RESEARCH ARTICLE

tRNA-derived short RNAs bind to *Saccharomyces cerevisiae* ribosomes in a stress-dependent manner and inhibit protein synthesis *in vitro*

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ABSTRACT

Recently, a number of ribosome-associated non-coding RNAs (rancRNAs) have been discovered in all three domains of life. In our previous studies, we have described several types of rancRNAs in *Saccharomyces cerevisiae*, derived from many cellular RNAs, including mRNAs, rRNAs, tRNAs and snoRNAs. Here, we present the evidence that the tRNA fragments from simple eukaryotic organism *S. cerevisiae* directly bind to the ribosomes. Interestingly, rancRNA-tRFs in yeast are derived from both, 5′- and 3′-part of the tRNAs and both types of tRFs associate with the ribosomes *in vitro*. The location of tRFs within the ribosomes is distinct from classical A- and P-tRNA binding sites. Moreover, 3′-tRFs bind to the distinct site than 5′-tRFs. These interactions are stress dependent and as a consequence, provoke regulation of protein biosynthesis. We observe strong correlation between tRF binding to the ribosomes and inhibition of protein biosynthesis in particular environmental conditions. These results implicate the existence of an ancient and conserved mechanism of translation regulation with the involvement of ribosome-associating tRNA-derived fragments.

Keywords: tRNA-derived small RNAs; tRFs; rancRNAs; ribosome; protein biosynthesis regulation; *Saccharomyces cerevisiae*

INTRODUCTION

As key elements of the protein biosynthesis machinery, tRNAs are highly stable, and therefore their rapid turnover would not be beneficial for the cell. Nevertheless, if tRNA is unable to accurately and efficiently play its role in protein synthesis by correctly charging its cognate amino acid or to perform other additional functions, its presence may be harmful for the cell. Consequently, the tRNA degradation pathways are switched on with the involvement of exonucleases acting on deleterious misfolded or hypomodified tRNAs. However, a processing of tRNAs might also lead to the production of stable, shorter RNA pieces. First assumed to be simply degradation products, there

is now growing evidence that specific tRNA-derived fragments (tRFs) may be implicated in important regulatory and biological processes.

Up to date, the generation of tRNA-derived fragments has been shown in multiple organisms (as reviewed in Gebetsberger and Polacek 2013). However, the biogenesis of tRFs seems to differ between organisms. In 2009, Thompson and Parker (2009) demonstrated that in *Saccharomyces cerevisiae* tRNAs are cleaved by the RNase T2 family member Rny1p, which is released from the vacuole into the cytosol during oxidative stress. In contrast, mammalian tRNAs have been shown to be processed to halves by angiogenin (Ivanov *et al.* 2011) and to shorter, AGO-associated fragments by Dicer (Cole *et al.* 2009; Haussecker *et al.*

2010; Maute *et al.* 2013). Similarly to their biogenesis pathways, the tRF induction seems to depend on the organism and growth conditions. Their expression was subsequently shown to be provoked in a wide variety of organisms by a variety of stress situations, e.g. oxidative stress, heat or cold shocks and UV irradiation (Li *et al.* 2008; Thompson *et al.* 2008; Fu *et al.* 2009; Yamasaki *et al.* 2009; Garcia-Silva *et al.* 2010; Saikia *et al.* 2012). Therefore, tRNA halves are also named as tRNA-derived stress-induced RNAs (Yamasaki *et al.* 2009), although they could be detected also under optimal growth conditions (Peng *et al.* 2012; Dhahbi *et al.* 2013). tRNA fragments have been detected in humans in a number of disease conditions, including cancer and, in some cases, they may function as biomarkers (as reviewed in Anderson and Ivanov 2014; Mleczko, Celichowski and Bąkowska-Żywicka 2014). In *S. cerevisiae*, as we have recently shown, the tRNA-derived fragments are generally widespread in multiple growth conditions (Bąkowska-Żywicka *et al.*, in press). Although we did not observe significant stress-dependent changes in the amounts of tRNA fragments pool, we have shown the differential processing of almost all individual tRNA isoforms.

The mode of gene expression regulation by tRNA cleavage is not well understood yet, but similarly to its biogenesis it seems to differ between higher eukaryotes and other organisms. One of the possible regulatory mechanisms is a decrease of tRNA availability. However, it has been shown that during stress conditions, formation of tRNA cleavage products does not influence significantly the pool of full-length tRNA (Saikia *et al.* 2012). Several observations, made already in 2009, suggested that tRNA-derived small RNAs can regulate protein synthesis: (i) tRNA fragments found in the phloem sap of pumpkin plants inhibited *in vitro* translation in wheat germ extracts (Zhang, Sun and Kragler 2009), (ii) transfection of 5′-tRNA, but not 3′-tRNA fragments triggered the assembly of stress granules, which are mainly composed of stalled pre-initiation translational complexes (Emara *et al.* 2010) and (iii) tRNA fragments have been found to interact with *S. cerevisiae* ribosomes (Zywicki, Bąkowska-Żywicka and Polacek 2012). The ribosome as cellular target for the molecular function of tRNA-derived fragments during initiation of protein biosynthesis has been recently confirmed in *Archaea* and human cells (Ivanov *et al.* 2011; Gebetsberger *et al.* 2012; Sobala and Hutvagner 2013). In the archaeon *Haloferax volcanii*, tRFs derived from the 5′-part of genuine tRNAs have been shown to directly bind to the ribosomes (Gebetsberger *et al.* 2012). The most abundant tRF, a 26 nucleotide long tRF from the Val-tRNA (GAC) locus, directly interacted with the ribosomes *in vitro* and *in vivo* and was capable of inhibiting translation. This Val-tRF exclusively associated with the small ribosomal subunit, especially upon alkaline stress conditions. It has also been shown that Val-tRF decreased translation efficiency of the total *H. volcanii* mRNA population by ∼45%, as well as the extent of peptide bond formation by about 60%. In 2013, Sobala and Hutvagner (2013) presented similar data showing that synthetic RNAs containing 5′-tRF sequences are able to repress the expression of two different reporter genes in mammalian cells *in vitro* and *in vivo*, in a process that does not require the existence of target sites in the mRNA. 5′-tRNA halves were also shown to inhibit translation, but by a different mechanism. Anderson group have found out that natural 5′-tRNA halves (but not the respective 3′-tRNA halves) purified from angiogenin-treated human osteosarcoma cell line (U2OS cells) significantly inhibited translation of uncapped luciferase transcripts in rabbit reticulocyte lysates (Ivanov *et al.* 2011). Consistently, they demonstrated that certain 5′ tRNA halves (Ala/Cys), possessing a putative terminal oligo-G motif containing four to five consecu-

