefly, 40 ng of total RNA was used as input. RNA was labeled for 2 h at 30°C using only dideoxy ATP (ddATP). Similarly, cDNA synthesis was conducted using only the RNA-DNA primer duplex with + 1T overhang. cDNA was recovered using the Minelute reaction cleanup kit (Qiagen, 28 204), then size selected on a 1 $\times$ Tris-borate-EDTA (TBE, 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) buffer, 8M urea, and 9%

acrylamide gel to remove adapter dimers. cDNA was recovered by diffusion at 70°C for 1 h with shaking in a solution of 10 mM Tris-HCl pH 8, 300 mM NaCl, 1 mM EDTA, and 0.25% SDS, followed by precipitation in 66% ethanol with 0.6 M ammonium acetate and 1 µg/mL glycoblue (Thermo Fisher, AM9515). cDNA was amplified with Q5 polymerase (NEB, M0493L) for 13 cycles using the Illumina NEBNext sRNA index adapters (NEB, E7600S) then size selected on a 1 × TBE, 6% acrylamide gel to remove remaining adapter dimer. Libraries were recovered from the gel by diffusion at 70°C for 1 h with shaking in a solution of 10 mM Tris-HCl pH 8, 300 mM NaCl, and 1 mM EDTA, followed by precipitation in 70% ethanol with 80 mM sodium acetate and 1 µg/mL glycoblue (Thermo Fisher, AM9515). The recovered libraries were quantified using the qubit dsDNA High Sensitivity kit (Invitrogen, Q32854), Agilent High Sensitivity DNA bioanalyzer kit (Agilent, 5067-4626), and finally by qPCR. qPCR was conducted using PrimeTime Gene Expression Master Mix (IDT, 1 055 772) according to a protocol published online (<https://www.protocols.io/view/illumina-truseq-library-quantification-with-qpcr-p-6qpvrddpzgmk/v1>). Libraries were sequenced on the NextSeq 1000/2000 platform to generate 100 bp single end reads. Each sample had a minimum of four million reads per sample.

Raw FASTA files were trimmed using Cutadapt with options `-j 8 -m 15 -a GATCGGAAGAGCACACGTC`. Reads were further processed to remove an 8nt UMI using `umi_tools extract` with options `-extract-method = string -bc-pattern = NNNNNNNN`. Trimmed reads were processed through the tRNA Analysis of eXpression pipeline (tRAX) [64], using a custom reference database generated for the *T. brucei* Lister 427 genome assembly. Resulting BAM files were split into reads above or below 60 bp in length, and those above 60 bp were processed through the tRAX pipeline again (using the `-lazyremap` option) to analyze the expression of mature tRNA transcripts. Modifications in mature tRNA were inferred from the presence of misincorporations at the site of known modifications in other eukaryotes (PMID: 38 015 436), using the `-coverage.txt` file output by tRAX and applying a conservative misincorporation cutoff of 10%. Changes in modification abundance were estimated by log2-fold change in misincorporation rate. Statistical significance of changes in misincorporation rate were calculated via a two-tailed T-test and adjusted for multiple testing via Benjamini/Hochberg procedure with a family-wise error rate of 0.001.

Results

Downregulation of TbLa expression leads to increased Q-tRNA levels

Previous studies showed that just like in humans, the La protein is essential in *T. brucei* [6, 30]. Most recently, a connection between La and the modification enzyme Trm1 in *S. pombe* was reported, demonstrating a non-catalytic function for Trm1 as an RNA chaperone, with synergistic regulation of Trm1 methyltransferase activity via La binding to pre-tRNA populations [38]. In this report we explore the connection between *T. brucei* (TbLa) and tRNA modifications at a global scale, given the nuclear colocalization of TbLa with various tRNA modification enzymes, and the ability of TbLa to bind tRNAs [36, 47]. For this, we downregulated TbLa expression

in *T. brucei* by establishing clonal lines in which a portion of the TbLa coding sequence was cloned into an RNAi plasmid vector under the control of a tetracycline operator. In this system RNAi is tetracycline inducible [54]. Upon tetracycline addition to the growth media, we observed a significant slow-down in cell growth as compared to a wild-type cell line or an uninduced control (where tetracycline was omitted from the growth media) (Fig. 1A). Downregulation of TbLa expression was further confirmed by reverse transcription followed by polymerase chain reaction (RT-PCR) with oligonucleotides specific for the TbLa mRNA, which showed a significant reduction in the steady-state TbLa mRNA levels as compared to RT-PCR with RNA samples from wild-type and/or uninduced cells (Fig. 1A). These results recapitulate previous observations about the importance of La for *T. brucei* growth [6, 30].

Prior to this work, we showed that tRNA-guanine transglycosylase (TGT), the enzyme responsible for Q₃₄ formation in tRNA, is uniquely nuclear in *T. brucei* [49, 50]. Thus, we used TGT and the Q system to explore what effect, if any, downregulation of TbLa has on Q₃₄ levels. For this, total RNA was extracted from RNAi-induced, wild-type and uninduced cells and Q₃₄ content in tRNA was assessed by separation on denaturing acrylamide gels containing 3-aminophenylboronic acid (APB) [57]. Because APB has affinity for *cis*-diols, such as those present in Q₃₄-containing tRNAs, it leads to an electrophoretic mobility shift if Q₃₄ is present [65], which we detect by northern blots using oligonucleotide probes specific for different tRNA species. These experiments showed that under “normal” growth conditions the steady-state levels of Q₃₄ in tRNA^{Tyr} range from 40 to 60% as shown previously (Fig. 1B and C) [48, 49]. To show that the observed band shift is indeed due to the presence of Q₃₄, we also probed total RNA isolated from a knockout of TbTGT2, the catalytic subunit of the heterodimeric TbTGT enzyme (TGTΔ). In this case no Q₃₄ modified tRNAs were detected and this sample serves as a reference control in this and subsequent experiments. When expression of TbLa was downregulated by RNAi, the levels of Q₃₄ in tRNA increased to nearly 90%, while the Q₃₄ levels in RNA from uninduced cells remained within the 40–60% range (Fig. 1B and C). Similar increases upon TbLa downregulation were also observed with the other Q₃₄-containing tRNAs (tRNA^{Asp}, tRNA^{His}, and tRNA^{Asn}, [supplementary Fig. S1](#)). These results indicate that TbLa plays an important role in regulating Q₃₄ levels of tRNA in *T. brucei*.

High Q₃₄ levels are not due to increased tRNA dwell time in the nucleus

We next explored possible mechanisms that lead to the observed increase of Q₃₄ in tRNA in the absence of TbLa. In a previous report, we showed increased Q₃₄ levels in tRNA upon reducing the levels of certain amino acids in the media including tyrosine [48], or upon downregulation of the tRNA exporter TbMex67 [51]. We established in both cases that the observed higher levels of Q₃₄-content in tRNA were partly due to increased dwell time of particular tRNAs in the nucleus [48, 51], indicating that there is competition between the rate of nuclear export and the rate of Q₃₄ formation in the nucleus. Therefore, we hypothesized that TbLa is required for efficient tRNA nuclear export, where absence of TbLa increases the nuclear dwell time of tRNA and consequently increases Q₃₄. To test this hypothesis, we analyzed the

