

Opinion

Origins and evolving functionalities of
tRNA-derived small RNAsQi Chen ^{1,*,@} Xudong Zhang,¹ Junchao Shi,¹ Menghong Yan,^{2,3} and Tong Zhou^{3,*,@}

Transfer RNA (tRNA)-derived small RNAs (tsRNAs) are among the most ancient small RNAs in all domains of life and are generated by the cleavage of tRNAs. Emerging studies have begun to reveal the versatile roles of tsRNAs in fundamental biological processes, including gene silencing, ribosome biogenesis, retrotransposition, and epigenetic inheritance, which are rooted in tsRNA sequence conservation, RNA modifications, and protein-binding abilities. We summarize the mechanisms of tsRNA biogenesis and the impact of RNA modifications, and propose how thinking of tsRNA functionality from an evolutionary perspective urges the expansion of tsRNA research into a wider spectrum, including cross-tissue/cross-species regulation and harnessing of the 'tsRNA code' for precision medicine.

On the ancientness of tsRNAs/tRNAs

With the wide application of high-throughput RNA sequencing (RNA-seq), tRNA-derived small RNAs (tsRNAs) (also called tDRs or tRNA-derived fragments, tRFs) are increasingly recognized as an emerging class of functional small noncoding RNAs (sncRNAs) in various fundamental biological and disease conditions [1,2]. tsRNAs have been identified in a wide range of species across all three domains of life, including Archaea, Bacteria, and some unicellular organisms (e.g., Protozoa), where other specialized small RNA pathways, such as miRNAs, siRNAs, and Piwi-interacting RNAs (piRNAs), are lacking [3–7]. These observations have positioned tsRNAs among the most ancient classes of sncRNAs for intra- and intercellular functionality and communication that may pre-date the emergence of more specialized sncRNAs.

The evolutionary conservation of tsRNAs is not entirely surprising because their tRNA precursors are among the most ancient and conserved RNA species that act as essential elements in all biological systems and were supposedly core players in the **last universal common ancestor (LUCA)**; see [Glossary](#) [8]. The main function of tRNAs is to decode the mRNA sequence and deliver specific amino acids from **aminoacyl-tRNA synthetases (aaRSs)** to the ribosome [2]. Beyond their canonical role in protein translation, tRNAs are also actively involved in other biological contexts such as being used as primers for reverse transcription (RT) of the retroviral genome [9]. Interestingly, many viral RNA genomes contain tRNA-like structures (TLSs) that can not only perform charging of amino acids but also function as telomeres for viruses, assist with viral replication, and interact with the ribosome – the translational machinery with a **ribozyme** at its core [10]. The intricate relationship between viruses and tRNAs (and TLSs) suggests that they may share a common evolutionary origin and have perhaps served as the drivers of early life on Earth ever since the LUCA [11] or even the 'RNA world' [12] ([Box 1](#)).

In the earlier mentioned evolutionary context, when tRNAs are fragmented through either non-specific metabolic turnover or specific cleavage, they give rise to diverse forms of tsRNAs that may interfere with or enhance the role of their precursor tRNAs (or TLSs) through basic molecular

Highlights

tRNA-derived small RNAs (tsRNAs) are present across all three domains of life: Archaea, Bacteria, and Eukarya.

The biogenesis of tsRNAs involves specific cleavage of mature tRNAs or tRNA precursors at preferred loci followed by further processing.

RNA modifications are essential for both tsRNA biogenesis and functionality.

tsRNA functionality is intricately linked to basic life elements including the ribosomal machinery and viral replication, in addition to binding to ribonucleoprotein and Argonaute proteins (i.e., AGO and PIWI).

tsRNAs can move across tissues/cells and may mediate intergenerational phenotype transmission and pathogen–host interplay and symbiosis.

tsRNAs and their RNA modifications can serve as disease biomarkers.

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interactions by competing with their target or by generating new functions via interacting with other RNA and proteins by sequence complementarity and structural effects. In other words, we argue that the fragmentation of tRNAs created a pool of tsRNA molecules for functional selection that have undergone Darwinian evolution ever since the RNA world and the LUCA, which would in theory result in heterogeneous and versatile functions of tsRNAs that penetrate fundamental biological machineries throughout evolution. Indeed, this overarching idea resonates well with the diverse tsRNA functions observed today, ranging from regulating translation, ribosome biogenesis, retrotransposition, cell–cell communication, and epigenetic inheritance, as well as with how tsRNA dysregulation is being linked to various human diseases (summarized in recent reviews [1,2]). From an evolutionary perspective, we first discuss the fundamental aspects of tsRNA biogenesis and regulation, and then propose a framework for the evolving principles of their mechanisms of action from which a wider spectrum of tsRNA functionality can be deduced. We also discuss the potential future integration and harnessing of different layers of tsRNA information (e.g., expression, cleavage patterns, and RNA modifications) for disease diagnosis and precision medicine.

