Ribosome-associated tDRs in yeast

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Abstract

The regulation of gene expression in response to environmental stress is a key process that ensures cellular survival across all three domains of life. The adjustment of protein synthesis appears to be one of the initial steps toward the response and adaptation to stress. Ribosome-associated non-coding RNAs (rancRNAs) efficiently regulate translation as an immediate response to stress by directly targeting the ribosome and fine-tuning translation. tRNA-derived RNAs (tDRs) are part of the RNA species that constitute the functionally diverse class of rancRNAs. Here we report a new experimental approach for creating deep sequencing libraries of ribosome-associated small RNAs in yeast utilizing state-of-the-art technologies. Our new strategy is supported by validating previously identified rancRNAs and discovering novel tDRs interacting with the *Saccharomyces cerevisiae* ribosome.

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1. Introduction

The crucial role of tRNAs in protein synthesis has been extensively studied. However, the full potential of these molecules, particularly their functions beyond their traditional role as adapter molecules in protein synthesis, has yet to be fully explored (Su, Wilson, Kumar, & Dutta, 2020). The production of tRNA-derived fragments (tDRs) has been established as a regulated process. tDRs are not merely byproducts of degradation, they are long-lived and play meaningful roles in various biological processes, including gene expression regulation, cell stress response modulation, and involvement in disease mechanisms (Polacek & Ivanov, 2020). Their functional versatility is attributed to their unique secondary and tertiary structures, or their specific sequences, which enable interactions with proteins and other RNAs. These interactions proved to be essential for the cellular functions that tDRs modulate. Moreover, recent studies started to shed light on their roles in diseases like cancer and neurodegenerative disorders (Kuhle, Chen, & Schimmel, 2023).

One of the processes in which tDRs have been shown to play a critical role is the regulation of protein synthesis. In the past decade a new class of non-coding RNAs was established, namely ribosome-associated non-coding RNAs (rancRNAs). These rancRNAs are capable of directly binding the ribosome and regulating translation. A significant proportion of tDRs have been identified among the rancRNAs uncovered so far (Pecoraro, Rosina, & Polacek, 2022).

One of the most studied tDR that acts as rancRNA is the tDR^{Val} (tDR-1:24-Val-GAC-1) (Holmes et al., 2023) isolated in the halophilic archaeal model organism *Haloferax volcanii* (Gebetsberger, Zywicki, Künzi, & Polacek, 2012). This rancRNA is generated during alkaline stress conditions and can decrease translation rates by directly targeting the ribosome's small subunit and thereby inhibiting mRNA loading (Gebetsberger et al., 2012; Gebetsberger, Wyss, Mleczko, Reuther, & Polacek, 2017). More recent structural studies have pinpointed the binding site of tDR^{Val} to the 30S subunit of the *H. volcanii* ribosome. The binding site of tDR^{Val} overlaps with that of translation initiation factor aIF1A, blocking the mRNA P-site and therefore preventing the binding of mRNAs (Wu et al., 2024).

The role of rancRNA, and in particular ribosome-targeted tDR, appears to be quite conserved since they have been discovered in more complex organisms too. In *Trypanosoma brucei* a cytosolic 3′-$tRNA^{Thr}$ half

(tDR-37:73-Thr-AGU-1) was shown to localize to the mitochondria upon starvation and stimulate translation by targeting the mitochondrial ribosome (Brogli, Cristodero, Schneider, & Polacek, 2023). Furthermore, a 5′-tRNA^{Pro} half (tDR-1:36-Pro-AGG-2-M2) was found to bind ribosomes of different mammalian cells. *In vitro* studies performed in HeLa cells demonstrated that this interaction led to global translation inhibition and the production and accumulation of peptidyl-tRNA (Gonskikh et al., 2020).

Saccharomyces cerevisiae is deficient of RNA interference pathways involving miRNA and siRNA, making it an excellent model for investigating new forms of ncRNA-based regulation (Harrison, Yazgan, & Krebs, 2009). Early investigations on the presence of tDRs in *S. cerevisiae* revealed that indeed some tRNAs are processed into stable tDRs, especially in response to environmental stress (Thompson, Lu, Green, & Parker, 2008). Subsequently, the RNY1 enzyme was identified as one key factor for the cleavage and generation of yeast tDRs (Thompson & Parker, 2009).