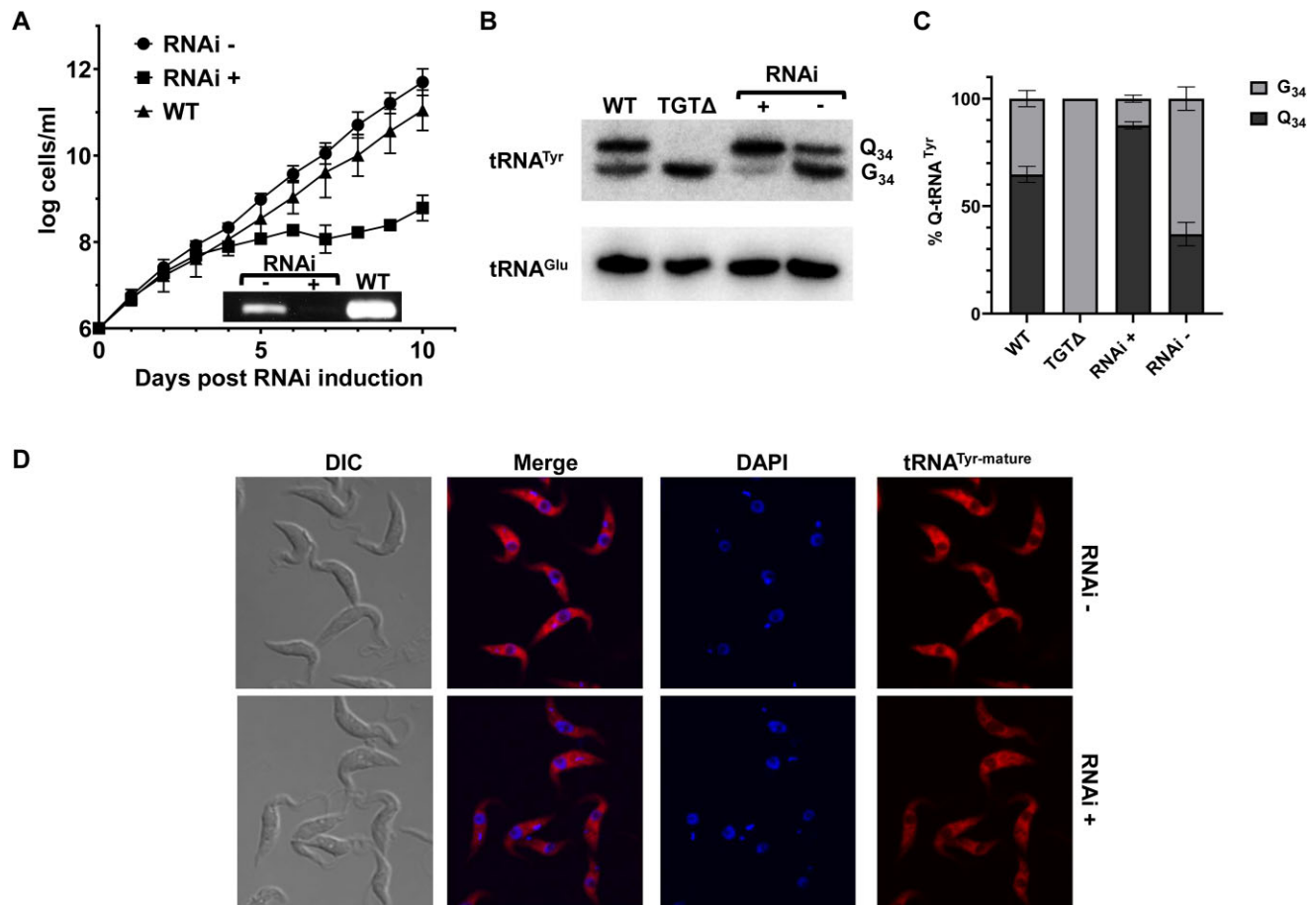


Figure 1. Knockdown of TbLa increases Q₃₄ levels in tRNA. **(A)** Growth curves of *T. brucei* wild type (WT, triangles), RNAi uninduced (RNAi-, circles) and TbLa RNAi induced cells (RNAi+, squares). The inset shows RT-PCR using total RNA extracted on day 5 of the growth curve. RNAi was induced by addition of tetracycline to the media (as described in the “Materials and methods” section). **(B)** An APB-northern blot with radioactive oligonucleotide probes specific for tRNA^{Tyr} and tRNA^{Glu} and total RNA samples as above from WT, TGT knockout (TGTΔ), TbLa RNAi- and RNAi+ cells, tRNA^{Glu} serves as an RNA input normalization control. **(C)** Graphical representation of percent of Q modified tRNA^{Tyr} shown in from **(B)**. **(D)** Fluorescence *in situ* hybridization of TbLa RNAi uninduced (RNAi-) and induced (RNAi+) cells, probed for mature tRNA^{Tyr} with a Cy3-labeled oligonucleotide probe specific for the spliced anticodon arm. DIC refers to the phase contrast image, DAPI stains nuclear and kinetoplastid (mitochondrial) DNA and merge shows the overlapped images of DAPI and Cy3 probes.

localization of mature tRNA^{Tyr} in *T. brucei* cells by fluorescent *in situ* hybridization (FISH) using a fluorescently labeled oligonucleotide probe specific for the spliced anticodon arm of tRNA^{Tyr}. We compared the localization of mature tRNA^{Tyr} between cells where TbLa expression is normal or as down-regulated by RNAi. These experiments revealed no difference in localization and no evidence of increased dwell time in the nucleus in the absence of TbLa, formally refuting the hypothesis that the observed increase in Q₃₄ levels is due to some role of TbLa in tRNA nuclear export (Fig. 1D). Q₃₄-tRNA species that are not encoded with an intron, for example tRNA^{Asp}, also localized similarly to tRNA^{Tyr} (Supplementary Fig. S2), with no evidence of increased nuclear dwell time in the absence of TbLa. Therefore, the observed increase in Q₃₄ must occur by a different mechanism.

The La motif and RRM1 domains are important for maintaining normal Q₃₄ levels

An alternative hypothesis is that tRNA binding by TbLa in the nucleus competes with TbTGT, thus reducing the availability of tRNA substrates to be modified. In this scenario, the binding domains of TbLa should be important to maintain

normal Q₃₄ levels. To test this hypothesis, we first generated a cell line expressing a recoded version of TbLa (TbLaRec), in which TbLa codons were swapped with synonymous codons in the region that is targeted by RNAi (Supplementary Fig. S3). The resulting recoded TbLa (TbLaRec) mRNA is thus impervious to RNAi of endogenous TbLa, yet generates an otherwise wild-type protein with identical amino acid sequence to endogenous TbLa. The TbLaRec was expressed from a plasmid in the endogenous La RNAi cells allowing degradation of the endogenous TbLa mRNA by RNAi and simultaneous expression of TbLaRec. Indeed, this construct could rescue the growth defect generated by La RNAi, upon addition of tetracycline to the media (Fig. 2A). Degradation of the endogenous TbLa mRNA in this background was confirmed by RT-PCR (Fig. 2B), while the mRNA for TbLaRec remains stably expressed (Fig. 2B). Expression of the recoded protein was also detected by western blots with antibodies against a Myc-tag placed at the N-terminus of the recoded protein (Fig. 2C). La proteins, including TbLa, are mainly targeted to the nucleus with small amounts found in the cytoplasm [22, 52, 66]. To confirm the proper localization of TbLaRec, we used anti-Myc antibodies for immunofluorescence microscopy, revealing that TbLaRec is localized to the nucleus (Fig. 2D) similar