tive guanosines, contribute in displacing translation initiation factors from both capped (eIF4E/G/A) and uncapped (eIF4G/A) mRNAs by cooperating with the translational silencer protein YB-1. Obviously, so far the regulatory functions of tRNA fragments during protein biosynthesis have been assigned exclusively to the 5′-part derived small RNAs. Concerning the molecular function of the 3′ tRFs, it has been shown that they function during cell proliferation (Lee *et al.* 2009; Maute *et al.* 2013) and associate with the small RNA effector machinery (Haussecker *et al.* 2010). In *S. cerevisiae*, there are no data suggesting possible mechanisms of tRF action, except their association with the ribosomes (Zywicki *et al.* 2012). The above mentioned findings suggest that ribosome inhibition by tRFs binding could be an evolutionary conserved and universal mechanism of tRFs action, we decided therefore to investigate this aspect in more detail in *S. cerevisiae*.

In this study, we reveal that in the yeast model species *S. cerevisiae* tRNA fragments are capable to directly bind to the ribosomes in a stress-dependent manner. We present experimental evidence that all investigated 3′-tRFs compete for the same binding site within the ribosome in contrast to 5′-tRF-His, which seems to bind to a different ribosomal region. Moreover, we demonstrate that all tested tRFs reveal stress-dependent inhibitory activity on *in vitro* translation.

MATERIALS AND METHODS

tRNA-derived fragments

tRNA-derived fragments for this analysis have been chosen based on our previous screens for ribosome-associated noncoding RNAs (rancRNAs) (Zywicki *et al.* 2012). We have chosen 6 tRFs with robust reads coverage (over 20 reads) observed in rancRNA library. Sequences and characteristics of the oligonucleotides used in this study are presented in Table 1. Five tRFs were derived from 3′-part of the corresponding tRNAs (3′-tRF-His (GTG), 3′-tRF-Ser (AGA), 3′-tRF-Gly (GCC), 3′-tRF-Leu (TAA), 3′-tRF-Thr (TGT) and one from the 5′-part (5′-tRF-His (GTG)). We have used scrambled 3′-tRF-Thr (TGT) sequence as a control for all experiments (scr-3′-tRF-Thr (GTG)). Secondary structures of *Saccharomyces cerevisiae* tRNAs with tRFs positions are presented in Fig. 1.

Strains and growth conditions

Saccharomyces cerevisiae wild-type strain BY4741 (MAT*α*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) was grown in YPD medium at 30°C. Cells were grown under 12 different growth conditions as described (Causton *et al.* 2001; Zywicki *et al.* 2012). Briefly, stress treatments were performed as follows: cells were grown to mid-log phase (optical density at 600 nm 0.7), the stress was applied for 15 min and the cells were harvested by centrifugation and stored at −20°C. The temperature shifts to 37°C (heat shock) or 15°C (cold shock) were carried out by the addition of an equal volume of YPD pre-warmed to 50°C or chilled to 4°C, respectively. The heat-shocked cultures were continued to grow for 15 min at 37°C, and cold-shocked at 15°C. The cultures were either supplemented with 1 M NaCl (high salt conditions), with 0.1 M Tris–HCl pH 8.3 resulting in a final pH of 7.9 (high pH conditions) or with 1 M citric acid (low pH conditions of pH 4.0). UV exposure was performed in a Stratalinker (Stratagene). Cells were grown to mid-log phase, and then moved into petri plates and exposed to 120 J/m^2 UV. Yeasts were returned to a flask and continued to growth for further 15 min. To induce hyperosmotic shock, the

Table 1. Oligonucleotides used in this study.