In 2012, the first transcriptome-wide screen for rancRNAs was performed in *S. cerevisiae* under normal and environmental stress conditions (Zywicki, Bakowska-Zywicka, & Polacek, 2012). Despite its limited sequencing depth, due to technological limitations at the time, this study resulted in the identification of functional novel rancRNAs, including tDRs and mRNA-derived fragments (Zywicki et al., 2012). Following studies revealed that both 5′ and 3′ fragments of specific tRNAs are associated with the small subunit of the *S. cerevisiae* ribosome. These works further found the tDR's binding sites to be distinct from the canonical tRNA binding sites. These interactions were detected upon stress conditions and determined global translation inhibition (Bąkowska-Żywicka, Kasprzyk, & Twardowski, 2016; Tyczewska & Grzywacz, 2023).

The initial libraries aiming to identify rancRNAs provided promising first insights into this fascinating class of biomolecules. However, they lack the depth to confidently identify less abundant rancRNAs and tDRs. Here, we describe a strategy, capitalizing on the advanced sequencing technology and an optimized workflow for the rapid and gentle isolation of rancRNAs from actively translating ribosomes. Using our new methodology, we successfully identified novel tDR that bind actively translating ribosomes and confirmed these findings by northern blot analysis. This method thus provides a new basis for studying rancRNAs, including tDRs.

2. Workflow for library preparation

For the isolation of rancRNAs, we have established an experimental pipeline that allows the preparation of high-quality libraries through a streamlined process (Fig. 1A). This pipeline has been designed to minimize contaminants and prevent the generation of degraded RNA products. The initial part of the protocol aims to yield yeast lysates extracted from stressed or unstressed cells (hyperosmotic, ethanol, and heat stress). Then the collected samples are subjected to size-exclusion chromatography (SEC). This procedure aims at isolating ribosomes actively engaged in translation, namely polysomes, and to exclude any free RNA molecules that are not directly involved in translation or do not bind to ribosomes (Krauer, Rauscher, & Polacek, 2021). Furthermore, it enables rapid separation of translating

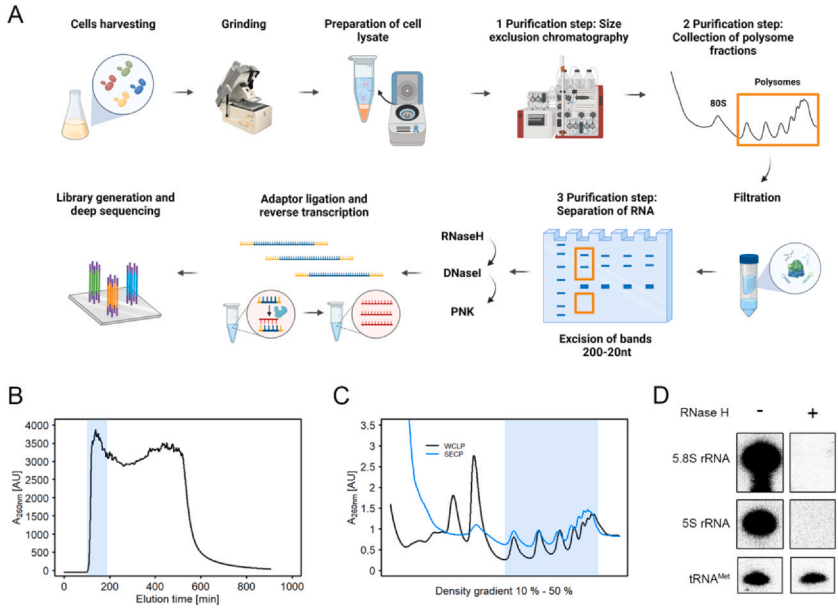

Fig. 1 The workflow for identifying ribosome-associated tDRs in yeast. (A) Schematic representation of the workflow for the isolation of rancRNAs binding to the *S. cerevisiae* polysomes. (B) Size exclusion chromatogram of whole cell lysate. Highlighted in blue are the fractions corresponding to the polysomes, which were further processed. (C) Polysome profiling of samples derived from whole cell lysate (in black, WCLP) compared to the profile of lysates subjected to size-exclusion chromatography (in blue, SECP). (D) Northern blot analysis of samples treated with RNase H (+) in the presence of DNA antisense probes against the 5.8S and 5S rRNA. Specificity is demonstrated by the unaltered $tRNA^{Met}$ abundance after RNAseH treatment.