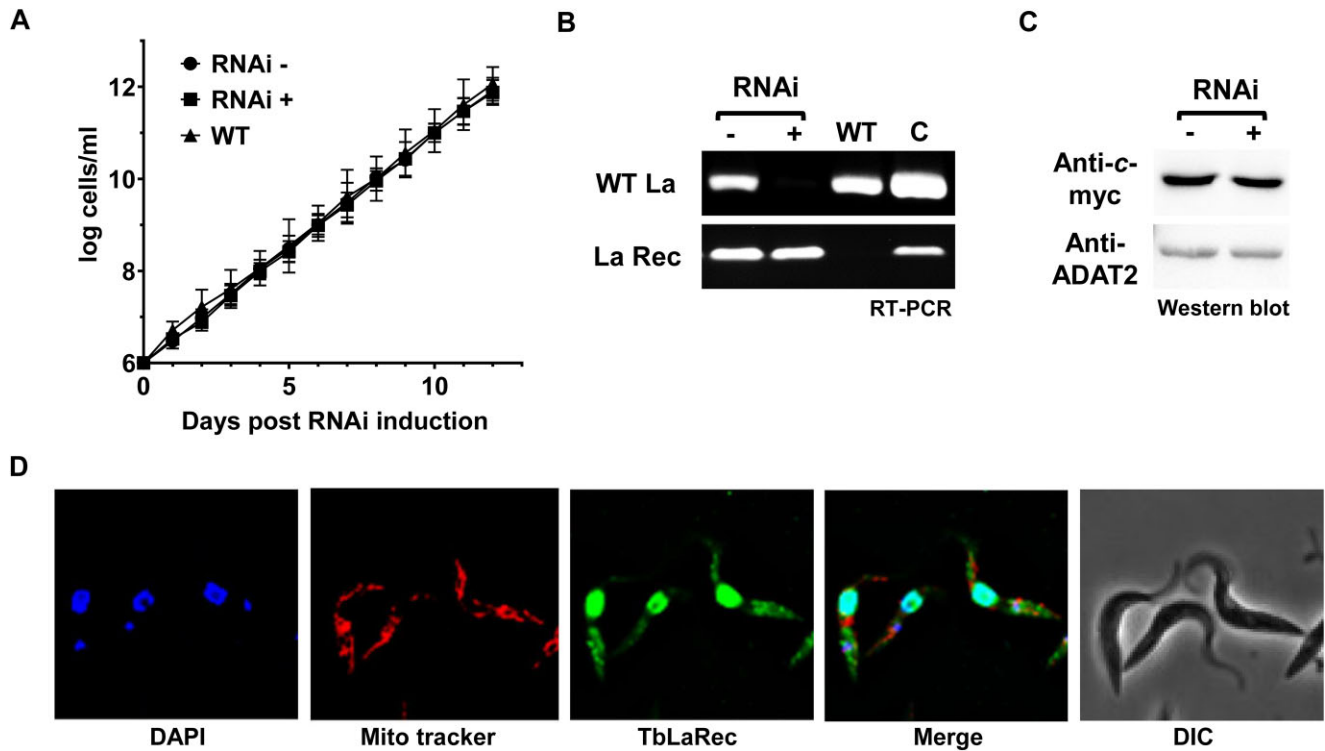


Figure 2. Recoded La rescues the growth defect caused by the knockdown of endogenous TbLa protein. **(A)** Growth curves of wild type (WT), uninduced (RNAi-) and induced (RNAi+) of endogenous TbLa cells expressing a constitutive myc-tagged recoded version of TbLa (myc-TbLaRec). **(B)** Agarose gels showing RT-PCR of endogenous TbLa mRNA (WT La) and recoded myc-TbLa mRNA (La Rec) using total RNA extracted on day 5 of the growth curve (as in Fig. 1). The “WT” lane indicates the RT-PCR of *T. brucei* total RNA from WT (29–13) cells and the “C” lane indicates the PCR using genomic DNA as the positive control. In this experiment RNAi refers to downregulation of endogenous TbLa. **(C)** Western blot with anti-c-myc antibodies and total protein extracted from uninduced (RNAi-) and induced (RNAi+) cells expressing myc-TbLaRec. The blot was stripped and probed for ADAT2 using anti-ADAT2 polyclonal antibodies as the loading control. **(D)** Immunofluorescence microscopy of TbLa RNAi cells expressing myc-TbLaRec, where Alexa 488-conjugated antibodies were used to detect myc-TbLaRec, while Mitotracker stains the mitochondria and DAPI nuclear and kinetoplast (mitochondria) DNA, merge shows the overlapping stains and DIC refers to a phase contrast image.

to what has been reported for the native protein [52]. We also isolated RNA from the La RNAi cells expressing TbLaRec when RNAi is induced and uninduced. The resulting total RNA was then analyzed by APB-northern blot hybridization as before and the levels of Q₃₄ in tRNA^{Tyr} were determined while comparing RNAi induced versus uninduced cells. Normal levels of Q₃₄ are restored with TbLaRec when endogenous La is downregulated by RNAi (Fig. 3A), in contrast to the almost 100% Q₃₄ increase in tRNA^{Tyr} samples from the parental La RNAi induced cells (Fig. 3A and B). These results show that TbLaRec completely rescues the endogenous TbLa function.

Using the TbLaRec cell line, we next explored the contribution of the different RNA binding domains of TbLa to regulation of Q₃₄ levels. Previous work showed that the La module is responsible for both tRNA and UUU-3′OH binding [47, 67]. *In vitro*, the RRM2 α domain binds structured RNAs [68] and the SBM is involved in 5′ triphosphate binding in pre-tRNA [8, 69]. Although the general function of these motifs is not well-established, it has been proposed that RRM2 α could be functionally significant due to the correlation between the presence of this domain and the essentiality of La proteins across different species including humans [8]. The most conserved RNA binding domains of TbLa, the LaM (between residues 5–90) and RRM1 (between residues 96–192) have been structurally and biochemically characterized and are essential for pre-tRNA-UUU-3′OH binding although

their ability to bind mature tRNA has not been tested before [67, 70]. While the RRM2 α (between residues 213–314) and SBM (between residues 315–330) domains of TbLa have not been biochemically characterized, the high sequence conservation of these domains with other well-characterized La proteins, like human La, has allowed the bioinformatic prediction of location and secondary structure of these domains [9]. We generated a series of cell lines expressing various versions of TbLaRec with each of the aforementioned RNA binding domains deleted (Fig. 4A). These were expressed in the endogenous La RNAi cells. Upon addition of tetracycline to the media to induce RNAi of endogenous TbLa, we observed that neither the recoded LaM Δ mutant nor the RRM1 Δ mutant could fully rescue the growth defect caused by TbLa downregulation while the RRM2 α Δ and SBM Δ deletions showed complete growth rescue (Fig. 4B). Both the expression of the recoded mutants and degradation of the endogenous TbLa mRNA were confirmed by RT-PCR (Fig. 4C). To rule out the possibility that the lack of rescue was due to poor expression and/or stability of some of the mutants, we also performed western blots, which after normalization to a control protein showed no significant differences in the relative levels of any of the mutants as compared to that of the recoded control (Fig. 4D). Altogether, these results suggest that LaM and RRM1 domains are essential for TbLa function, while RRM2 α and SBM domains are not, thus, ruling out the importance of the two C-terminal domains for TbLa essentiality.