Name	Sequence	Length	Copies in Zywicki *et al.* (2012)
3′-tRF-His (GTG)	GAUGAAACCCUGGUUCGAUUCUAGGAGAUG	30 nt	111
5′-tRF-His (GTG)	GCCAUCUUAGUAUAGUGGUUAGUACACAU	29 nt	109
3′-tRF-Ser (AGA)	GGUUCGAGUCCUGCAGUUGU	20 nt	155
3′-tRF-Gly (GCC)	CAUCGUUGGGCCCCCGGUUCGAUUCCGGGCUUGCGCA	37 nt	65
3′-tRF-Leu (TAA)	UUGUCCGCGCGAGUUCGAACCUCGCATCCUUCA	33 nt	47
3′-tRF-Thr (TGT)	GCAAAGGUCGUUAGUUCAUUUCUGACAGGUGGCA	44nt	46
scr-3′-tRF-Thr(TGT)	AUAGGCCAUAAGGAGUCUCGGUACGUCUUGUAUG	44nt	–
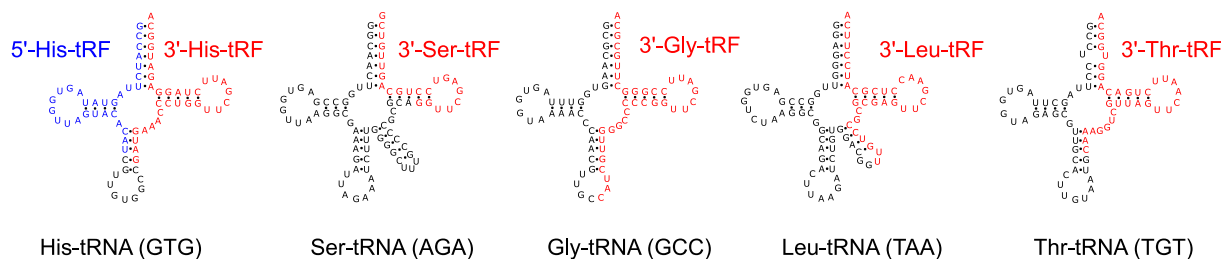
Figure 1. Secondary structures of *S. cerevisiae* tRNAs with marked positions of tRFs used in this study. Secondary structures His-tRNA (GTG), Ser-tRNA (AGA), Gly-tRNA (GCC), Leu-tRNA (TAA) and Thr-RNA (TGT) are presented. tRFs sequence is depicted in colors.

cultures were supplemented with 1 M sorbitol. For hypoosmotic conditions, the cells were grown to mid-log phase in YPD supplemented with 1 M sorbitol, then collected by centrifugation and resuspended in YPD without sorbitol. For amino acid and sugar starvation stresses, cells were collected by centrifugation at mid-log phase, washed in starvation medium and further grown in medium lacking amino acids or sugar, respectively. In parallel, anaerobic and normal growth of *S. cerevisiae* was performed.

Ribosome isolation

Yeast ribosomes were prepared as described (Velichutina *et al.* 2000). Briefly, cell cultures were grown at 30°C to early log phase. Sodium azide (1 mM NaN$_3$) was added to the culture 15 min before the harvest. Cell pellets were washed with water and resuspended in 1 mL of buffer A (10 mM MgCl$_2$, 100 mM KCl, 50 mM Tris/HCl, pH 7.5, 0.4 mM PMSF) at 4°C. An equal volume of glass beads (400 μm in diameter) was added and cells were broken by 10–15 pulses of vortexing (15 s each), punctuated with cooling on ice. Cell debris was precipitated at 27 000 × g for 15 min at 4°C. Lysate was clarified by centrifugation at 30 000 × g for 20 min at 4°C. After clarification, the ribosomes were pelleted from the lysates by centrifugation at 160 000 × g for 90 min at 4°C. Pelleted ribosomes were resuspended in buffer B (2 mM MgAc$_2$, 100 mM KAc, 20 mM HEPES/KOH pH 7.4, 0.1 mM PMSF, 1 mM DTT, 20% glycerol) and stored at –70°C.

In vitro filter-binding assays

Non-covalent binding of tRNA-derived fragments to the ribosomes was determined by the so-called binding assay. *Saccharomyces cerevisiae* ribosomal particles (80S) were incubated with 5′-[^{32}P]-end-labeled synthetic RNA (Table 1) in 30 μL binding buffer (40 mM Tris/HCl pH 7.5, 5.5 mM MgAc$_2$, 80 mM NH$_4$Cl, 3 mM 2-mercaptoethanol, 1 mM spermidine, 0.2 mM GTP). After 15 min of incubation at 30°C, the reactions were filtered

through a nitrocellulose membrane (0.45 μm diameter) using a vacuum device or dot-blot apparatus (GE Whatman Acrylic Minifold I), followed by two washing steps with ice-cold binding buffer. After the membrane was dried, the radioactivity retained was monitored by liquid scintillation counting or exposed to phosphor imaging screens. In the latter case, the signals were monitored with a phosphor imager (FLA-3000; Fuji Photo Film) and analyzed quantitatively with the densitometric program Multi Gauge Image Analyzer. The binding measurements were performed in triplicate. The values reported are corrected for the control samples lacking ribosomes, which were typically 1% to 3% of the total counts of a probe applied.

Saturation assays

The increasing amounts of 5′-[^{32}P]-end-labeled synthetic RNA probes (5–50 pmol) were denatured by incubation for 2 min at 90°C and incubated with 5 pmol of 80S *S. cerevisiae* ribosomes for 15 min in 30 μL binding buffer. The reaction mixtures were filtered under vacuum in the nitrocellulose filter-binding assay.

Translation of poly(U) templates *in vitro*

Translation of poly(U) templates was carried out as described (Synetos, Frantziou and Alksne 1996) using 5 A$_{260}$ units of ribosomes, 25 mg poly(U), 100 mg of soluble protein factors, 25 mg deacylated yeast tRNA and 0,3 nmol [^{3}H]-phenylalanine. The reaction was performed at 30°C for 30 min. The products were precipitated in 10% TCA, and the ester bond between the tRNAs and poly(Phe) peptides was disrupted at 95°C for 10 min, followed by cooling on ice for 10 min in order to release precipitated polypeptide chains. Then the filtration through the Whatmann glass fiber GF/C filters was performed. Filters were washed with 1 mL of 5% TCA and 1 mL of 95% ethanol and subjected to scintillation counting. The *in vitro* translation assays were performed in triplicate. The values reported are corrected for the control
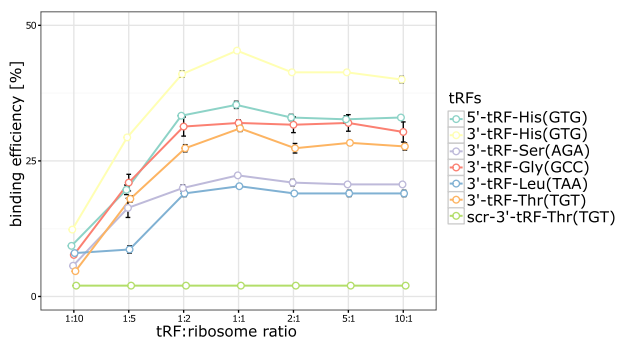
Figure 2. tRFs associate with *S. cerevisiae* ribosomes. Saturation assays of radio-labeled synthetic tRFs binding to 80S ribosome. tRF/ribosome ratio is presented (1:10, 1:5, 1:2, 1:1, 2:1, 5:1 and 10:1). Binding efficiency is shown in [%].