tsRNA biogenesis: from degradation to regulation

From a structural perspective, the cloverleaf-shaped secondary structure of tRNA is folded into an L-shape in 3D (Figure 1A) [2]. This L-shaped structure is overall tightly condensed but has two relatively exposed sites: the anticodon at one end of the L and the **tRNA elbow** at the bending site of the L, where the D-loop and the T-loop meet and interact with each other. The exposed sites of the tRNA structure could be 'points of attack' in an ancient cellular (and perhaps early proto-cell) environment, being fragmented by either nonspecific stress signals such as radiation and reactive oxygen species (ROS), specific recognition by enzymes or ribozymes, or a combination of both.

This simple view coincides with prevailing observations that the most abundantly detected tsRNAs are fragmented at the anticodon, and are derived from the 5' half of the tRNA (~30 nt), whereas shorter 3' or 5' tsRNAs (~18–22 nt) fragmented at the T-loop or D-loop, respectively, or internal tsRNAs derived from sequences between these loops, are less abundant [13,14] (Figure 1A).

This observation may support the assumption that, in early life forms, the biogenesis of tsRNAs directly originated from tRNA degradation processes starting with these loops, perhaps including multistep degradation and the generation of different intermediates [15], accompanied by regulatory elements such as RNA modifications (Figure 1B) and specific RNases targeting these loops (Figure 1C) that emerged during evolution.

Notably, tRNA cleavage can also occur independently of the loop site, such as by targeting a specific tRNA stem position by RNase P that recognizes a specific (e.g., GC-rich) sequence [16] or, more generally, by enzymes targeting double-stranded (ds) RNA regions. For example, although it is well-known that the RNase Dicer cleaves dsRNAs to generate siRNAs and miRNAs, Dicer is also responsible for the biogenesis of some tsRNAs from tRNAs [17–19]. Since tsRNAs are present in all domains of life, whereas Dicer has not been identified in prokaryotes [20], this supports the notion that Dicer-based canonical small RNAs (i.e., siRNAs and miRNAs) emerged later than tsRNAs during evolution, and Dicer may have recognized the ds stem region in a tRNA-like structure when it first emerged. Currently known enzymes that cleave tRNA (and pre-tRNAs) to generate tsRNAs are well-summarized in a recent review [1], and are shown in Figure 1C. The list of enzymes mediating tsRNA biogenesis is expected to expand in the future [21,22].

Glossary

Aminoacyl-tRNA synthetases

(aaRSs): also termed tRNA ligases, aaRSs are enzymes that catalyze the acylation of a specific amino acid or its precursor to one of its cognate tRNAs to form an aminoacyl-tRNA.

Apoptosome: a large quaternary protein structure formed in the process of apoptosis, the formation of which is triggered by the release of cytochrome *c* from the mitochondria.

Arginylation: a post-translational modification mediated by arginyl-tRNA–protein transferase (ATE1) that transfers arginine from tRNA onto proteins.

Argonaute family proteins:

evolutionarily conserved proteins in both prokaryotes and eukaryotes that play an essential role in gene silencing. These can be subdivided into the AGO subfamily and the PIWI subfamily according to their phylogenetic classification.

Artificial neural network: algorithms that aim to recognize underlying relationships in a dataset through a process that mimics the way the human brain operates.

Cytochrome *c*: a small hemoprotein that is loosely associated with the inner membrane of the mitochondrion, and which triggers cell apoptosis on being released into the cytosol in response to apoptotic stimuli.

Deep learning: a part of the broader family of machine learning methods.

Hepatitis delta virus (HDV): the smallest virus that is known to infect animals; HDV has a single-stranded circular RNA genome.

Last universal common ancestor

(LUCA): the most recent common ancestor (most likely a single-celled organism) of all current life on Earth.

Protocell: a self-organized, endogenously ordered, spherical collection of lipids that has been proposed as a stepping-stone toward the origin of life.

Ribosome heterogeneity: ribosomes of distinct compositions within a cell or across tissues that generate biased translational specificity for different mRNA subpopulations.

Ribotoxins: extracellular RNases that can enter host cells and cleave essential RNAs such as rRNAs and tRNAs.