ribosomes from free cellular RNases that could generate possible false positives of ribosome-bound small RNA fragments in a conventional slower procedure. The comparison between the polysome profile obtained from the whole cell lysate and the samples derived from size-exclusion chromatography highlighted the successful enrichment of polysomes by this approach (Fig. 1B). Moreover, this chromatography step successfully removed the majority of free small RNAs that did not enter the sucrose gradient. Subsequently, the polysome-enriched heavy fractions (Fig. 1C) were pooled and subjected to sucrose gradient centrifugation. In this second step of purification, we selected once more for the actively translating ribosomes. The second extraction from the polysomes further ensures that only those RNAs that were stably bound to actively translating ribosomes were maintained. For the last purification step, small RNA molecules (20 nt to 200 nt) that comigrate with the polysomes were separated and size-selected on a polyacrylamide gel (Fig. 1A). In this phase, full-length tRNAs were excluded to avoid the production of unspecific tRNA fragments. Finally, since rRNA is a common contaminant in deep sequencing experiments that can saturate the majority of reads, RNase H treatment was performed to reduce rRNA content before library generation and deep sequencing (Fig. 1D).

2.1 Yeast strains, cultivation, and harvesting

2.1.1 Equipment

- Petri dishes
- Inoculating loops
- 13 mL snap cap tubes
- 250 mL Erlenmeyer flasks
- 2 L Erlenmeyer flasks
- 30 °C incubator and shaker
- 37 °C incubator and shaker
- Centrifuge (Eppendorf)
- 50 mL Falcon tubes
- Metal spatulas/scrapers
- Membrane filters (0.45 μm, 110 mm diameter, cytiva, Whatman™)
- Filter unit
- −80 °C freezer

2.1.2 Materials

- *S. cerevisiae* KAY488 strain bearing pNOY373 plasmid (Wai, Vu, Oakes, & Nomura, 2000)

- SC-Leu agar plates (6.7 g/L Yeast Nitrogen Base without amino acids, 1.62 g/L drop out medium supplement (w/o leucine), 2 % (w/v) glucose, 2 % (w/v) agar)
- SC-Leu media (6.7 g/L Yeast Nitrogen Base Without Amino Acids, 1.62 g/L Yeast Synthetic Drop-out Medium Supplements (w/o leucine), 2 % (w/v) glucose)
- YPD 0.55 M NaCl (1 % (w/v) yeast extract, 2 % (w/v) tryptone, 2 % (w/v) glucose)
- 100 % Ethanol
- Liquid N_2
- Polysome Buffer: 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL cycloheximide (CHX), 1 mM DTT
- Vanadyl ribonucleoside complex 200 mM (VRC)

2.1.3 Protocol

- Streak out cells from cryo-stocks on SC-Leu agar plates.
- The next morning pick a colony with an inoculating loop and inoculate in 5 mL of SC-Leu media culture in a 13 mL snap cap tube and grow during the day at 30 °C on a rotating wheel.
- Expand the culture to 50 mL SC-Leu media and grow overnight at 30 °C shaking 220 rpm.
- The next morning set up a 400 mL SC-Leu media culture diluting the cells to $OD_{600} = 0.1$ and let grow at 30 °C shaking at 220 rpm.
- Grow cells until they reach $OD_{600} = 0.8$ (exponential phase).
- Divide the culture into eight 50 mL Falcon tubes and centrifuge for 2 min, 4000 xg at room temperature.
- Decant supernatant and resuspend pellets in fresh media:
  - Unstressed: SC-Leu media
  - Hyperosmotic stress: YPD media + 0.5 M NaCl
  - Ethanol stress: SC-Leu media + 12 % EtOH
  - Heat stress: SC-Leu media at 37 °C
- Grow cells for unstressed, hyperosmotic stress, and ethanol stress conditions at 30 °C shaking at 220 rpm, while for heat stress grow them at 37 °C shaking at 220 rpm.
- In the meantime, cool down a 50 mL falcon tube in liquid N_2 and assemble the filter unit.
- Fill the falcon tube with liquid N_2 and drop in 450 μL of Polysome Buffer and 50 μL of VRC (20 mM final concentration).