samples lacking ribosomes, which were typically 1%–2% of the total counts of a probe applied.

RESULTS

tRFs bind to *Saccharomyces cerevisiae* ribosomes *in vitro*

In our previous studies, we have identified 41 tRNA-derived fragments that interact with yeast ribosomes (rancRNAs; Zywicki *et al.* 2012), out of which 26 were derived from the 3′-part of particular tRNAs. The most abundant tRFs investigated in this study constitute mostly 3′-tRFs. An interesting case was observed with His-tRNA (GTG) processing, when both derivatives (5′-part and 3′-part) were interacting with *S. cerevisiae* ribosomes with similar efficiency. To investigate the tRF interaction with the yeast ribosomes in detail, *in vitro* binding assays have been performed. Inasmuch as there may be multiple sites that can interact with a portion of a given tRNA-derived fragment, it is essential to determine the involved binding site(s). We have

performed a set of experiments to access these sites, namely the saturation assays.

We performed the saturation assays by increasing the amount of tRF probe present until the ribosome saturation occurs. If the binding is non-specific, it is expected that a tRF probe binding continues to increase linearly with the probe concentration. However, if there is a single site, a saturation occurs (Schmitt *et al.* 1997), wherein the number of the ribosome with attached probe does not increase with the higher tRF probe concentration. According to our results, the tRF probe binding begins to saturate the ribosome when the tRF probe: ribosome ratio reaches 1:1 (Fig. 2). Increasing the concentration of the tRF probe higher than 10 pmol for 5 pmol of ribosomes did not affect the hybridization ratio. Importantly, a 44-mer composed of a scrambled sequence of the 3′-tRF-Thr (TGT) showed minor interactions with the ribosomes, thus highlighting the specificity of tRF–ribosome interaction. Therefore, the saturation assays are clearly indicative of binding specificity.

Yeast 3′-tRFs occupy the same ribosomal binding site which is distinct from the classical tRNA binding sites

To investigate particular binding sites within the ribosomes for different 3′-tRFs, we have performed *in vitro* competition binding assays. We have first pre-incubated the ribosomes with radioactively labeled 3′-tRF-Leu (TAA) in 1:1 molar ratio (as the optimized amount in the saturation assay, see Fig. 2) and then subjected these complexes for an additional binding of increasing quantity of other 3′-tRFs (from 5 to 50 pmol). Consequently, we have measured the fraction of 3′-tRF-Leu (TAA) remaining on the ribosome after incubation with the competitive 3′-tRF. We have observed a clear dose-dependent decrease in 3′-tRF-Leu (TAA) binding to the ribosomes in the presence of all tested 3′-tRFs (Fig. 3A and C). This result strongly suggests that
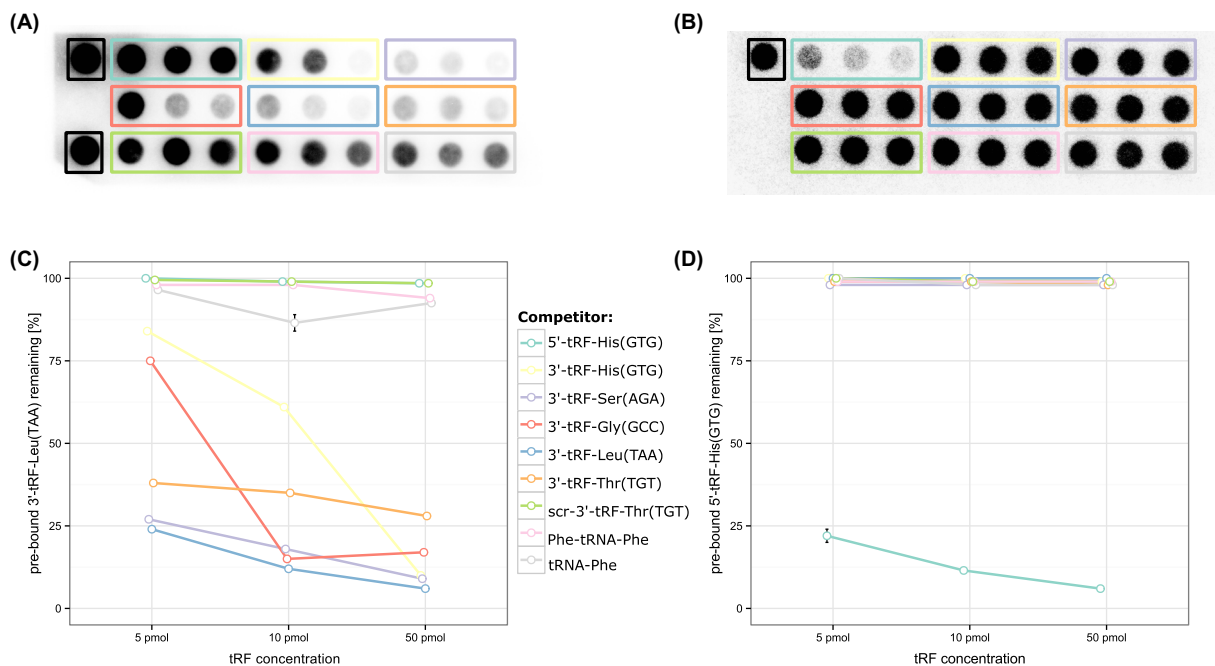