Ribozyme: RNA molecules that catalyze specific biochemical reactions similarly to the action of protein enzymes.

tsRNA termini

RNA cleavage by several classes of self-cleaving ribozymes [23] produces RNA fragments with 5'-hydroxyl (5'-OH) and 2',3'-cyclic phosphate (2',3'-CP) termini, whereas ancient endonucleases in the protein world, including tRNA splicing endonuclease [24], RNase T2 [25], RNase L [26], and RNase A [27], also similarly generate 5'-OH and 2',3'-CP RNAs. Interestingly, the RNases T2 [28,29] and L [26], and the vertebrate-specific angiogenin (RNase A family) [30,31], can all cleave tRNAs at the anticodon loop, resulting in their fragmentation into tsRNAs. Cleavage of tRNAs by these ribozymes and enzymes provides unique differences in tsRNAs from other sncRNAs such as those generated by Dicer, which bear a 5'-phosphate (5'-P) and a 3'-hydroxyl (3'-OH) [32], or piRNAs (and plant miRNAs) that bear 2'-O-methylation at the 3' terminus as a result of additional enzymatic processing [33].

Notably, some tsRNA sequences can be found in the piRNA database, which could be partially due to misannotation or contamination (and not to PIWI-binding) [34]. However, some of them may represent genuine piRNAs (i.e., tRNA^{AspGUC} and tRNA^{HisGUG} in *Bombyx*) because they bind to PIWI, and have 3' terminal 2'-O-methylation but not 2',3'-CP; these piRNAs are further processed from tsRNAs and are termed 'tRNA-derived piRNAs' [35]. Although the different sncRNA termini represent the chemical properties of their processing enzymes, they may also have functional consequences. Different termini might be harnessed as intra- or intercellular sorting signals that determine protein-binding potential and subcellular compartmentalization (e.g., cytoplasmic versus nuclear), which is an exciting area that awaits further investigation.

Impact of tRNA modifications

Both tsRNAs and their precursor tRNAs are heavily modified. More than 150 types of modifications are found in tRNAs [36], and their evolutionary origin remains an interesting question. Hypothetically, in early life forms on Earth, RNA modifications might have been first added by random chemical reactions or by base-modifying ribozymes [37,38], which in turn expanded the functionality of ribozymes. In this regard, the RNA modifications on ancient RNAs, such as tRNAs, rRNAs, and spliceosomal small nuclear RNAs (snRNAs), may represent the vestiges of the RNA world at a time when RNA modifications were maximally exploited to increase the functional diversity of ribozymes, before being replaced by the emergence of functionally more versatile protein-based enzymes. The present-day tRNA modifications contribute to multiple aspects of tRNA function, including stability, amino acid charging, and translational accuracy, as well as to tsRNA biogenesis [39,40].

It has been demonstrated that DNMT2- and NSUN2-dependent addition of a 5-methylcytosine (m⁵C) modification to several tRNAs (e.g., tRNA^{Asp}, tRNA^{Val}, tRNA^{Gly}, and tRNA^{Leu}) increases tRNA stability in flies and mice, whereas deletion of *Dnmt2* and/or *Nsun2* abolishes m⁵C on these tRNAs, making them more likely to be cleaved into tsRNAs under stress conditions [41–43]. The queuosine (Q) modification by QTRT1 occurs in the wobble anticodon position of several tRNAs (tRNA^{His}, tRNA^{Asn}, tRNA^{Tyr}, and tRNA^{Asp}) and protects tRNAs against cleavage into tsRNAs in human HEK293T cells [44]. Interestingly, recent reports showed that C38 Q-modified tRNA promotes DNMT2-mediated m⁵C on C38 of tRNA^{Asp} [45,46]; these discoveries resonate with findings that the establishment of one RNA modification can depend on the existence of another [47]. Recent evidence also shows that deletion of *Alkbh1* [48] or *Alkbh3* [49] increased the levels of *N1-methyladenine* (m¹A) in tRNAs, preventing tRNA cleavage and resulting in less tsRNA production. TRMT10A-mediated *N1-methylguanine* (m¹G) modification also leads to increased tRNA^{Gln} stability and less production of tsRNA^{Gln} [50]. Moreover, 2'-O-methylation of C34 in human tRNA^{Met} can prevent site-specific cleavage of tRNA^{Met} by angiogenin and reduce tsRNA production [51].