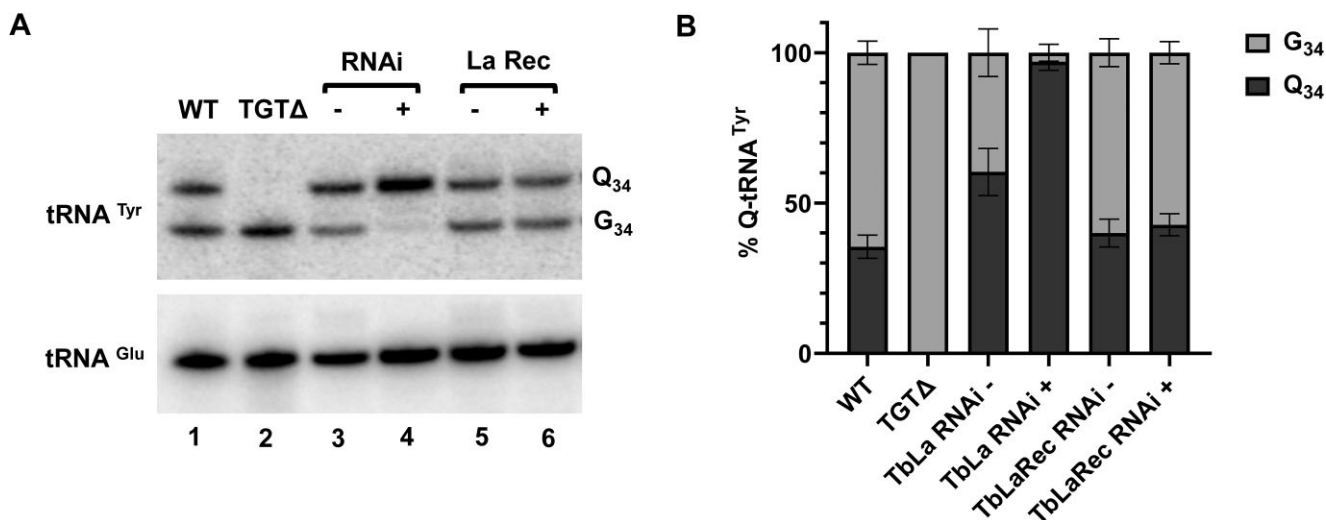


Figure 3. Recoded La protein restores Q₃₄ levels in tRNA. **(A)** Levels of Q₃₄ in tRNA^{Tyr} determined by APB-northern blot. ³²P-oligonucleotide probes specific for tRNA^{Tyr} or tRNA^{Glu} were incubated with membranes from APB-PAGE separated total RNA isolated from WT, TbTGT knockout (TGTΔ), TbLa RNAi uninduced (-) and induced cells (+) (lanes 1–4), as well as the endogenous TbLa RNAi cell line constitutively expressing the recoded version of TbLa (LaRec) with RNAi uninduced (-) or induced (+) (lanes 5 and 6). **(B)** Graphical representation of the percentage of Q₃₄ modified tRNA^{Tyr} as shown in **(A)**. Error bars obtained by measurement from three independent biological samples.

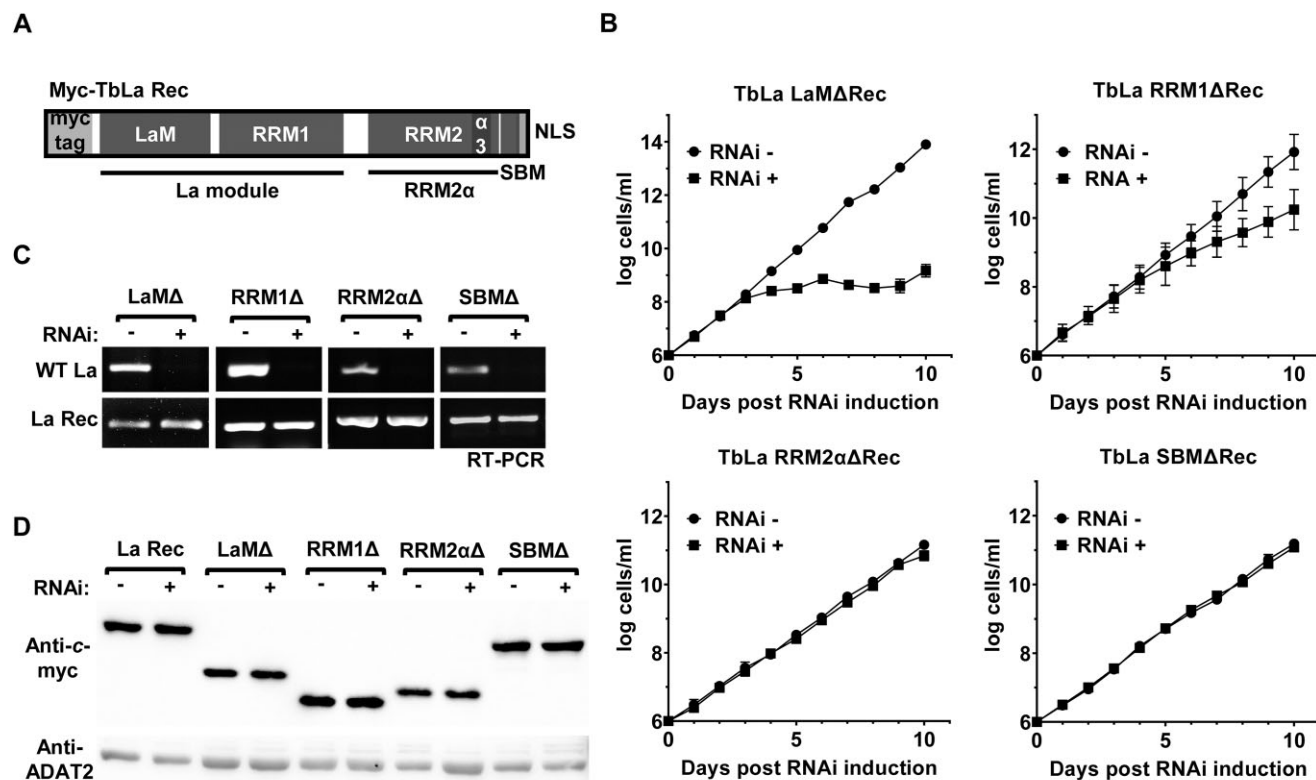


Figure 4. Only LaM and RRM1 domains of TbLa are important for normal cell growth. **(A)** Schematic representation of each domain of myc-TbLaRec, showing the La motif (LaM), RNA recognition motif 1 (RRM1), RNA recognition motif 2, which includes the α3 helix (RRM2α), Short basic motif (SBM), and the nuclear localization signal (NLS). **(B)** Growth curves of TbLa RNAi uninduced (RNAi -) and induced (RNAi +) cells constitutively expressing TbLaRec domain deletion mutants as indicated. **(C)** Agarose gels showing RT-PCR of endogenous TbLa mRNA (WT La) and recoded myc-TbLa mRNA (La Rec) using total RNA extracted on day 5 of the growth curves shown in **(B)**. **(D)** Western blot of total protein extracted from RNAi uninduced (-) and induced (+) cells expressing each myc-TbLaRec mutant. The blot was stripped and probed for ADAT2 using anti-ADAT2 polyclonal antibodies as the loading control.

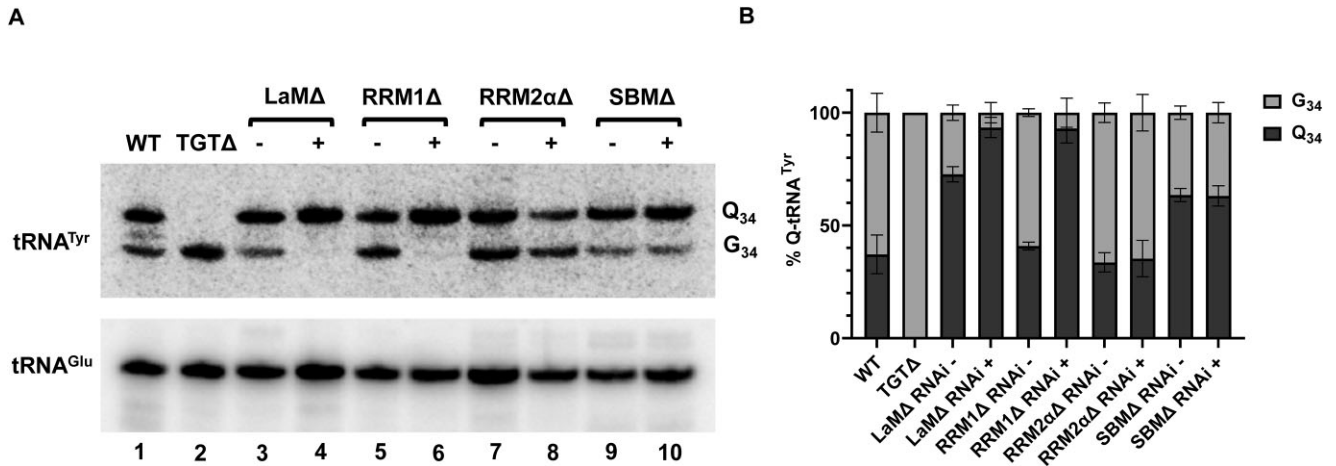


Figure 5. The key RNA-binding domains of TbLa (LaM and RRM1) are important for maintaining normal Q₃₄ levels. **(A)** APB-northern blot with radioactive oligonucleotide probes specific for tRNA^{Tyr} and tRNA^{Glu} and total RNA from WT, TGT knockout (TGTΔ) (lanes 1 and 2), and the endogenous TbLa RNAi cell line constitutively expressing deletion mutants of each domain of TbLaRec as indicated: La motif (LaM) (lanes 3 and 4), RNA recognition motif 1 (RRM1) (lanes 5 and 6), RNA recognition motif 2, which includes the α3 helix (RRM2α) (lanes 7 and 8), Short basic motif (SBM) (lanes 9 and 10) when TbLa RNAi is uninduced (–) and RNAi induced (+). The signal from tRNA^{Glu} served as a normalization control. **(B)** Graphical representation of the percentage of Q₃₄ modified tRNA^{Tyr} as shown in **(A)**. Error bars obtained by measurement from three independent biological samples.