- After 15 min pour the culture into the filtering system and, once all the media is filtered out, scrape the cell pellet from the filter with a spatula and add it immediately to the falcon tube containing liquid N_2.
- Store at −80 °C.

2.2 Preparation of cell lysate

2.2.1 Equipment

- Freezer mill (SPEXSamplePrep 6775)
- Grinding vials, impactors, and lids
- Metal spatulas/scraper
- Water bath
- Centrifuge (Eppendorf)
- Eppendorf tubes 1.5 mL

2.2.2 Materials

- Liquid N_2
- H_2O_{mq}
- Polysome Buffer: 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL cycloheximide (CHX), 1 mM DTT
- Vanadyl ribonucleoside complex 200 mM (VRC)

2.2.3 Protocol

- Pour liquid N_2 into the freezer mill tank.
- Cool down in liquid N_2 the grinding vials, and impactors.
- Once cold, add the sample (cell pellet, Polysome buffer and VRC droplets) and the impactor into the tube. Close it with the metal lid and cool down again in liquid N_2.
- Insert the vial in the freezer mill and run (add parameters).
- In the meantime, fill the 50 mL Falcon tube with liquid N_2.
- After the run, open the vial and with a cold metal spatula transfer the powder to the falcon tube.
- Store at −80 °C or, once all the liquid N_2 is evaporated, proceed with the next steps.
- Add to the powder 450 μL of Polysome buffer and 50 μL of VRC (20 mM final concentration).
- Thaw the sample in a water bath at 30 °C shaking the tube.
- Transfer the sample to an ice-cold 1.5 mL Eppendorf tube and centrifuge for 3 min at 5000 × g, 4 °C.

- Transfer the supernatant to a new ice-cold 1.5 mL Eppendorf tube and centrifuge for 5 min at 14,000 × g, 4 °C.
- Again, transfer the supernatant to an ice-cold 1.5 mL Eppendorf.
- Proceed with the next steps.

2.3 Size exclusion chromatography and polysome profiling

2.3.1 Equipment

- Sepharose-4b column for size exclusion
- ÄKTA purifier chromatography system (GE Healthcare)
- 96 well plate
- 1.5 mL Eppendorf tubes
- Centrifuge (Eppendorf)
- Open-top polyclear centrifuge tubes (14 × 89 mm, SETON scientific)
- Ultracentrifuge rotor SW41Ti (Beckman)
- Ultracentrifuge buckets (Beckman)
- Ultracentrifuge (Beckman)
- Piston gradient fractionator (Biocomp)
- Column filter tubes (Pierce™ Protein Concentrator PES, 100 K MWCO)

2.3.2 Materials

- Sepharose-4b
- Polysome Buffer (20 mM Tris-HCl pH7.5, 100 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL CHX, 1 mM DTT)
- Vanadyl ribonucleoside complex 200 mM (VRC)
- 10 % Sucrose in Polysome Buffer
- 50 % Sucrose in Polysome Buffer

2.3.3 Protocol

- Wash and equilibrate the Sepharose-4b column with Polysome Buffer.
- Load the sample on the column and elute with Polysome Buffer.
- Collect fractions containing polysomes (Fig. 1A and B), pool them and add again VRC (final concentration 20 mM)
- Directly load the sample onto a 10–50 % sucrose gradient and ultracentrifuge for 3 h at 35,000 rpm (210.100 × g), 4 °C.
- Pump out gradients with the piston gradient fractionator.
- Collect fractions containing polysomes (Fig. 1A and C), pool them, and dilute 1:1 with Polysome Buffer.

- To concentrate the sample, load it onto a column filter tube and centrifuge at 5000 × g, 4 °C until it reaches ~ 300 μL. Wash with 1 mL of Polysome Buffer and centrifuge again until it reaches ~ 200 μL.
- Transfer the sample to an ice-cold 1.5 mL Eppendorf tube and dilute it 1:1 with Polysome Buffer.
- Snap-freeze the sample and store at −80 °C.

