

and indirect impacts of CAPAM. In other studies using reporter constructs and RNAs, the presence of the m7G(5')ppp(5')m6Am cap had the opposite or minimal effect on translation [5,9]. Thus the m7G(5')ppp(5')m6Am may have gene- and cell-specific impacts. Sendinc *et al.* report that first-nucleotide adenosine methylation decreases binding to eIF4E, a cap-binding protein that promotes ribosomal subunit recruitment [5]. The list of cap-binding proteins which impact on translation is continually increasing, and it may be that cap adenosine methylation alters the relative affinity for these proteins, which could explain the observed differential effects of CAPAM on translation [2,3,5].

In summary, the identification of CAPAM as the first-nucleotide adenosine N6-methyltransferase is a major finding which will allow the biological function of this modification to be uncovered. Once the physiological processes in which CAPAM has an influential role are identified, the impact of this enzyme and the m7G(5')ppp(5')m6Am cap on gene expression may be clarified.

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Forum

tsRNAs: The Swiss Army Knife for Translational Regulation

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tRNA-derived small RNAs (tsRNAs, or tRFs) are a new category of regulatory noncoding RNAs with versatile functions. Recent emerging studies have begun to unveil distinct features of tsRNAs based on their sequence, RNA modifications, and structures that differentially impact their functions towards regulating multiple aspects of translational control and ribosome biogenesis.

The Expanding Functions of tsRNAs

tRNAs are a type of highly modified and structured RNA that have a well-defined role in mRNA translation. The fragmentation of tRNAs at different loci gives birth to a new species of small RNAs: tsRNAs (also known as tRNA-derived fragments, tRFs) with unexpected complexity, which is due, in part, to the numerous types of RNA modifications inherited from tRNAs as well as to the RNA interaction potential (e.g., RNAs and proteins) endowed by RNA modifications and novel structures [1]. tsRNAs show diverse functions, ranging from stress response, tumorigenesis, stem cell biology, and epigenetic inheritance [1]. At the molecular level, recent converging studies have begun to provide evidence that different tsRNAs interplay with multifaceted aspects of translational regulation and ribosome biogenesis, which involve their sequence specificity, RNA modifications, and structural effects. Since tsRNAs are at relatively low abundance compared with their corresponding full-length tRNAs, these emerging studies reinforce the idea that tRNA fragmentation in translation interference merely due to tRNA destruction is an oversimplified model, instead indicating a novel layer of regulation repurposed by the generation of various functional tsRNAs.

Interfering Translational Initiation and the Role of tsRNA Structure and/or Modification

The function of tsRNA in translational inhibition was documented in early studies by Paul Anderson's group (reviewed in [1]). They found that, under stress, the cleavage of tRNAs at the anticodon by the nuclease angiogenin generates 5' and 3' tsRNAs and that the 5'tsRNAs, but not 3'tsRNAs, could inhibit global protein synthesis [1]. Recently, it was further found that two tsRNAs [5'tsRNA-Ala and -Cys, ~30 nucleotides (nt)] with a terminal oligo-G motif (TOG),

can form intermolecular RNA G-quadruplexes (RG4), displacing the translation initiation factor eIF4E/G/A from m⁷G-capped mRNAs [2]. In addition, TOG-5' tsRNAs bind to the cold shock domain of Y-Box Binding Protein 1 (YBX1) to facilitate the assembly of stress granules (ribonucleoprotein complexes), resulting in the sequestration of initiation factors and adding to the effect of global translation repression, although YBX1 does not directly displace translation-initiating factors from the m⁷G-capped mRNAs [3] (Figure 1A,B). However, knocking down YBX1 only partially reverses the translation repression [1], suggesting that other mechanisms are also involved in 5' tsRNA-induced translational inhibition.