Stress granules (SGs):

ribonucleoprotein (RNP) granules in the cytoplasm which aggregate stalled translational preinitiation complexes and nontranslating mRNAs that accumulate during stress.

tRNA elbow: a conserved structure arising from the interaction between the D- and T-loops of a tRNA.

Viroids: the smallest known infectious pathogens which are composed solely of a circular, single-stranded RNA that has no protein coating.

Box 1. The emergence, existence, and exit of the 'RNA world'

Ever since the discovery of ribozymes in the 1980s, the hypothesis that early life forms were dependent solely on RNA to both store genetic information and catalyze chemical reactions for self-propagation has gained momentum, making an 'RNA world' before DNA-protein-dominated life highly probable [118]. Recent triumphs in the RNA world hypothesis include the demonstration that the fundamental macromolecular machineries – the ribosome [119] and spliceosome [120] that are essential for protein translation and mRNA processing – both possess ribozymes at their cores. Furthermore, some ancient RNA viruses (e.g., **hepatitis delta virus, HDV**) and **viroids** are circular RNAs with ribozyme functions that replicate their genomes without a protein enzyme [121]. *In vitro* evolution experiments [122,123] have also generated an increasing repertoire of synthetic ribozymes with essential functions, such as a ribozyme with RNA polymerase (or replicase) activity to direct RNA-catalyzed RNA polymerization [124] and self-sustaining RNA replication through cross-catalytic reactions between different ribozymes [125]. Interestingly, tRNA-like sequences can replicate by self-assembly, and this may be a relic of self-replicating RNAs from the RNA world [126,127]. Ribozymes can also add site-specific RNA methylations [38], and the ribozyme-mediated RNA modifications may in turn modulate the functionality of ribozymes. Finally, ribozymes that can catalyze the aminoacylation of tRNA [128] may have triggered exit from the RNA world by entering into an RNA–amino acid hybrid form leading to the formation of RNPs that may outperform the function of ribozymes solely based on RNA [129], eventually moving towards protein-dominated functionality.

Pre-dating the ribozyme-dominated RNA world, earlier propagating systems could have consisted of nucleic acids other than the present-day RNA and DNA molecules [130]. Indeed, nonenzymatic template-directed RNA synthesis can be catalyzed by the coordination of Mg^{2+} and citrate inside fatty acid membranes – a hypothetical **protocell** [131] – and this provides the wider possibility that metal ions and other mineral cofactors may have assisted with the formation, polymerization, and propagation of ribose-based nucleic acid structures before the emergence of ribozymes. Notably, many active molecules in extant cellular life, including ATP, NAD^+ , acetyl-CoA, and vitamin B12 (Figure 1), are structural derivatives of ribonucleosides. These molecules are deeply incorporated into cellular metabolism and RNA and DNA modifications [132] where they may represent chemical 'fossils' that were concurrent with or pre-dated the RNA world. Finally, the existence of the RNA world might also be accompanied by DNA through chemical coproduction [133], where DNA could either have a catalytic function (e.g., RNA ligation [134]) or be harnessed for genetic information storage.