2.4 RNA extraction and separation on polyacrylamide gel

2.4.1 Equipment

- 2 mL Eppendorf tubes
- 1.5 mL Eppendorf tubes
- 0.5 mL Eppendorf tubes
- Thermomixer
- Centrifuge (Eppendorf)
- Electrophoresis apparatus
- Blue light gel cutting thing (check)
- Razor Blades

2.4.2 Materials

- H_2O_{mq}
- Phenol (pH 4.3) (Thermo Fisher Scientific)
- Chloroform (Thermo Fisher Scientific)
- NaOAc pH 5.3 (3 M)
- GlycoBlue™ Coprecipitant (15 mg/mL) (Invitrogen)
- Ethanol 100 %
- 8 % Polyacrylamide (7 M Urea) electrophoresis gel
- 2x RNA loading dye (Thermo Fisher Scientific)
- RiboRuler Low Range RNA Ladder (Thermo Fisher Scientific)
- 20 nt RNA oligo
- SYBR™ Gold Nucleic Acid Gel Stain
- Elution buffer (100 mM EDTA, 50 mM NaOAc)
- SUPERase·In™ RNase Inhibitor (20 U/μL) (Invitrogen)
- 15 mL Falcon tubes
- Spin-X centrifuge tube filter (0.45 μm, 2 mL tube, costar)

2.4.3 Protocol

- For RNA extraction add to the sample 1/10 vol of 10 % SDS and 1volume of hot Phenol (pH 4.3) pre-warmed at 65 °C and incubate under a fume hood in a thermomixer at 65 °C, 1200 rpm for 5 min.

- Afterwards incubate the sample on ice for 5 min.
- Centrifuge at for 5 min at room temperature.
- Transfer aqueous phase to a new 1.5 mL Eppendorf tube and add 1 vol of Phenol (pH 4.3) at room temperature, vortex 30 s and centrifuge again at 14,000 × g for 5 min at room temperature.
- Transfer aqueous phase to new tube and add 1 vol of Chloroform, vortex 30 s and centrifuge again at 14,000 × g for 5 min at room temperature.
- Transfer aqueous phase to new tube and add 1/10 vol of 3 M NaOAc (pH 5.3) and 1 μL of GlycoBlue, vortex 10 s
- Add 2.5 volumes of 100 % EtOH or 1 vol of 100 % Isopropanol.
- Incubation overnight at −80 °C.
- The day after centrifuge at full speed for 40 min at 4 °C.
- Remove supernatant.
- Add to the pellet 1 mL of 80 % EtOH, vortex and centrifuge again at full speed for 10 min at 4 °C.
- Remove supernatant and dry the pellet for 5 min under the fume hood.
- Resuspend the RNA pellet in 20 μL of $H2O_{mq}$ and add one volume of 2 × RNA loading dye.
- Boil the sample for 3 min at 95 °C and then cool down on ice for 3 min.
- Load the sample on an 8 % Polyacrylamide (7 M Urea) gel alongside RiboRuler Low Range RNA Ladder with spiked in a single-stranded 20 nt RNA oligo.
- Electrophorese the gel for 2 h, 400 mA and 250 V.
- Afterward, stain the gel with SYBR™ Gold Nucleic Acid Gel Stain.
- Slice gel bands with a sterile razor blade from 200 nt until 20 nt excluding the bands corresponding to the full-length tRNAs.
- Transfer the gel pieces into a 0.5 mL Eppendorf tube with a pierced bottom. Place this tube inside a 2 mL Eppendorf tube. Crush the gel pieces by centrifuging (5000 × g, ~ 10 min, 4 °C), which will force them through the holes into the larger tube.
- Transfer the crushed gel into a 15 mL Falcon tube, add 3 mL of Elution Buffer and 1 μL of SUPERase·In™ RNase Inhibitor.
- Elute overnight shaking at 4 °C.
- The day after filter the slurry gel by loading it on a Spin-X centrifuge tube filter.
- Centrifuge 4000 × g, ~10 min, 4 °C.
- Divide the volume eluted into three 2 mL Eppendorf tubes.
- To each tube add 1/10 vol of 3 M NaOAc (pH 5.3), 1 μL of GlycoBlue, and 1 vol of 100 % EtOH.