Recently, work by Cristian Bellodi's group further expanded our understanding of 5' tsRNA-mediated translational control by emphasizing the role of RNA modification [4]. They found that the pseudouridine (Ψ) synthase PUS7 is enriched in embryonic and/or hematopoietic stem cells, and that it binds to distinct tRNAs and modifies U into Ψ at the U8 position (Ψ 8). PUS7 deletion leads to significantly decreased levels of TOG-5' tsRNAs around 18 nt, which is associated with increased global protein synthesis [4]. Transfecting TOG-5' tsRNAs with Ψ 8, but not those with U8, can restore the protein synthesis of *PUS7-KO* hESCs and, thus, impact the process of stem cell commitment.

Mechanistically, Ψ 8-containing TOG-5' tsRNA preferentially bind to polyadenylate-binding protein 1 (PABPC1), another initiation factor integral to the formation of the translational initiation complex, resulting in displacement of PABPC1 and eIF4A/G from m⁷G-capped mRNAs. Moreover, depletion of PABPC1 by small interfering (si)RNA can decrease global protein synthesis in *PUS7-KO* hESCs, phenocopying the

effect of Ψ 8-containing TOG-5' tsRNA [4]. These data demonstrate the novel role of Ψ 8 in fine-tuning the function of 5' tsRNAs in translational regulation (Figure 1C).

Notably, the 18-nt U8-TOG-5' tsRNAs show strong binding affinity to YBX1, but cannot displace eIF4A/G, which is distinct from their longer version (30-nt-TOG-5' tsRNAs, which form RG4), as reported previously [3,4]. These results suggest a different secondary RNA structure mediated by tsRNAs length and, thus, a context-dependent binding preference. Given that human embryonic stem cells (hESCs) contain both modified and unmodified TOG-5' tsRNAs (while most bear Ψ 8), they may function synergistically to exert optimized effects in translational regulation.

In another example, hypoxic stress induced a specific population of tsRNAs with a distinct motif that can bind to YBX1, displacing YBX1 from the pro-oncogenic, cancer-promoting mRNAs it protects, resulting in suppression of cancer metastasis [5]. This suggests context-dependent translational regulation mechanisms mediated by versatile tsRNA species in a tissue- and cell-dependent manner.

AGO-Dependent Translational Inhibition by Targeting Specific mRNAs

Previous studies on individual 21–22-nt 3' tsRNAs with 3'CCA-end (suggestive of cleavage from mature tRNAs) revealed their miRNA-like behavior in downregulating gene expression. These 3'CCA-tsRNAs show DICER-dependent biogenesis, binding with AGO, and repressing mRNA translation in a sequence-specific manner [6]. The effects of 3'CCA-tsRNAs are primarily at the translational level, because the mRNA of the target genes is not affected [6]. Despite these individual reports, a general understanding of

AGO-dependent tsRNA-targeting is still lacking.

Recently, by analyzing 495 public small RNA-sequencing libraries, combined with mRNA sequencing and ribosome profiling after tsRNA transfection, Jian Lu's group provided further insight into the principles of mRNA targeting under this type of tsRNA-mediated, AGO-dependent translational inhibition via antisense pairing [7]. Their bioinformatic analyses showed that shorter tsRNAs (20–22 nt) preferentially associate with AGO1/2, whereas longer tsRNAs (23–29 nt) associate with AGO3/AUB/PIWI (the absence of longer tsRNAs of 30–34 nt found in mammals may be due to the size limitation (18–30 nt) of library construction in the analyzed data sets, or to species differences). They also found that tsRNAs tend to be derived from 5' halves of tRNAs in most fly tissues.

By transfecting S2 cells with 12 tsRNA mimics that have been shown to be abundant in *Drosophila* (5' tsRNAs and middle-derived tsRNAs), the authors found that the polysome:monosome ratio decreased by 20–50% after transfection, indicating that tsRNAs repressed translational activity. By comparing the mRNA-seq data with ribo-seq data generated by three tsRNA transfections (which show the most prominent effect), they found that the genes with more tsRNA target sites were more likely to be translationally arrested; meanwhile, tsRNAs did not affect the mRNA level of target genes. Notably, 7-mer motifs in tsRNAs can perfectly antisense match conserved target sites in mRNAs; these targeted mRNAs are associated with a reduced translational activity. Interestingly, tsRNA-targeting sites are located not only in the 3' untranslated regions (UTRs), but also in 5'UTRs or CDSs, consistent with a previous analysis of CLASH data showing the small RNA-mRNA interactome [8].