We then isolated total RNA from each recoded mutant to assess the contribution of each domain to the regulation of Q₃₄ levels by APB-northern blot hybridization. It showed that neither the LaMΔ nor the RRM1Δ recoded mutants could restore normal Q₃₄ levels, while Q₃₄ remained at wild-type levels in the cells expressing the RRM2αΔ or the SBMΔ mutants (Fig. 5A and B).

To confirm that the deletions of LaM and RRM1 affect tRNA binding, as observed for other organisms [47], we recombinantly expressed those domain deletion mutants in *E. coli* and tested binding of each mutant to mature tRNA^{Tyr} by Electrophoretic Mobility Shift Assays (EMSAs) while comparing their binding behavior to reference wild-type TbLa protein. We found that while the apparent dissociation constant (K_{Dapp}) for wild-type TbLa is 216 ± 3 nM, binding affinity was reduced with both mutants, with a K_{Dapp} of 855 ± 14 nM and 446 ± 20 nM for LaMΔ and RRM1Δ respectively (Fig. 6D and E). Interestingly, in all cases we observed a sigmoidal curve for binding. We thus transformed the data into a Hill plot, and in all cases positive cooperativity was observed (hill coefficients > 1), with highest cooperativity imparted by the LaM domain deletion. This observation suggests that these domains contribute to tRNA interaction despite not being previously reported as required for tRNA binding [67]. Although we do not currently fully understand the basis nor the importance of cooperativity, these results confirm that both LaM and RRM1 domains significantly contribute to mature tRNA binding by TbLa. Further, these results support our hypothesis that TbLa may regulate Q₃₄ levels in tRNA through direct binding via LaM and RRM1 domains. It should be noted that at least in the case of tRNA^{Tyr}, substrate recognition does not involve a tRNA UUU-3'OH motif, as tRNA^{Tyr} processing requires 3' end-maturation, transit to the cytoplasm to be spliced, and finally return to the nucleus as an end-matured spliced molecule via retrograde transport to get Q₃₄ [49, 51].

TbLa globally impacts tRNA modifications

Given the observed effect of TbLa on Q₃₄ formation, we further explored if this phenomenon is specific to Q₃₄ or

could also impact other tRNA modifications. To this end, total tRNA was purified from TbLa RNAi induced and uninduced cells, followed by digestion to nucleosides for global modification profiling by LC-MS/MS. The results show a significant increase in Q₃₄ levels upon TbLa knockdown as previously seen on APB gels. The levels of ms²t⁶A, Am, Gm, cm⁵U, and OHyW modifications were also significantly increased while a decrease was observed with pseudouridine (ψ), hm⁵C, m¹G, t⁶A, ncm⁵U, m¹I, m⁶t⁶A, D, m⁶A, acp³U, and i⁶A. (Fig. 7). These results indicate that TbLa downregulation has a broad effect on the tRNA modification landscape beyond Q₃₄ formation. However, one of the caveats with total nucleoside analysis using LC-MS/MS is that in cases where a particular modification occurs in different tRNAs (isoacceptors or isodecoders), changes in the observed abundance of a modification on a single specific tRNA species may be obscured by the lack of change on the rest of the tRNA population bearing the same modification. This all depends on the absolute concentration of each tRNA species amongst the sampled group. To explore the impact of TbLa on modifications of specific tRNAs and at specific sites, we implemented OTTR-seq comparing modification levels between RNA samples from TbLa RNAi-induced and uninduced cells. Alas, OTTR-seq is not perfect and it is limited to modifications which lead to a particular nucleotide mis-incorporation signature during reverse transcription. Thus, OTTR-seq permits quantification of up to 7–9 modifications at single-nucleotide resolution including m²G, m²G₂, m¹G, m¹A, m¹I, m³C and acp³U/D/ψ [63, 64]. Among the subset of modifications above, that are detectable via OTTR-seq, only m¹A₅₈ levels were significantly decreased (P value < 0.01) in some but not all tRNAs upon TbLa downregulation (Fig. 8A, B and C). The observed decrease in m¹A₅₈ for select tRNAs is reminiscent of what has been described in other organisms, and could result from the observed RNA chaperone function of TbLa [33, 34, 36, 37, 41]. It is possible that the changes in modifications observed with either technique (be it increases or decreases) were due to relative changes in the concentration of various transcript. To rule out the possibility, we performed northern blots probing for particular tRNAs and we observed not significant