Figure 3. Yeast 3′-tRFs occupy the same ribosomal binding site which is distinct from the classical A site, P site and 5′-tRF-His (GTG) binding site. (**A** and **B**) Dot-blot representatives and (**C** and **D**) graphical representation of *in vitro* competition binding studies on the ribosomes with 3′-tRF-Leu (TAA) pre-bound (A and C) or with 5′-tRF-His (GTG) pre-bound (B and D). 5–50 pmol of corresponding competitor were used, marked with color boxes on (A and B) and color lines on (C and D). Dot blots in the black boxes correspond to the input 3′-tRF-Leu (TAA) (A) or input 5′-tRF-His (GTG) (B).

3′-part derived tRFs from *S. cerevisiae* possess the same or very close binding site within the ribosome. The specificity of the observed competition has been verified by using a control RNA molecule composed of a scrambled sequence of the 3′-tRF-Thr (TGT), which did not induce the 3′-tRF-Leu (TAA) dissociation. Interestingly, the 5′-derived tRF-His (GTG) was also unable to compete with 3′-tRF-Leu (TAA) for its binding site, revealing the pattern of action of the scrambled control.

Since investigated 3′-tRFs span large portion of the tRNAs, they could theoretically associate with the classical A- and P-tRNA binding sites, which are accessible in the 80S ribosomes. In order to verify this possibility, we have used *S. cerevisiae* full-length tRNA^Phe (binding the P site) and its aminoacylated variant (binding additionally the A site when the P site is occupied) as competitors. Interestingly, in both cases, even in the highest concentration of the competitor tRNAs, the pre-bound 3′-tRF-Leu (TAA) was not dissociated from the ribosomes. This result strongly suggests that yeast 3′-tRFs do not bind to the classical A- nor P-tRNA binding sites within the ribosomes.

5′-tRF-His (GTG) occupies different ribosomal binding site than 3′-tRFs

In order to further investigate if all 3′-tRFs possess different ribosomal binding site than 5′-tRF-His (GTG), we have performed the reciprocal *in vitro* competition binding assays. We have first pre-incubated the ribosomes with radioactively labeled 5′-tRF-His (GTG) and then subjected these complexes for an additional binding of 3′-tRFs. None of investigated 3′-tRFs was able to compete with the 5′-tRF-His (GTG) for its binding site, in contrast to 5′-tRF-His (GTG), which served as a positive control for this assay (Fig. 3B and D). This result confirmed that the binding site which is common for all tested 3′-tRFs is different for 5′-tRF-His (GTG). Moreover, by using full-length tRNA^Phe and its aminoacylated variant as a competitor, we have verified that also 5′-tRF-His (GTG) binding site is distinct from the classical A- and P-tRNA binding sites.

tRFs bind to the ribosomes and inhibit translation in a stress-dependent manner

It has been shown that protein composition of the ribosomes can vary in stress conditions (Byrgazov, Vesper and Moll 2013). In order to verify if observed association of tRFs to *S. cerevisiae* depends on ribosome composition, we have compared the tRFs binding efficiencies to the ribosomes isolated from yeast grown under 12 different environmental conditions: heat shock, cold shock, high salt conditions, high pH conditions, low pH conditions, UV exposure, hypoosmotic or hyperosmotic shock, amino acid and sugar starvation stresses, anaerobic and normal growth. Interestingly, we have observed significant differences in tRF–ribosome association. The binding efficiency was oscillating between 8% and 45% for most of the tested tRFs (Fig. 4). However, both derivatives (5′ and 3′) of tRNA-His (GTG) most efficiently associated with the ribosomes in all tested conditions, especially under low pH conditions, amino acid or sugar starvation conditions (62%–100%).

The observation that tRFs bind to ribosomes in a stress-dependent manner led to the speculation of their potential function as regulatory ncRNAs during protein biosynthesis. To clarify this, we set up a simple *in vitro* translation system for *S. cerevisiae* and quantified the amount of synthesized polypeptides in the presence or absence of tRFs. Repeatedly, all tested tRFs de-

creased translation efficiency of the poly(Phe) mRNA in a dose-dependent manner (Fig. 5A). In contrast, a 44-mer composed of a scrambled 3′-tRF-Thr (TGT) sequence did not show any inhibition of protein synthesis. We did not observe any increase in the inhibitory potential of tRFs when increasing the dose to over 100 pmol per translation reaction.

Based on the observation that the efficiency of tRF binding differs for ribosomes isolated from yeast grown under different environmental conditions, we decided to test whether their inhibitory effect reveals the same dependence. We have performed *in vitro* translation assays with the employment of a similar set of stress-altered ribosomes as in case of tRF binding assays. Although as we have shown, the tRF binding was independent from the proteins within the post-ribosomal supernatant (S100 fraction), in order to exclude the influence of potential stress-related protein factors from the S100 fraction, the proteins necessary for the *in vitro* translation reactions for all assays were obtained from non-stressed yeast. As a result, we have indeed observed stress-dependent effect of selected tRFs on *in vitro* translation (Fig. 5B). Besides the regulation of polypeptide synthesis down to 24%–36% in most cases (compared to no tRF control), we have observed a complete shutdown of translation by 5′-tRF-His (GTG) and 3′-tRF-His (GTG), when the ribosomes were derived from yeast grown under amino acid starvation or low pH conditions, respectively. Very high inhibitory potential of tRFs-His (GTG) was also observed for the sugar starvation conditions (5% of translation efficiency with 5′- or 3′-tRF-His). Under these environmental conditions, tRFs-His (GTG) were most strongly associated with the ribosomes. Similar correlation between tRF association and translation efficiency could be drawn for basically all tested tRFs (Fig. 6). 3′-tRF-Ser (AGA), 3′-tRF-Gly (GCC) as well as 3′-tRF-Thr (TGT) revealed constant inhibitory potential within all tested conditions, with the mean values of the poly(Phe) synthesis efficiency of 27%, 36% and 32%, respectively. In case of these three tRFs, also the association with the ribosomes derived from yeast grown under certain physiological conditions did not change significantly and oscillated with the same value as for the optimal conditions.