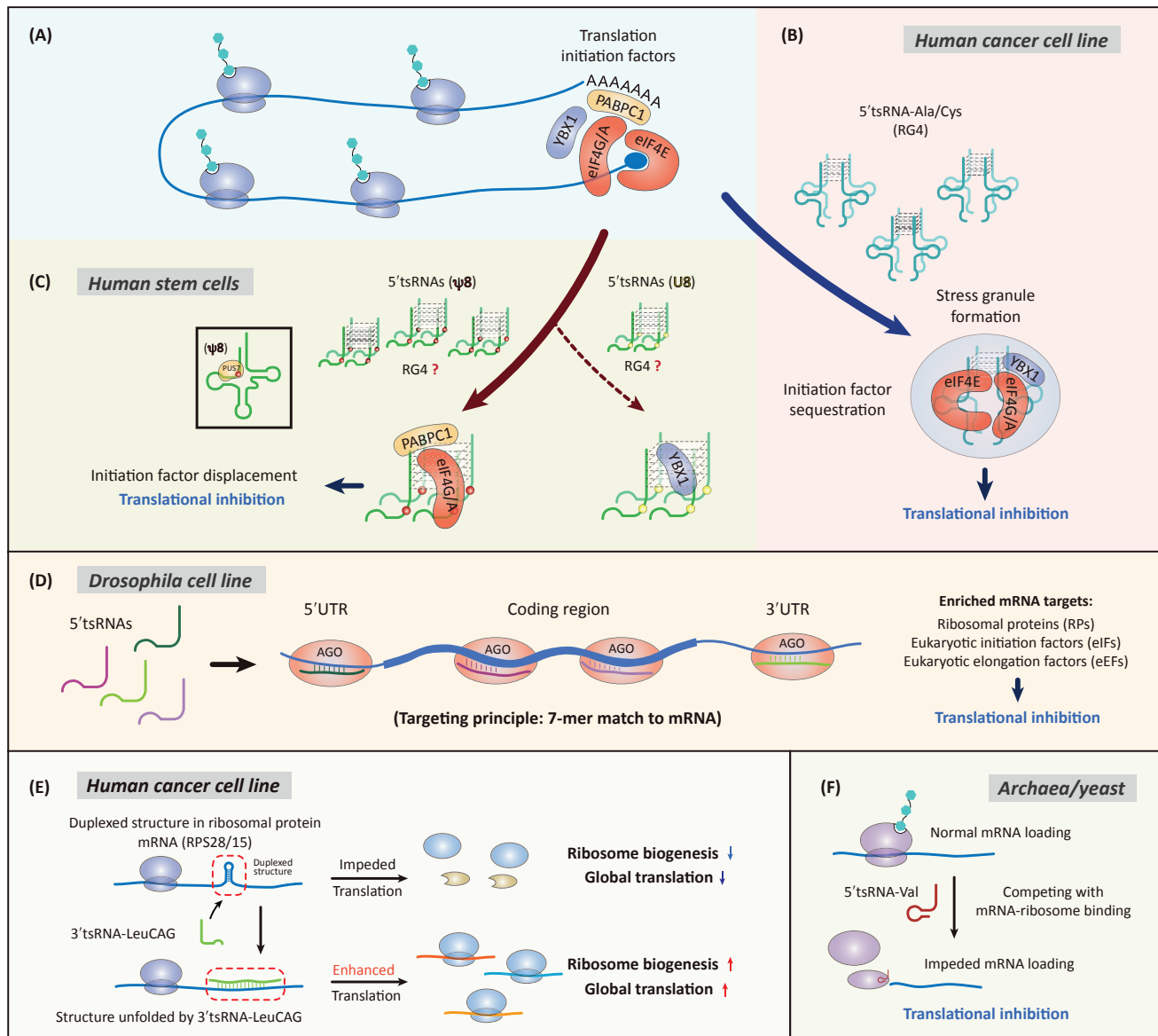


Figure 1. Multifaceted Functions of tRNA-Derived Small RNAs in Translational Regulation. (A) Illustration of mRNA translation and the key translation initiation factors. (B) 5'tsRNAs with terminal oligo-G motifs (TOG) [e.g., 5'tsRNA-Ala or 5'tsRNA-Cys, ~30 nucleotides (nt)] form an RNA G-quadruplex (RG4) structure, which interacts with translational initiation factors [eukaryotic initiation factor (eIF)-4E, G, and F], displacing them from the m⁷G-capped mRNAs and sequestering them in stress granules, leading to global translational inhibition. (C) Shorter forms of TOG-5'tsRNAs (~18 nt), which may also form RG4, show different binding affinities with translational initiation factors, depending on whether they are modified at the Ψ8 position, mediated by PUS7 (converting U to Ψ). The 18-nt Ψ8-TOG-5'tsRNAs inhibit translation, while the 18-nt U8-TOG-5'tsRNAs do not. (D) 5'tsRNAs recognize target mRNAs through conserved 7-mer complementary sequence matches, and inhibit target mRNA translation in an AGO-dependent manner. 5'tsRNAs preferentially match to the mRNAs of key components of the general translation machinery [i.e., the ribosomal proteins (RPs), eIFs and eukaryotic elongation factors (eEFs)]. Inhibition of these key proteins leads to global translational inhibition. (E) The 22-nt 3'tsRNA-LeuCAG binds to the mRNA of ribosome protein of small subunit (RPS28/15), unfolding the duplexed RNA structure at the target site, which increases the translation of the RPS18/15 protein, allowing increased ribosome biogenesis and, thus, enhancing global translation. (F) The 5'tsRNA-Val competes with mRNA for binding to the 16S rRNA of small ribosomal subunit (30S), interfering with mRNA loading into the ribosomal machinery and, thus, inhibiting translation. Abbreviation: UTR, untranslated region.