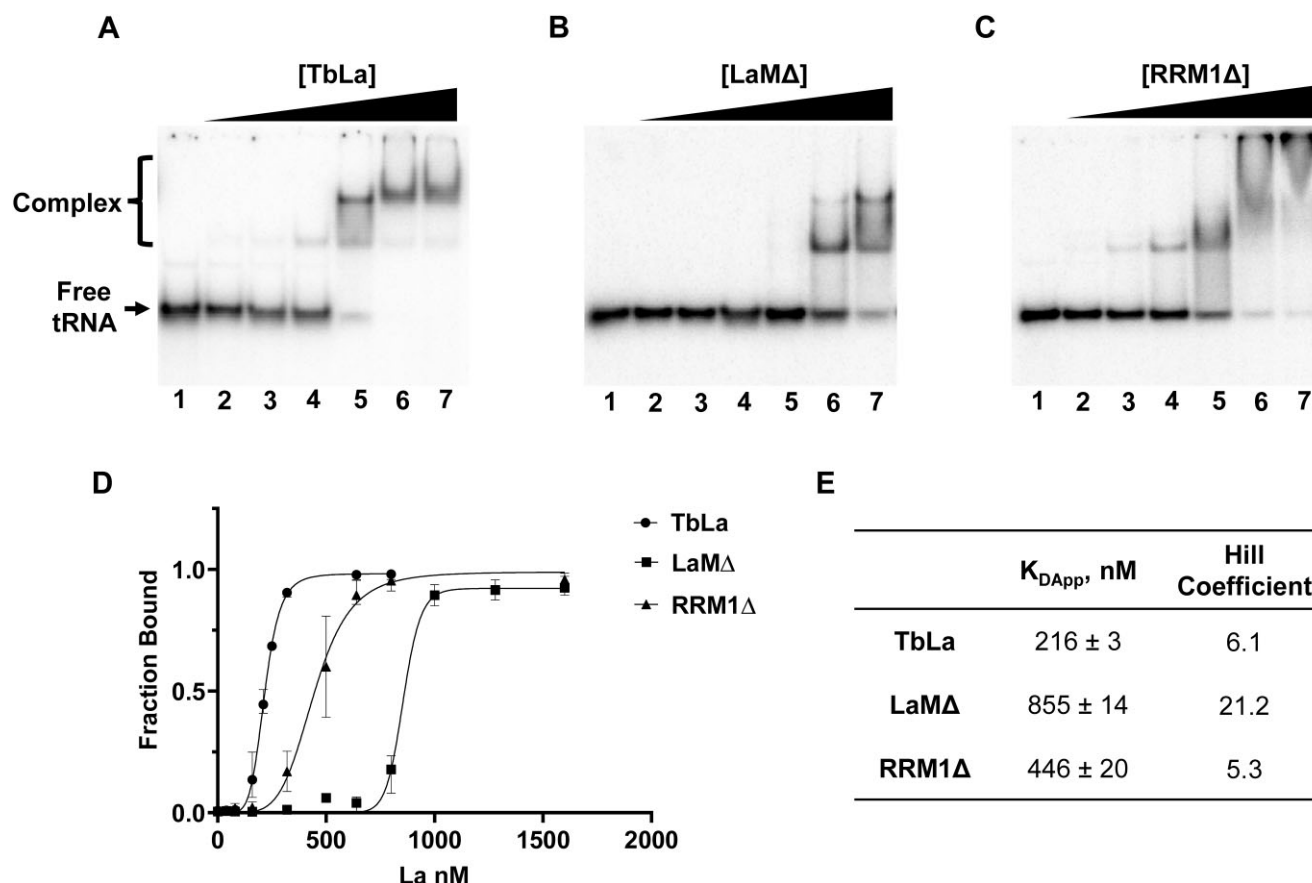


Figure 6. LaM and RRM1 are important for tRNA binding *in vitro*. Electrophoretic mobility shift assay (EMSA) performed on native, mature tRNA^{Tyr} isolated from *T. brucei*, which was T4 PNK 5' ³²P-end labeled prior to incubation with increasing concentrations of TbLa variants (**A**) wild type (**B**) LaM domain mutant (LaMΔ), or (**C**) RRM1 domain mutant (RRM1Δ). Protein-tRNA complexes were separated from unbound tRNA (free tRNA) on non-denaturing polyacrylamide gels. In all cases, lane 1 contains a no protein control, and lanes 2–7 contain TbLa, or domain deletion mutants at 40, 80, 160, 320, 640, and 800 nM, respectively. (**D**) Plots of fraction bound tRNA graphed against increasing concentration of TbLa (circles), LaMΔ (squares), or RRM1Δ (triangles). (**E**) The apparent dissociation constants (K_{DApp}) expressed in nM, and hill coefficients for TbLa and mutants derived from graph (**D**). All the EMSAs, plots, and calculations are representative of three independent replicates.

changes in tRNA levels upon TbLa downregulation by RNAi (Supplementary Fig. S5). As expected, one exception was elongator tRNA^{Met}, which decreased in levels upon TbLa RNAi as has been previously reported [6]. Likewise, estimating tRNA abundance based on read-count during OTTR-seq showed that with the exception of few species (i.e. tRNA^{Ser}_{GCT-2}), in a majority of cases transcript levels were comparable between the RNAi induced and uninduced samples (Fig. 8D). But notably, for reasons that are not currently clear, elongator tRNA^{Met} does not appear to change in levels using OTTR-seq.

Discussion

Post-transcriptional RNA modifications occur in most types of RNAs in cells, but the largest chemical diversity is found in tRNA [71–73]. tRNA modifications are catalyzed by numerous enzymes, some performing bewildering chemistry [74]. Implied in such chemical diversity is an array of metabolites that can be used as both substrates and co-factors for modification reactions, effectively connecting tRNA modification to metabolism and in turn to translational outputs [48, 72, 75]. In fact, fluctuations in the modification content of tRNAs, in response to environmental changes, have been documented

for over 50 years [76–80]. These include responses to changes in temperature [78], amino acid availability [48, 77], and more recently across a range of phosphate concentrations in growth media [81]. Likely, the number of external factors that affect modification levels does not end with this brief list and examples will continue to increase as the field progresses; less clear are the molecular underpinnings that lead to modification dynamics. We, and others, have shown that intracellular transport informs modification levels, for example, the existence of competing rates between tRNA export and modification in the nucleus [48, 51].

Clearly, modifications can be affected by changes in the levels of modification enzymes and/or tRNAs themselves, but beyond this, tRNAs interact with a multitude of factors in their path from maturation to function. In eukaryotes for example, in the nucleus tRNAs interact with enzymes that mature their 5' and 3' ends, a subset of tRNA modification enzymes, tRNA splicing factors (in many organisms), tRNA export factors, etc. Following nuclear export, mature tRNAs interact with another subset of modification enzymes, aminoacyl tRNA synthetases, the translation machinery and eventually with factors that lead to their degradation and recycling. Any of these interactions could potentially affect modification levels. However, for all the known interactions, the complete repertoire of

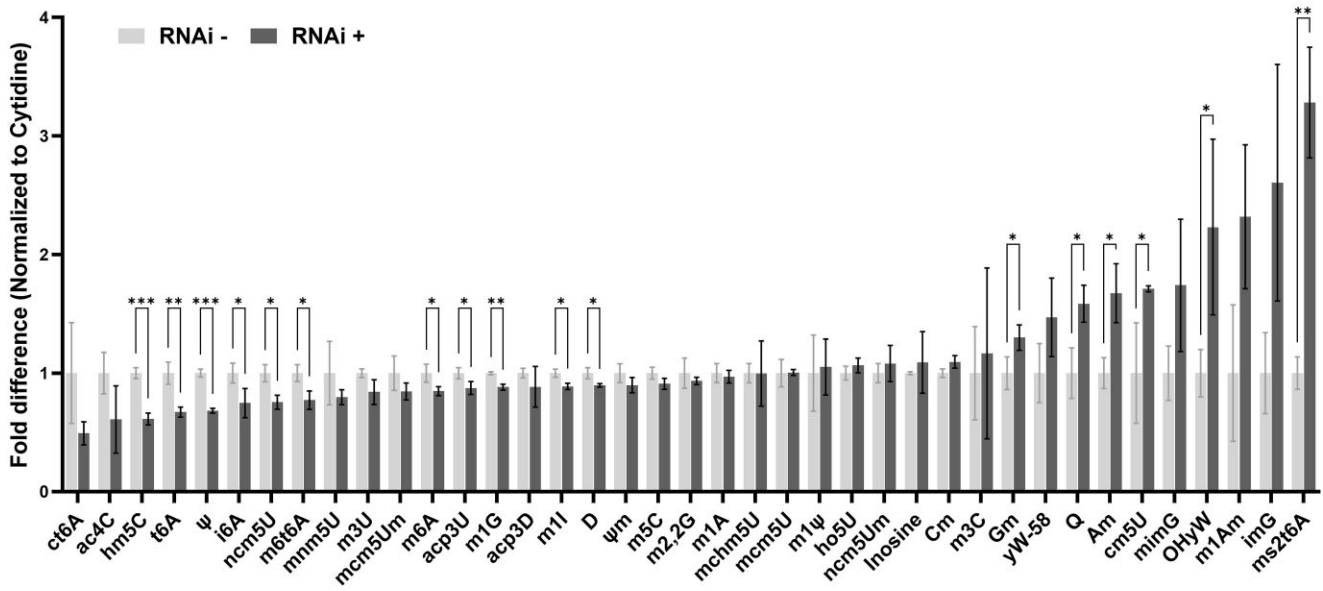


Figure 7. Global tRNA modification changes upon TbLa downregulation are not limited to Q₃₄. Total tRNA samples from uninduced (RNAi-) and induced (RNAi+) were digested to nucleosides and analyzed by LC-MS/MS. Measurements were performed on triplicate biological samples. To calculate the fold difference, the raw values of each sample, were internally normalized to the values of Cytidine in each sample. Each sample quotient was then divided by the triplicate average of RNAi- samples. Asterisks (*) denote the *P*-value thresholds calculated using two-tailed unpaired t-test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001. (ct⁶A: Cyclic N⁶-threonylcarbamoyladenine, ac⁴C: N⁴-acetylcytidine, hm⁵C: 5-Hydroxymethylcytidine, t⁶A: N⁶-threonylcarbamoyladenine, ψ: pseudouridine, i⁶A: N⁶-isopentenyladenine, ncm⁵U: 5-Carbamoylmethyluridine, m⁶t⁶A: N⁶-methyl-N⁶-threonylcarbamoyladenine, mnm⁵U: 5-Methylaminomethyluridine, m³U: 3-Methyluridine, mcm⁵Um: 5-Methoxycarbonylmethyl-2'-O-methyluridine, m⁶A: N⁶-methyladenine or 6-methyladenine, acp³U: 3-(3-Amino-3-carboxypropyl)uridine, m¹G: 1-Methylguanine, acp³D: 3-(3-Amino-3-carboxypropyl)-5,6-dihydrouridine, m¹I: N¹-inosine or 1-methylinosine, D: dihydrouridine, ψm: 2'-O-methylpseudouridine, m⁵C: 5-Methylcytidine, m^{2,2}G: N², N²-dimethylguanine, m¹A: N¹-methyladenine or 1-methyladenine, mchm⁵U: 5-Carboxyhydroxymethyluridine methyl ester, mcm⁵U: 5-Methoxycarbonylmethyluridine, m¹ψ: 1-Methylpseudouridine, ho⁵U: 5-Hydroxyuridine, ncm⁵Um: 5-Carbamoylmethyl-2'-O-methyluridine, I: inosine, Cm: 2'-O-methylcytidine, m³C: 3-Methylcytidine, Gm: 2'-O-methylguanine, yW-58: 7-Aminocarbonylpropylwyosine methyl ester, Q: Queuosine, Am: 2'-O-methyladenine, cm⁵U: 5-Carboxymethyluridine, mimG: Methylwyosine, OHyW: Hydroxywybutosine, m¹Am: N¹, 2'-O-dimethyladenine, imG: wyosine, ms²t⁶A: 2-Methylthio-N⁶-threonylcarbamoyl-adenine)