- Incubate overnight at −80 °C.
- The day after centrifuge at full speed for 40 min at 4 °C.
- Remove supernatant.
- Add to the pellet 1 mL of 80 % EtOH, vortex and centrifuge again at full speed for 10 min at 4 °C.
- Remove the supernatant and dry the pellet for 5 min under the fume hood.
- Resuspend the RNA pellet in 10 μL $H2O_{mq}$.
- Proceed with next steps.

2.5 RNase H, DNase I, and PNK treatments

2.5.1 Equipment

- 1.5 mL Eppendorf tubes
- Thermomixer
- −80 °C freezer

2.5.2 Material

- RNase H (5 U/μL) (Thermo Fisher Scientific)
- 10X RNase H Buffer (Thermo Fisher Scientific)
- DNase I (1 U/μL) (Thermo Fisher Scientific)
- T4 Polynucleotide Kinase (10 U/μL) (T4 PNK) (Thermo Fisher Scientific)
- 10 X PNK Buffer A (Thermo Fisher Scientific)
- SUPERase·In™ RNase Inhibitor (20 U/μL) (Invitrogen)
- RNA Clean & Concentrator – 5 kit (Zymo Research)

2.5.3 Protocol

- To the previously isolated RNA add the mix of DNA probes (1 μg total) for RNase H treatment aimed at the depletion of the 5S and 5.8S rRNA.
- Allow annealing of the DNA probes to the RNA for 3 min at 65 °C in a Thermomixer.
- Cool samples in ice for 3 min.
- Add the RNase H Buffer and the RNase H and incubate at 37 °C for 20 min.
- To eliminate the DNA probes from the samples, immediately add 1 μL of DNase I and incubate at 37 °C for 10 min.
- Perform RNA extraction using the RNA Clean & Concentrator – 5 kit.
- Elute RNA in 40 μL of H_2O_{mq}.
- Denature RNA at 80 °C for 2 min and then cool down on ice for 5 min.
- Add to the sample the following mix:

- 5 μL of 10 X T4 PNK buffer
- 1 μL of T4 PNK
- 1 μL of RNase inhibitor
- 3 μL of H_2O_{mq}.

- Incubate at 37 °C for 60 min.
- Perform RNA extraction using the RNA Clean & Concentrator – 5 kit.
- Elute RNA in 10 μL of mqH_2O, snap freeze the sample and store it at −80 °C.

2.6 Library generation and deep sequencing

The libraries were prepared using the Small RNA-Seq Library Prep Kit (Lexogen) and then sequenced by NovaSeq 6000 System (Illumina).

3. Deep sequencing data analysis

The pipeline described in the previous chapter ends with the deep sequencing of the putative rancRNAs colocalized with the actively translating polysomes. The deep sequencing data was then analyzed, and its quality was assessed. Subsequently, rancRNA candidates were chosen based on their fulfillment of the criteria to be classified as rancRNAs.

3.1 Bioinformatic analysis

The Bioinformatic analysis of the deep sequencing data was performed with missRNA (Method for Identification of Small Stable RNAs) developed by Prof. Marek Zywicki (Adam Mickiewicz University Poznan) (Chełkowska-Pauszek & Żywicki, 2019). This bioinformatic tool was designed to process RNA sequencing data and identify stable small ncRNAs. The method is specifically suitable for our analysis since it is able to detect small RNAs originating from longer transcripts with high confidence. Moreover, missRNA proves to be extremely accurate in assigning the starting and ending positions (5′ and 3′) of the RNA sequence. This is crucial for the discrimination between RNA fragments produced in a controlled way, for example, fragments processed by an enzyme, and random degradation products.

First, we assessed the read length distribution in each library. In our experimental pipeline, we specifically selected RNAs ranging from 20 nt to 200 nt, a selection that was evident in our sequencing results. Notably, there was a consistent enrichment of reads between 30 nt and 50 nt across

all libraries (Fig. 2A). This observation aligns with previous findings on the majority of characterized rancRNAs and suggests a potential enrichment of tDRs.

Subsequently, we stratified RNA fragments based on their biotypes. In the libraries for the different conditions the majority of reads belonged to mRNA-derived small RNAs. Other candidates belonged to further classes of primary transcripts such as snRNAs, snoRNAs, transposable elements, and tRNA (Fig. 2B).