DISCUSSION

Previous studies by Thompson *et al.* (2008) demonstrated that *Saccharomyces cerevisiae* contains small RNA populations consisting primarily of tRNA halves and rRNA fragments. By the employment of high-throughput sequencing, we have found similar tRNA-derived RNAs to interact with yeast ribosomes (Zywicki *et al.* 2012). In these studies, we have identified 41 tRNA-derived fragments, out of which 26 were derived from the 3′-part of particular tRNAs and 15 from the 5′-part. We have observed an interesting processing of four tRNAs: tRNA-His (GTG), tRNA-Val (AAC), tRNA-Glu (TTC) and tRNA-Glu (CTC), where both 5′-part derived and 3′-part derived tRFs were present in the ribosome-derived library. All these results hint into possible regulatory potential during protein biosynthesis of not only 5′-tRFs (as in *Haloferax volcanii* and human) but also 3′-tRFs. Herein, we show that indeed both 3′-tRFs and 5′-tRF from *S. cerevisiae* bind to the ribosomes *in vitro* and their binding influence protein biosynthesis. The fact that not only archeal but also eukaryotic tRFs are capable of inhibiting protein biosynthesis through direct interaction with the ribosomes suggests the existence of evolutionary conserved mechanism of translation regulation. Concerning *H. volcanii* and *S. cerevisiae* as simple unicellular organisms, we
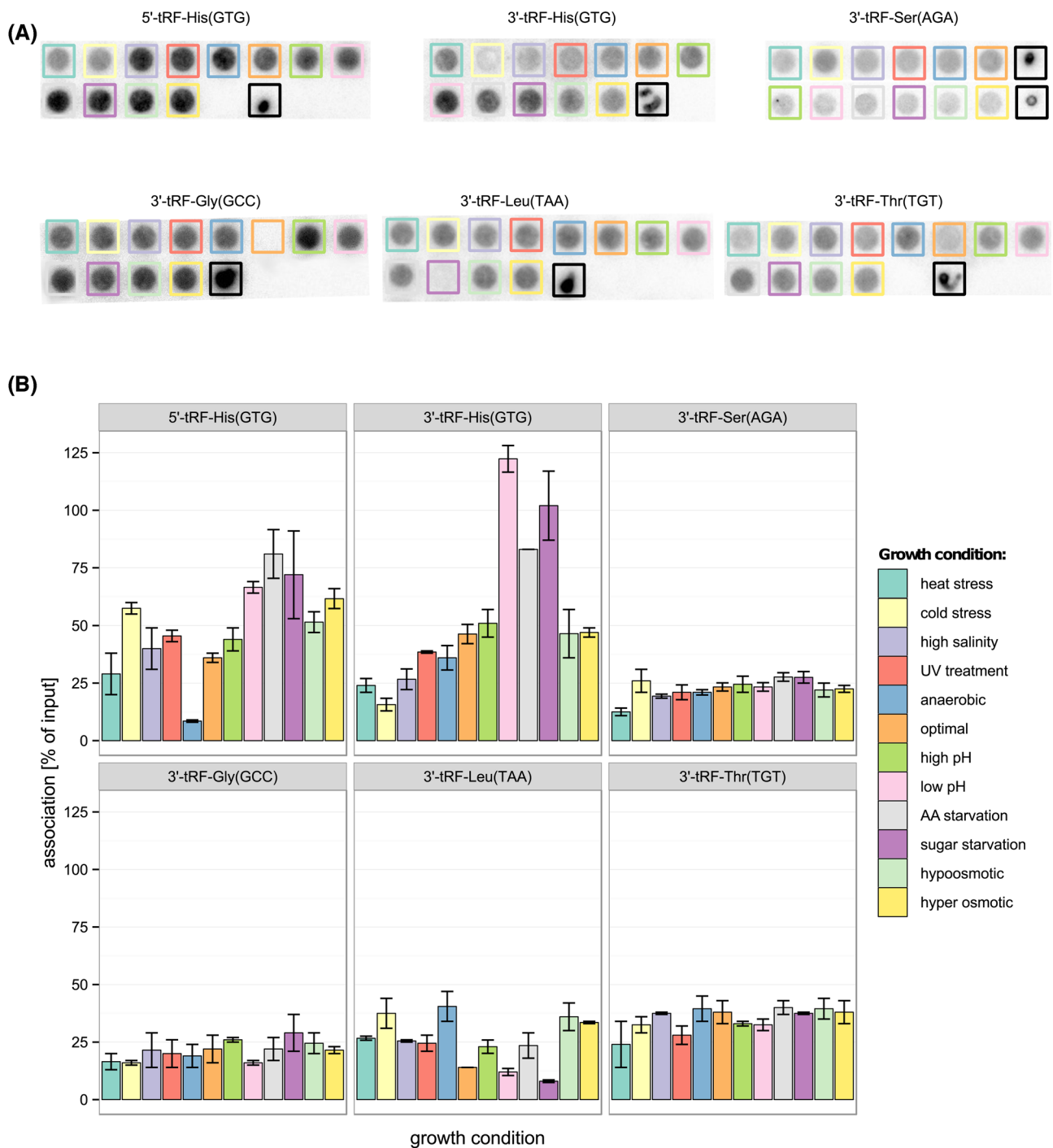
(A)