proteins that, for one reason or another, bind tRNA is not currently known.

Perhaps, no “professional” tRNA-binding protein (a protein that is not an enzyme) has been better studied than the La protein, which is well-known to bind tRNAs containing a 3'-poly U tail, resulting from pol III transcription [24, 25]. Bayfield and Maraia has demonstrated using *in vitro* binding assays that La also binds mature tRNA with high affinity to support their argument that La makes contact with the tRNA body in addition to 3' trailer while chaperoning pre-tRNA [47]. However, the biological significance of such observations was not formally established. In this work we showed that downregulation of TbLa increases Q₃₄ levels to almost 100% in all the TGT tRNA substrates (tRNA^{Tyr}, -His, -Asp, and -Asn). Among these, tRNA^{Tyr}, the only intron-containing tRNA in *T. brucei*, gets modified with Q₃₄ in the nucleus following retrograde transport after undergoing cytoplasmic splicing [49]. Prior to this work, we showed that the version of tRNA^{Tyr} that undergoes retrograde transport to the nucleus bears a mature 3' end (devoid of a poly-U tail) [49, 82], supporting the view that at least for tRNA^{Tyr} the observed Q₃₄ increase during TbLa downregulation is independent of TbLa poly-U binding function. To our knowledge, this is the first evidence for a La protein impacting an end-mature tRNA *in vivo*, and in doing so affecting the levels of a modification.

We explored several non-mutually exclusive models for how TbLa may influence Q₃₄ levels in tRNA. In a previous report, we showed that increased dwell time of tRNAs in the

nucleus, during conditions of reduced amino acid availability, led to increase in their Q₃₄ content [48], suggesting that nuclear export and Q₃₄ modification have competing rates which contribute to the observed 40–60% steady-state levels of Q₃₄. Downregulation of TbLa had little impact on the dwell time of tRNAs in the nucleus, ruling out the possibility that the observed increase in Q₃₄ was due to a slowdown in nuclear export caused by the absence of TbLa. We also considered the alternative hypothesis that TbLa may compete with TGT for binding fully spliced and 3' end matured tRNA^{Tyr}, as well as the other tRNAs that receive Q₃₄ in the nucleus. In this model, downregulation of TbLa increases the amount of free tRNA substrate available for TGT leading to the observed increases in Q₃₄. To test this hypothesis, we analyzed deletion mutants of the various RNA binding domains of La. First, we performed *in vitro* binding studies and confirmed that only LaM and RRM1 are important for mature tRNA binding. We then used the LaM and RRM1 mutants, separately, to complement the La RNAi cell line; in both cases, the levels of Q₃₄ in tRNA remained high, phenocopying what was observed with the downregulation of TbLa alone. An alternative explanation for the observed phenotype in the absence of TbLa is that under normal conditions, TbLa itself interacts with TGT regulating its activity. However, we did not observe a stable interaction between TbLa and TGT in immunoprecipitation experiments (Supplementary Fig. S4). An additional question is, why in the presence of TbLa only 40–60% of those tRNAs get modified. This could be related to the relative concentra-