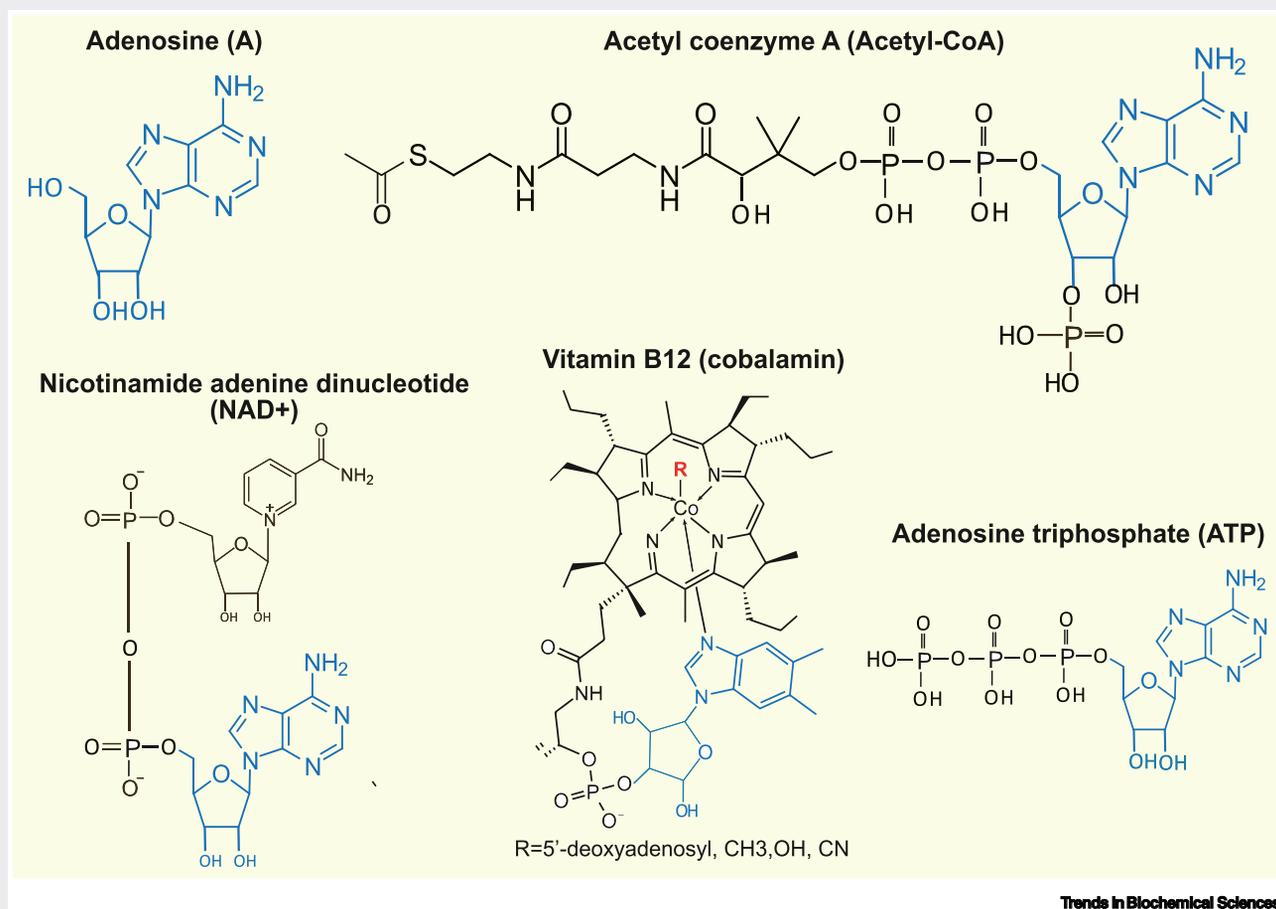


Figure 1. Chemical structures of ATP, NAD^+ , acetyl-CoA, vitamin B12, and adenosine (A).

In addition to preventing tRNA cleavage, some RNA modifications can also promote tsRNA biogenesis. For example, PUS7-mediated pseudouridine (Ψ) at the U8 position has been shown to affect tsRNA biogenesis in stem cells, where deletion of *Pus7* leads to a decreased levels of several types of 5'-tsRNAs with terminal oligo(G), suggesting that (Ψ) at the U8 position increases the cleavage of these tRNAs to generate tsRNAs [52]. In another example in yeast, 5-methoxycarbonylmethyl-2-thiouridine (mcm^5S^2) at the anticodon wobble position can promote the cleavage of tRNA into tsRNAs [53].

In an intriguing scenario, when bacterial tRNAs ($tRNA^{Asp}$ and $tRNA^{Arg}$) are cleaved in half by **ribotoxins** that recognize the Q-containing anticodon of tRNA [54], the newly generated two tsRNA halves can be reunited/repared into a full tRNA by a Pnkp/Hen1 heterotetramer while adding a 2'-O-methylation to the previous RNA cleavage site [55]. The added 2'-O-methylation can protect the repaired tRNA from being recut by ribotoxins. This example shows how dynamic RNA modifications are harnessed to balance tRNA stability and tsRNA biogenesis, and also raises the possibility that tsRNAs can gain additional modifications after being cleaved from precursor tRNAs.

Importantly, it should be noted that the changes in tRNA modifications not only affect tsRNA biogenesis but may also affect the function of the resulting tsRNAs owing to altered modification status [56]. In fact, the RNA modifications of tsRNAs have posed challenges for RNA-seq library preparation and for functional studies of tsRNAs (Box 2), and this will need to be resolved to permit new waves of tsRNA research.