(B)

Figure 4. tRFs associate with *S. cerevisiae* ribosomes in a stress-dependent manner. tRF/ribosome *in vitro* binding efficiency is shown as percentage of input tRF used for the binding reaction. Individual stress conditions used for ribosome isolation are marked on both **A** and **B** with corresponding colors.

might reason that this regulation is ancient but possibly also present in higher eukaryotes.

tRNAs are abundant non-coding RNAs and well-known substrates for protein biosynthesis. Already in the 1980s it has been shown that the ribosomes can bind up to three molecules of deacyclated tRNA^{Phe} (Rheinberger, Sternbach and Nierhaus 1981; Grajevskaja, Ivanov and Saminsky 1982). The classical tRNA binding sites within the ribosome comprise of the A site (the aminoacyl-tRNA binding site), the P site (the peptidyl-tRNA binding site) and the E site (the exit site). The accuracy of trans-

lation strictly depends on the ability of the ribosome to properly select cognate tRNA molecules. Moreover, it has been shown that also minimal tRNA molecules composed exclusively of anticodon stem-loop (ASL) of tRNA^{Phe} can successfully occupy the tRNA binding sites in *E. coli* and compete with the full-length tRNA^{Phe} for the same binding site (Rose, Lowary and Uhlenbeck 1983). Such interactions appeared to be sufficient for the translocation (Joseph and Noller 1998). Recent crystal structures of the ribosomes revealed the possibility of ASL positioning in the classical tRNA binding P site (Berk *et al.* 2006) or A site (Phelps, Jerinic
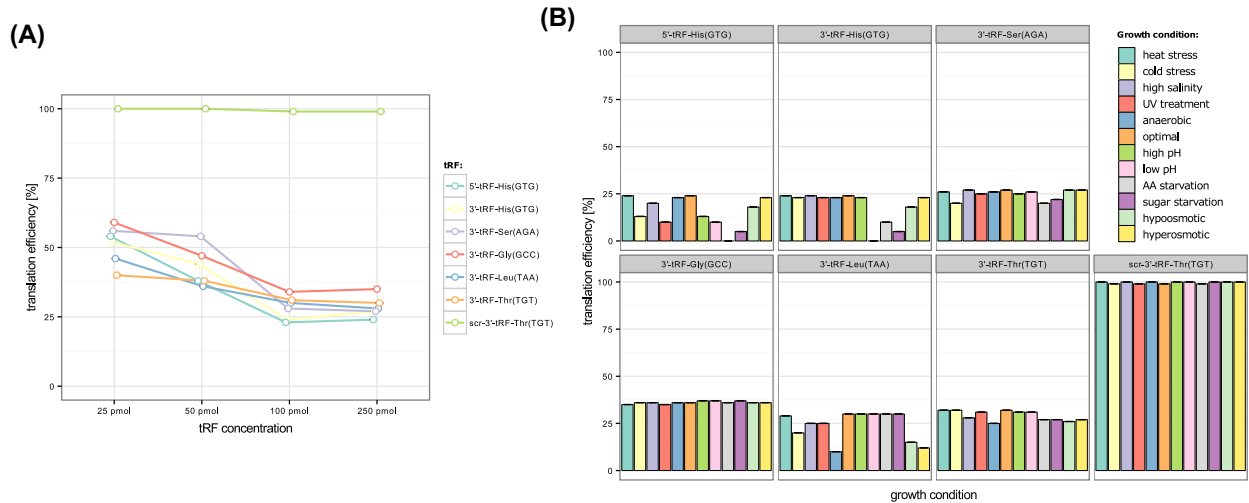
Figure 5. tRFs inhibit polypeptide synthesis in *S. cerevisiae.* Translational efficiency is shown as percentage of the activity of control experiment without tRFs. (**A**) Dose-dependent tRF effect on yeast poly(Phe) synthesis in the isolated *in vitro* translation system. (**B**) Stress-dependence of tRFs inhibitory effect on yeast poly(Phe) synthesis in the isolated *in vitro* translation system.
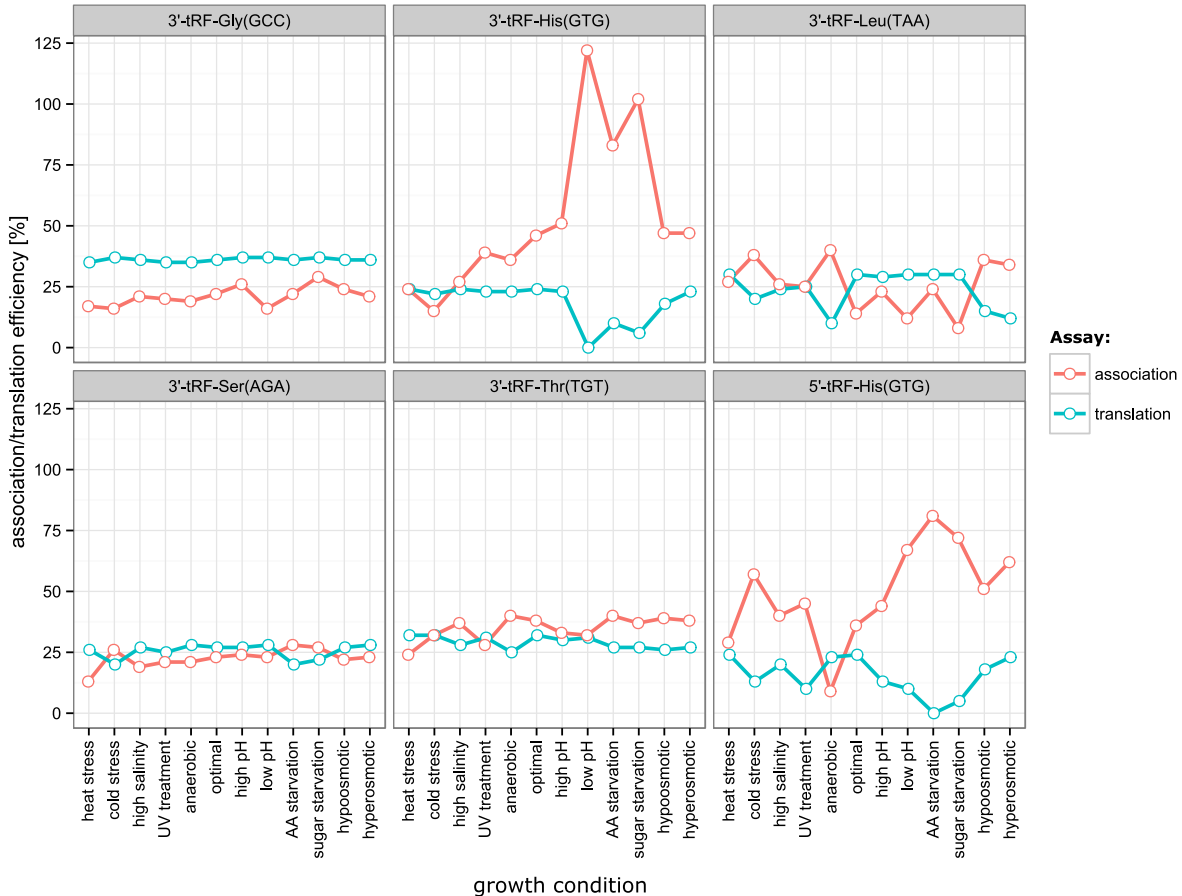