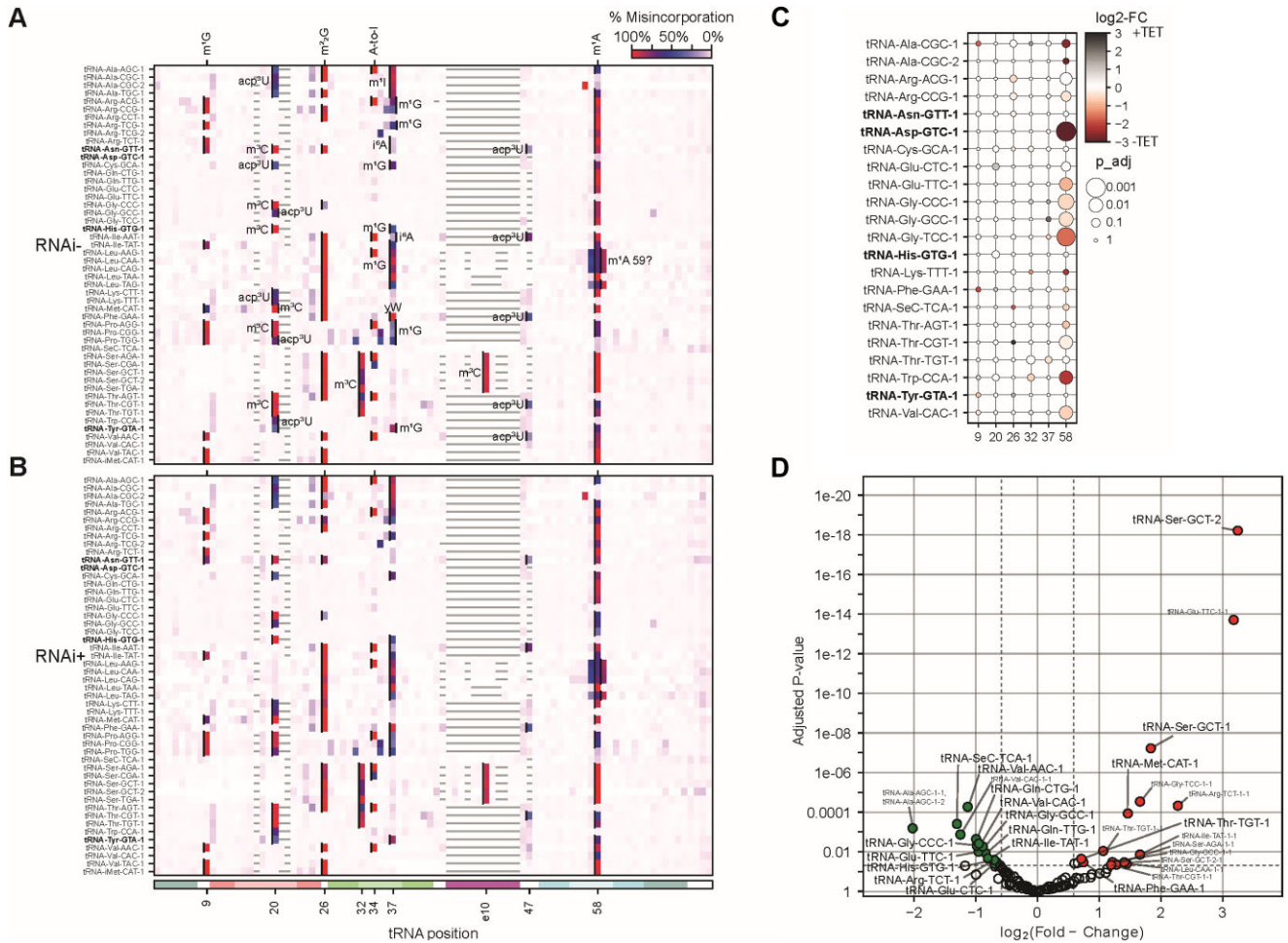


Figure 8. OTTR sequencing reveals m^1A_{58} modification is decreased in a subset of tRNA upon TbLa downregulation. (A) Reverse Transcription (RT) % misincorporation across all *T. brucei* tRNA isodecoders in tRNA samples from TbLa RNAi uninduced (RNAi-) or (B) TbLa RNAi induced (RNAi+) cells. (C) Fold-change difference for % RT misincorporation for a subset of tRNA positions on select tRNAs (color spectrum log₂ scale). TGT substrate tRNAs, which can be modified with Q_{34} , are indicated in bold text. Circle areas indicate adjusted P -value thresholds as indicated. (D) The volcano plot comparing the log₂ fold change of tRNA species between RNAi induced (RNAi+) and uninduced (RNAi-) conditions. The red dots indicate the tRNA species with a significant increase ($P < 0.05$) above a fold change threshold of 1.5 ($\log_2(0.59)$) and green dots indicate the tRNA species with a significant decrease ($P < 0.05$) below a fold change threshold of 1.5 ($\log_2(0.59)$) when TbLa is downregulated (RNAi+). Pre-tRNA species are labeled in smaller text while mature tRNA species are labeled in bigger text.

tions of TbLa and TGT, in addition to the aforementioned competing rates of export. Taken together, the data supports our proposal that tRNA binding by TbLa serves as a key determinant of Q_{34} levels in tRNA. Along these lines, our findings are in agreement with the observation that *in vitro*, La can occlude access of the anticodon loop to chemical modification by kethoxal [7], with one significant difference, in our studies deletions of RRM2 α or the SBM domain of TbLa had little effect on Q_{34} levels. It is possible that our mutations did not completely destroy the intrinsically disordered region of TbLa. However, the most likely explanation is that the various La domains act in concert to regulate modification levels and that indeed there are idiosyncratic differences between TbLa and La from other organisms, for example that of *S. cerevisiae*, the subject of the aforementioned study [7].

A dichotomy in the importance of genuine La in different organisms has been highlighted, whereby genuine La is essential in organisms where La has two RNA-binding motifs (RRM1 and RRM2) [8]. These include Humans and *T. brucei*, but in certain other cases, where La only has one RRM, it

is not essential (i.e. *S. cerevisiae*, *S. pombe*) [8, 9]. Despite these observations it is not fully understood why La is essential. RRM2 α has been suggested as the reason for essentiality [8]. We found that RRM2 α of TbLa is dispensable for its essential function, thus ruling out the suggestion above. Despite its high degree of evolutionary conservation, it is not clear what the general function of RRM2 α is. It remains possible that this domain makes contacts with tRNA and facilitates binding even though it is not a functional determinant for essentiality. This is supported by the cooperative binding behavior observed in binding curves of TbLa, and for the LaM Δ and RRM Δ TbLa mutants.

The ways that TbLa impacts modification may not be limited to our simple competition model. LC-MS/MS analysis of total nucleosides in the presence or absence of TbLa showed its role as a more global regulator of tRNA modification and not strictly a specific regulator of Q_{34} content. Some modifications significantly decreased, such as t^6A , m^6t^6A , ncm^5U and m^1G ; these are precursors or derivatives of modifications that significantly increased upon TbLa downregulation, for exam-

ple, $\text{ms}^2\text{t}^6\text{A}$, cm^5U and OHyW [73, 76, 83, 84]. This observation suggests that TbLa is important in maintaining balance in complex modification pathways and fine-tuning the levels of numerous tRNA modifications. Interestingly, all the modifications that significantly increased upon TbLa knockdown except for Am are found in the anticodon loop of tRNA. Gm and cm^5U occur at position 34 similar to Q while OHyW and $\text{ms}^2\text{t}^6\text{A}$ occur at position 37 [73, 76, 83, 84]. It is worth investigating if any of these modifications are regulated by TbLa in a similar mechanism to Q_{34} . It is also possible that some of these modifications change in a Q_{34} -dependent fashion similar to m^5C_{38} modification in tRNA^{Asp} in *S. pombe*, human cells and mice [85–87]. Additionally, six out of eleven modifications that significantly decreased (t^6A , $\text{m}^6\text{t}^6\text{A}$, i^6A , m^6A , m^1I and m^1G) are found in position 37 of anticodon loop while pseudouridine and ncm^5U also occurs in the anticodon at position 34 [73, 76].

Changes in levels of modifications observed in the anticodon loop may have direct and complex effects on translation, not limited to selection of a specific synonymous codon over another. It is also possible that the modifications that decrease are dependent on the known chaperoning activity of La [36, 37, 39–42] and as such those effects may be more prevalent in structurally “troublesome tRNAs”, while tRNAs which easily fold naturally may not be under such dependency. OTTR-seq revealed that m^1A_{58} went down in a subset of tRNA isoacceptors, most significantly in tRNA^{Gly}_{TCC} and surprisingly in tRNA^{Asp}_{GTC} (one of the Q-containing tRNAs). A_{58} methylation occurs in pre-tRNA and is important for tRNA folding and structural stability [2, 32–35], thus we speculate that in this particular case, TbLa does not serve as a competitor but may provide chaperone activity. Alternatively, TbLa may regulate the levels of the TRM6/61 enzyme itself.

The limitations of total nucleoside analysis by LC-MS/MS are that whereas global changes in modifications can be assessed, the signal for such changes becomes suppressed with modifications that occur in many tRNA species but change only in a small subset. Our analysis of tRNA modification changes via OTTR-seq, which permits single tRNA/ single-nucleotide resolution, circumvents the limitations imposed by total nucleoside analysis on quantification of the specific tRNA modifications that are detectable via OTTR-seq. Exemplifying this, the clear effect on m^1A_{58} modification that is observed via OTTR-seq, is not apparent in the net change of m^1A levels quantified via LC-MS/MS based total nucleoside analysis. Further, although the modifications like i^6A , Acp^3U , m^1G , m^1I are seen significantly decreased via total nucleoside analysis by LC-MS/MS, they are not seen significantly decreased below the P value threshold of 0.01 via OTTR-seq. The significant decrease of these modifications in the total nucleoside analysis could be due to the combinatorial effect of minor changes in individual tRNA species that do not appear as significant via OTTR-seq. Additionally, high sensitivity of LC-MS/MS allows detection of imbalances in modifications such as the family of modifications related to the m^1G_{37} ‘core’ modification such as wybutosine and derivatives (i.e. yW , OHyW). Even though this position is identified as modified via OTTR-seq, this technique cannot formally differentiate between wybutosine (and derivatives) and m^1G , both generate similar nucleotide mis-incorporation signatures during reverse transcription. The two different techniques for assessing tRNA modification levels are thus complementary, both

with inherent benefits and limitations, which further emphasizes the need for technological improvements in new methods that can detect RNA modifications with high sensitivity as well as specificity [88].

The La protein can be described as an evolutionary hallmark of eukaryotes due to its conservation throughout the eukaryotic domain of life [8]. The early La protein that emerged in ancestral eukaryotes diverged into a family of La related proteins (LARPs) specialized for different substrates and functions [3, 4, 9]. Remarkably, phylogenetically deep-rooted *T. brucei* possesses only one La protein while multiple LARPs are present in other non-early diverging eukaryotes [3]. This suggests that the more ancestral TbLa may carry out multiple roles that are otherwise split amongst multiple LARPs in other systems. Interestingly, LARP7, the protein that has most similarities with genuine La proteins [9], has been linked to promoting 2'-O-methylation in U6 snRNA in *S. pombe* and mammals which is required for pre-mRNA splicing [89–91]. Presented with this work is one of the few instances where a member of the LARP family is involved in regulating RNA modifications. Our study also demonstrates for the first time a role for the La protein in affecting the function of end-matured tRNAs *in vivo*. Collectively, the present work constitutes a pivotal finding for understanding how a tRNA-binding protein globally affects modification levels.

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Supplementary data

Supplementary data are available at NAR online.

Conflict of interest

None declared.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

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