Evolving tsRNA functionalities

Research on the functions of tsRNAs continues to expand, and roles have been reported in regulating stem cell maintenance [57,58], cancer [59–61], viral infection [9,62], neurological diseases

Box 2. Technical challenges centered on tsRNA modifications

Fully understanding the impact of RNA modifications on the function of each tsRNA remains experimentally challenging, as synthetic tsRNAs may not fully mimic the modified tsRNAs that exist in tissues/cells. Two recent reports have provided valuable protocols to isolate individual tsRNAs *in vivo* [135,136], enabling the future possibility of comparing the functions of modified tsRNAs versus synthetic unmodified tsRNAs – this might permit the engineering of individual tsRNAs by adding site-specific modifications to mimic or enhance their functions. In addition, when performing functional studies (such as cellular transfection) of either synthetic tsRNAs or isolated tsRNAs *in vivo*, quantitative and stoichiometric factors should aim to reflect the intracellular situation. Blockade of tsRNAs using complementary sequences such as antisense oligonucleotides (ASOs) or locked nucleic acids (LNAs) should be carefully validated so as to not affect the levels of precursor tRNAs [72].

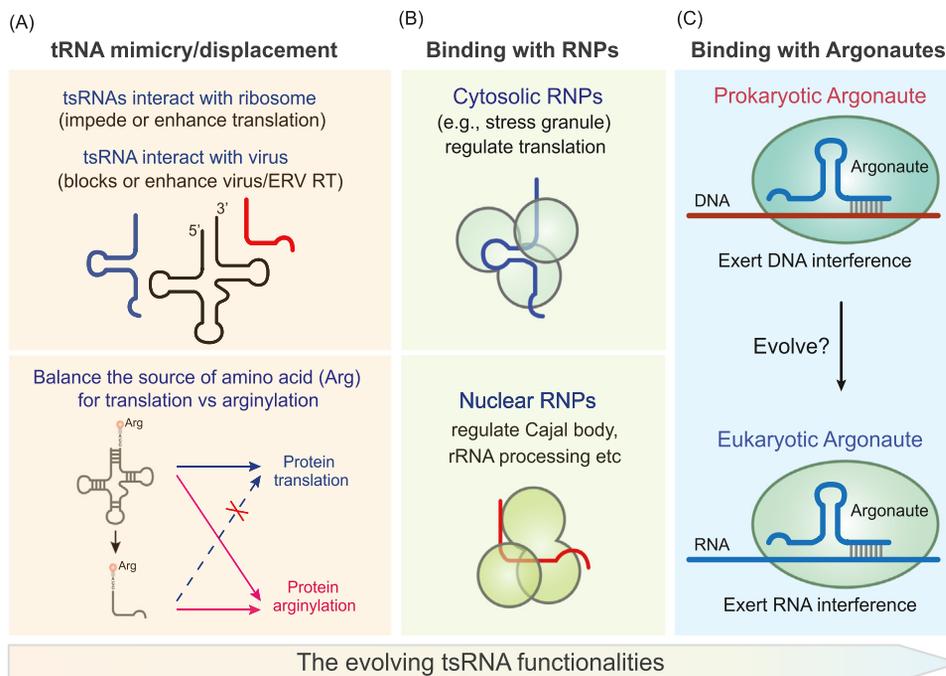
When profiling tsRNAs using RNA-seq, the specific tsRNA termini (e.g., 3'-phosphate and 2',3'-cyclic phosphate) and RNA modifications (e.g., m^1A , m^3C , m^1G , and *N2-dimethylguanosine*, m^2_2G) have created challenges to prevent efficient and complete conversion of tsRNAs into cDNA libraries during standard RNA-seq protocols, resulting in biased detection and quantitation during deep sequencing [14]. Recently improved RNA-seq methods such as panoramic RNA display overcoming RNA modification aborted sequencing (PANDORA)-seq [14] and Cap-Clip acid pyrophosphatase, PNK, and AlkB (CPA)-seq [137] aim to overcome these problems by removing key RNA modifications that block adapter ligation and RT by using consecutive enzymatic treatment; these techniques await further optimization such as combination with other strategies focusing on the 5'-termini [138].

Finally, some RNA modifications of tsRNAs/tRNAs may affect probe binding efficiency during northern blot (NB) examination, which may lead to incorrect interpretations when an altered NB signal is associated with altered levels of tRNA/tsRNA modifications. For example, in a cell/tissue with altered RNA modifications caused by mutation or specific environmental stimuli, changes in the observed NB signal may reflect altered tsRNA levels, or altered probe hybridization efficiency owing to RNA modification changes, or both. In addition, in studies involving the knockdown or knockout of tRNA modification enzymes, the resulting phenotypes will represent a mixed effect of altered levels of tRNAs, tsRNAs, and potentially other molecules. These challenging situations in tsRNA research await clarification and resolution.