Figure 6. Correlation between tRF association with the ribosomes and tRF influence on translation. Values are means of replicates which are fully presented on Figs 5 and 6. Ribosomes were isolated from *S. cerevisiae* grown under 12 different environmental conditions.

and Joseph 2002). Also 33 nt-long mini-tRNA molecules composed of the acceptor stem and DHU stem-loop or TΨC stem-loop of tRNA[Phe] were shown to efficiently bind to the P and E sites within the eukaryotic ribosomes (Bąkowska-Żywicka *et al.* 2008). It might be therefore expected that tRNA-derived fragments observed in multiple species could possibly bind to the ribosomes. So far, such case was only observed for the *H. volcanii* 5′-tRF (Gebetsberger *et al.* 2012). In these studies, tRFs were observed to interact with the small ribosomal subunit; thus, it might be possible that they occupy the classical tRNA binding sites

within the ribosomes. In our studies, however, we observed that neither 3′-tRFs nor 5′-tRF was competing with the full-length tRNAs (aminoacylated Phe-tRNA^{Phe} and deacylated tRNA^{Phe}) for the ribosomal binding. This result strongly suggests that *S. cerevisiae* tRNA-derived fragments bind to the ribosome in locations distinct from the classical A and P sites. All of tested 3′-tRFs compete with each other for the ribosome binding; therefore, they obviously occupy the same binding sites or located in a close proximity. Nonetheless, 3′-tRFs and 5′-tRF-His (GTG) possess specific binding sites which are located separately within the ribosome structure.

Recent studies have shown that human osteosarcoma cell line U2OS treated with 5′-tRFs derived from tRNA-Ala, Gly and Val tends to form stress granules (Emara *et al*. 2010). Stress granules are dense aggregations in the cytosol composed of proteins and RNAs mainly as stalled translation pre-initiation complexes. These studies confirmed that one role of 5′-tRFs is a rapid response to downregulate protein biosynthesis during stress. In our studies, we showed that *S. cerevisiae* tRFs associate with the ribosomes in a stress-dependent manner which could be further correlated with their regulatory potential during translation process. We have observed the most prominent ribosome association *in vitro* for two tRFs derived from tRNA-His (GTG). These interactions were increased during harsh environmental changes, such as low pH conditions, sugar depletion or amino acid starvation. When eukaryotic cells are deprived of amino acids, uncharged tRNAs accumulate and activate the conserved GCN2 protein kinase (Dong *et al*. 2000). Activated Gcn2p upregulates the general amino acid control pathway through phosphorylation of the translational initiation factor eIF2. On the other hand, the accumulated tRNAs might be susceptible for the processing to shorter, stable forms. Our recent results also show that during amino acid starvation tRNA-His (GTG) is not processed preferentially and the formation of both 5′- and 3′-tRFs does not differ when compared to the optimal growth conditions (Bąkowska-Żywicka *et al*. 2016). In this study, however, the tRFs-His (GTG) possess the highest association with the ribosomes during amino acid stress, meaning that these tRFs might be specifically selected by the ribosomes under certain environmental conditions. Both tRFs-His (GTG) presented the highest regulatory potential in *in vitro* translation assays.

Taken together, all of these data strongly suggest that specific interactions between the ribosomes and 5′- and 3′-tRF-His (GTG) downregulate translational activity during selected non-optimal growth conditions. In this aspect, 5′- and 3′-tRF-His (GTG) could be classified as a second example of rancRNAs, next to previously described by us an mRNA exon-derived 18-residue-long ncRNA, capable of adjusting translation rates by interacting with polysomes under hyperosmotic growth conditions (Pircher *et al*. 2014).

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