Finally, the missRNA tool provided a list of putative rancRNA candidates. The potential rancRNA candidates were selected based on different criteria including the length of the fragment, the presence in at least one of the libraries with a high number of reads, and the stable processing of both the 5′ and 3′ ends.

To verify the performance of our library generation pipeline, we investigated the expression pattern of rancRNA_18, a well-characterized *S. cerevisiae* rancRNA. This 18 nt-long RNA derives from the TRM10 mRNA and has been shown to downregulate translation rates by interacting

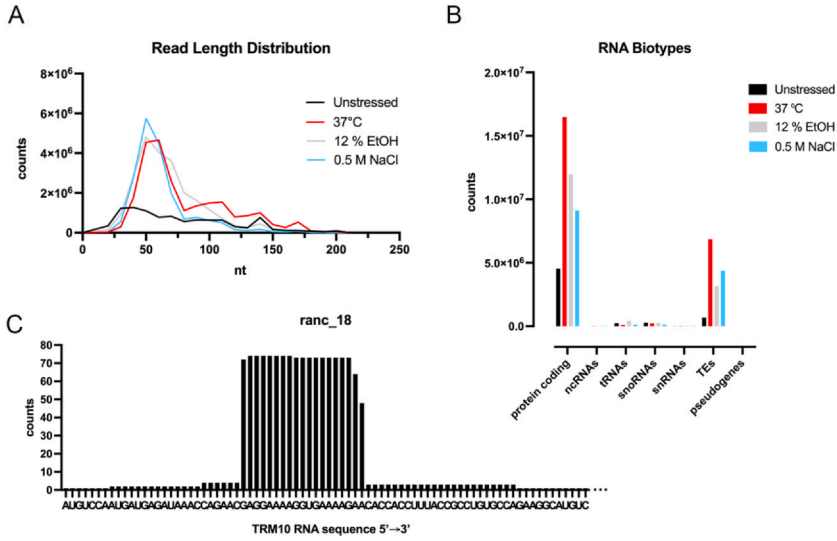

Fig. 2 Biocomputational analysis of the rancRNA library reads. (A) Read length distribution analysis of RNAs present in the libraries. Lines indicate the number of reads of specific sizes ranging from 20 to 200 nt in different stress conditions. (B) Biotype classification of the RNA detected in the different libraries. (C) Alignment to the TRM10 mRNA of read counts for a previously functionally characterized ribosome-associated mRNA fragment ranc_18 (Pircher et al. 2014) in the hyperosmotic stress library (0.5 M NaCl). The height of the bars corresponds to the number of reads in the library.

with polysomes under hyperosmotic growth conditions (Pircher, Bakowska-Zywicka, Schneider, Zywicki, & Polacek, 2014; Reuther et al., 2021), whereas it did not associate with translating ribosomes in normal conditions (Fig. 2C). Our libraries precisely mirrored this already described behavior of rancRNA_18, suggesting that we can specifically enrich for known functional rancRNAs.

4. Validation of the pipeline results and confirmation of tDR rancRNA candidates

Among the small RNAs found in the libraries an average of ~2 % of reads derived from the 5′ and 3′ parts of tRNAs and are thus promising functional tDR candidates. They were abundant and by nature contain at least one sharp processing end, depending on whether they are 3′ or 5′ fragments. To validate if these tDRs represent rancRNA candidates, we experimentally verified their expression in *S. cerevisiae* cells. We used the same four stress conditions as for the library preparation. For this purpose, we employed the northern blot technique as described (Gebetsberger et al., 2012), and we compared RNA samples obtained from polysome fractions (P) and whole cell lysate (L). Here we report three examples of tDRs originating from various primary tRNA molecules that we were able to confirm as rancRNAs. Northern blot analysis confirmed the presence and the association with polysomes of a 3′-tDR generated from the $tRNA^{Arg}$ (tDR^{Arg}, tDR-33:T3-Arg-CCT-1–1) and 5′-tDRs deriving from $tRNA^{Asp}$ (tDR^{Asp}, tDR-1:33-Asp-GTC-1) and $tRNA^{Gln}$ (tDR^{Gln}, tDR-1:29-Gln-CTG-1-M2) (Fig. 3B–D). While the tDR^{Arg} and tDR^{Gln} are newly discovered tDRs, tDR^{Asp} was identified in a previous study. In this study, the molecule was discovered by northern blot analysis of *S. cerevisiae* total RNA samples. Its expression was upregulated during aminoacidic starvation, with the highest accumulation upon methionine starvation (Thompson et al., 2008). Our research builds on this early finding by revealing for the first time that tDR^{Asp} is associated with ribosomes.