[63,64], epigenetic inheritance [42,65–68], and symbiosis [69]. Unlike other canonical snoRNAs such as miRNAs, siRNAs, and piRNAs, which usually function through strict molecular mechanisms by binding to **Argonaute family proteins**, the mechanisms of action of tsRNAs appear to be more diverse. Instead of listing known tsRNA functions and mechanisms that have been well summarized elsewhere [1,2,70], we propose to streamline these tsRNA functions and mechanisms in a new narrative to dissect/propose three possible major steps regarding how the function of tsRNAs has emerged and evolved: (i) tsRNAs function via mimicry/replacement of tRNAs with sequence/structure effects (Figure 2A), (ii) tsRNAs function via association with ribonucleoproteins (RNPs) (Figure 2B), and (iii) tsRNAs function via Argonaute proteins (i.e., AGO, PIWI) (Figure 2C). In addition, tsRNAs (and other mobile RNAs) can travel between cells and organisms, enabling long-distance regulation inside and between organisms, and even mediate cross-kingdom regulation between prokaryotes and eukaryotes (Figure 3).

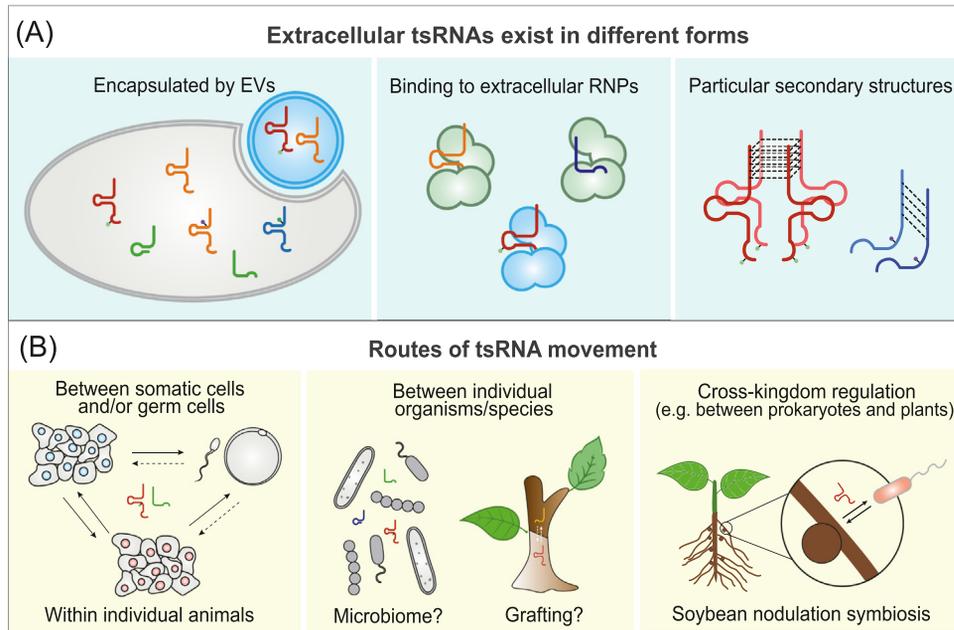
Mimicry/displacement of tRNAs

Since the 1960s it has been reported that a tRNA fragment from tRNA^{fMet} can interact with ribosomal subunits in a fashion similar to that of its precursor mature tRNA^{fMet} [71]; this suggests that tsRNAs may naturally compete with the function of tRNAs under some circumstances. The tRNA:tsRNA ratio may reach an equilibrium under normal cellular conditions, whereas the waxing and waning of this ratio under changing environments (e.g., stress conditions that cleave more tRNAs) enables regulatory effects. Supporting this idea, recent evidence has shown that, in Archaea (e.g., *Haloferox volcanii*), a stress-induced 5'tsRNA^{Val} can interact with the rRNA of the ribosomal subunit, and interferes with the loading of mRNAs into ribosomes, thus inhibiting global protein



Trends in Biochemical Sciences

Figure 2. The evolving principles of tRNA-derived small RNA (tsRNA) functionality from an evolutionary perspective. We propose three possible major steps regarding how tsRNA function has emerged and evolved; the arrow at the bottom shows evolution of tsRNA functionality towards more specialized roles. (A) tsRNA function by tRNA mimicry/displacement and structural effects. (B) tsRNA function via cytosolic and nuclear ribonucleoproteins (RNPs). (C) tsRNA function through binding to prokaryotic or eukaryotic Argonaute proteins. Abbreviations: ERV, endogenous retrovirus; RT, reverse transcription.