Importantly, the mRNA of ribosomal proteins (RPs) and translational initiation or elongation factors (IEFs) have the highest target density of AGO2-bound tsRNAs. Under serum starvation, the levels of some 5'tsRNAs are significantly increased, which are correlated with decreased translational activities of RPs and IEFs. Further AGO2 knockdown experiments indicated that AGO2 is indispensable for tsRNA-mediated translational repression under conditions of serum starvation. Interestingly, tsRNA- and miRNA-mediated gene targets are largely independent [7].

These data suggest that tsRNAs preferentially repress genes that are essential for ribosome biogenesis (i.e., RPs) and translation regulation (i.e., IEFs), thus enabling the repression of global protein synthesis (Figure 1D). The mechanism for the targeting preference of tsRNAs to RPs and IEFs are not well understood but may result from the long-term coevolution of tsRNAs and target sites.

AGO-Independent Translational Regulation by tsRNAs: Structural Effects

In addition to AGO-mediated translational repression, recent studies have begun to provide novel modes of action for tsRNA-mediated translational regulation by exerting structural effects on mRNAs or rRNAs, independent of the AGO protein.

Mark Kay's group recently reported that a 22-nt-3'tsRNA-LeuCAG did not bind to any of the known AGO proteins and could not repress luciferase expression with perfectly complementary target sites. Instead, 3'tsRNA-LeuCAG increased cell viability and its inhibition induced apoptosis in rapidly dividing cells *in vitro* as well as in a hepatocellular carcinoma mouse model [9]. The effect of 3'tsRNA-LeuCAG was specific, because inhibition of other 3'tsRNAs (3'tsRNA-Asp, 3'tsRNA-Ser, or 3'tsRNA-Met) did not reduce cell viability.