As shown in the northern blot analysis of the whole cell lysates, all the tDRs appear to be expressed at high levels in all the different stress conditions, except during hyperosmotic stress. Importantly however, the association with polysomes was more prominent upon heat stress. This evidence suggests that these tDRs might have an increased affinity to the ribosome upon heat stress.

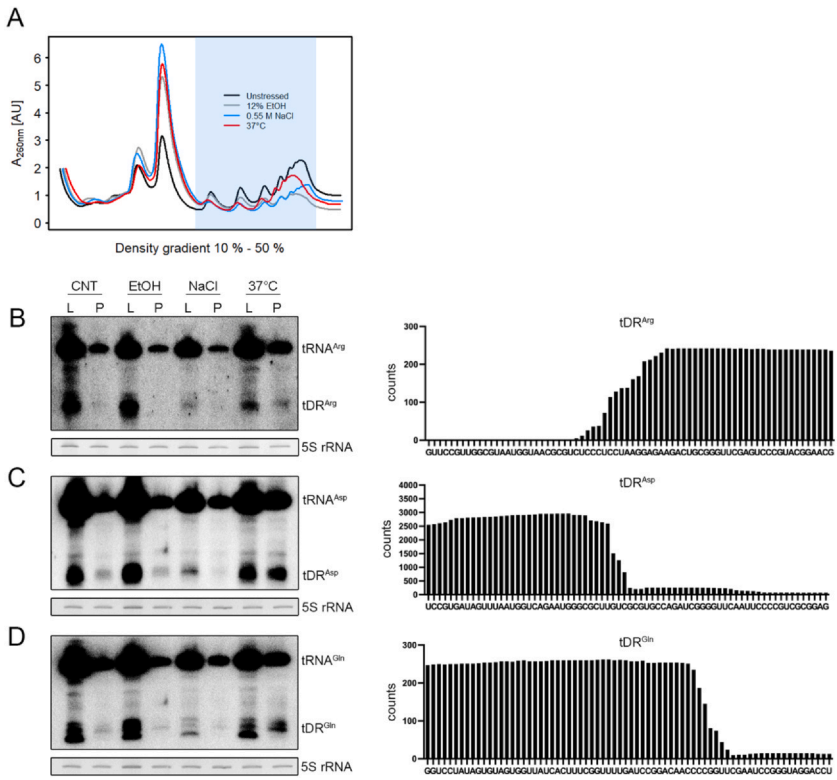

Fig. 3 Verification of tDR candidates as possible rancRNAs. (A) Cells were stressed and lysates were separated by density gradient centrifugation utilizing 10–50 % sucrose gradients. The blue bar highlights the polysome fractions from which RNA was isolated for northern blot analyses. (B–D) Northern blot analyses of tDR rancRNA candidates in whole cell lysates (L) and polysome fractions (P) in four different conditions. 5S rRNA was used as a loading control. tDR expression patterns detected in the libraries are displayed on the right.

These findings substantiate the efficiency of the library generation strategy. Indeed, we successfully identified new tDR rancRNAs and validated this discovery through an alternative methodology, namely by northern blot analysis. This data gives but a glimpse of possible rancRNAs in yeast. Their diverse mode of action could further be investigated by the enrichment of other ribosomal species such as free subunits, or the often hibernating monosomes. To validate that the ribosome-bound tDRs identified via this size-exclusion chromatography-based method are indeed of physiological relevance, dedicated functional assays need to follow. We have in the recent past adapted and applied a plethora of various experimental

tools in *S. cerevisiae* to characterize rancRNAs. These include in vitro translation, in vivo metabolic labeling utilizing yeast spheroplasts, in vitro binding competition and UV cross-linking assays, polysome-Seq-based approaches to study effects on the translatome, and cryoEM (Pircher et al., 2014; Reuther et al., 2021). In our opinion, only the combination of RNA-seq-based tDR identification methods combined with functional assays provides confidence for assigning a biological role to a tDR candidate.

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