Outstanding Questions

- Why are some tsRNA species preferentially produced from specific tRNA precursors, and how is the length preference of tsRNAs from a single tRNA precursor regulated in a tissue and cell type-specific manner?
- Why do reported tsRNAs with similar sequences show distinct functional mechanisms in a cell- and tissue-dependent manner? Is this related to their RNA modifications or the different proteins they are binding with, or both?
- To what extent can RNA modifications impact the structure and function of tsRNAs? Does this mean that previous conclusions based on RNA sequences alone require re-examination?
- In addition to regulating translational initiation, do tsRNAs impact other steps of mRNA translation (e.g., elongation or termination)?
- What are the different molecular mechanisms involved in tsRNA-mRNA and miRNA-mRNA targeting and the functional outcome? For example, how do these differences affect the impact on translation regulation and ribosome biogenesis?
- Could a specific alteration in tsRNA composition result in ribosome heterogeneity that directs the cell to a specific functional state?

In addition, transfection of longer 27-nt-3'tsRNA-LeuCAG rather than 22-nt-3'tsRNA-LeuCAG had no effect on cell viability, suggesting that the effect is related to both sequence specificity and RNA structure.

Ribosome gradient analysis indicated that the inhibition of 3'tsRNA-LeuCAG decreased the abundance of 40S and 80S ribosomal complexes and increased that of the 60S ribosomal complex, suggesting a reduction in the number of 40S ribosomes and impaired assembly of the 80S ribosome. Additionally, inhibition of 3'tsRNA-LeuCAG resulted in 30S pre-rRNA accumulation and the subsequently decreased level of mature 18S rRNAs. This led to the discovery that 3'tsRNA-LeuCAG inhibition decreased the level of specific ribosomal proteins of the small subunit (RPSs), RPS28 and RPS15, at the translational level, while their mRNA abundance was not affected.

By using RNA secondary structure prediction, target-site mutation, and an *in vivo* click selective 2'-hydroxyl acylation and profiling experiment (icSHAPE), the authors convincingly showed that 3'tsRNA-LeuCAG unfolds the duplexed mRNA (*RPS15* and *RPS28*) structure at the target site (Figure 1E). Importantly, 3'tsRNA-LeuCAG cannot increase translation of other RPS mRNAs (*RPS9* and

RPS14) that have similar target sequence but do not have the secondary RNA structure. These data further support the idea that 3'tsRNA-LeuCAG is involved in unfolding the duplexed secondary structures of RPS mRNAs at the targeting-site, thus facilitating ribosome protein biogenesis.

Another study by Norbert Polacek's group showed a tsRNA-induced structure effect in regulating translation. They found that stress-dependent 5'tsRNA-Val can bind to the 16S rRNA of the small ribosomal subunit (30S) near the site of mRNA entrance, thus inhibiting global protein synthesis by displacing mRNA from the translational initiation complex [10] (Figure 1F). Similar translational control based on tsRNA-rRNA interactions may also account for other reports showing tsRNA-mediated global translational inhibition without complementary targeting on mRNA [11].

Concluding Remarks

The recent emerging studies discussed above have promoted the idea that various tsRNAs produced by tRNA fragmentation can engender acrobatic ways to regulate multiple aspects of translation machinery. In particular, the function of tsRNAs is augmented by unexpected roles of RNA modifications and RNA secondary structure (see Outstanding

Questions). Similarly, recent studies found that mammalian Nsun2- and Dnmt2-mediated m⁵C in tRNAs can profoundly affect tsRNA biogenesis [12–14] and the structure of the resulting tsRNAs [14], suggesting tsRNA-mediated translational control in stem cell function, embryo development, and intergenerational epigenetic inheritance of specific acquired phenotypes. Recent systematic analyses of tRNA-modifying enzymes in budding yeast also revealed the widespread impact of noncoding RNA modifications in translational regulation and gene expression [15]. Detailed molecular mechanisms involved in these circumstances may go beyond our current knowledge and deserve case-by-case studies in the future.

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