Trends in Biochemical Sciences

Figure 3. Mechanisms and routes of tRNA-derived small RNA (tsRNA) movement between cells, individual organisms, and species. (A) Extracellular tsRNAs can be encapsulated in extracellular vesicles (EVs), bind to ribonucleoproteins (RNPs), or form specific secondary/3D structures with the aid of RNA modifications. (B) Known and predicted (?) movement of tsRNA between somatic cells or between somatic and germ cells in a single individual, or between organisms/species (e.g., between microbiomes, grafted plants), where tsRNAs may mediate cross-kingdom regulation such as between bacteria and plants/animals.

synthesis [4,5] (Figure 2A, top). By contrast, in another example, in *Trypanosoma brucei*, a unicellular protozoan, a stress-induced 3'tsRNA^{Thr} can be incorporated into ribosomes to enhance mRNA loading during stress recovery, and thus increases global translation [6]. These opposing examples suggest that tsRNAs can function flexibly in a context-dependent manner, possibly depending on where they interact with the ribosome and how they might alter the local structure of the rRNAs. In another example, 3'tsRNA^{Leu} can bind to specific ribosomal protein mRNAs (e.g., RPS28 and RPS15) to enhance their translation by changing their mRNA structure [72,73]; depletion of 3'tsRNA^{Leu} causes decreased translation of RPS28, which impairs pre-18S ribosomal RNA processing and ribosome biogenesis [72].

In addition to tsRNA–ribosome interactions, many RNA viruses and retrotransposons (e.g., endogenous retroviruses, ERVs) can duplicate themselves by using the 3' terminus of mature tRNA as primers for their RT [9] (Figure 2A, top). In a recent study, transfection of exogenous 18 nt 3'tsRNAs led to competition with the mature tRNAs for the primer binding sequence (PBS) of the ERVs, leading to RT blockade and thus impeding retroviral cDNA synthesis [74]. A similar tsRNA blockade effect at the PBS site may also take place in HIV-infected T cells [75]. By contrast, in other scenarios, tsRNAs can be directly used as primers for RT in human T cell leukemia virus type 1 (HTLV-1) [76] and *copia* retrovirus-like particles [16]. These opposite roles of tsRNAs in retroviral RT processes suggest adaptation of the viral world to specific tsRNAs that either block or mimic the function of tRNAs.

In another interesting case, ATE1-mediated post-translational **arginylation** of proteins directly competes with protein translation involving the use of arginine-charged tRNA^{Arg} [77]. In this

context, it was found that when arginine-charged tRNA^{Arg} (Arg-tRNA^{Arg}) is fragmented into tsRNA, Arg remains bound to the 3'tsRNA^{Arg} (Arg-3'tsRNA^{Arg}) and the translation-incompetent Arg-3'tsRNA^{Arg} can still be efficiently used as a donor for arginylation by the ATE1 enzyme [77] (Figure 2A, bottom). Although this is only one piece of evidence, these data suggest a scenario in which the balance between translation and arginylation might be regulated by the tsRNA^{Arg}: tRNA^{Arg} ratio.

We anticipate the discovery of more tsRNA functions under the principle of mimicking/displacing precursor tRNAs because we believe this scenario may represent the most ancient regulatory mode of tsRNAs – simply based on sequence/structural competition.

Forming RNPs

Another well-studied mode of tsRNA functionality is through the formation of tsRNA-RNP complexes. For example, under stress conditions, angiogenin-induced tsRNAs can induce **stress granule (SG)** formation in a portion (~4%) of cells and the tsRNAs could be incorporated into SGs [78,79] (Figure 2B, top). The ability of tsRNAs to induce SG formation requires the action of YB1 [79], and the role of tsRNAs in suppressing translation is dependent on their binding with eukaryotic initiation factors (eIFs) to sequester them from the translational machinery [79,80]. The formation of this tsRNA-RNP complex involves tsRNA secondary structures such as intermolecular RNA G-quadruplexes (RNA-G4) [81], and RNA modifications such as PUS7-mediated Ψ modifications at U8 [52]. Angiogenin-induced tsRNA has also been shown to form an RNP complex with **cytochrome c** that is released from the mitochondria, where the tsRNA-mediated sequestration of cytochrome c protects the cells from **apoptosome** formation and apoptosis under cell stress [82]. Interestingly, the binding of tsRNAs to cytochrome c to inhibit apoptosis resembles the function of some tRNAs [83].

In addition to functioning with cytosolic RNPs, tsRNA can also interact with spliceosomal proteins (e.g., hnRNPF/H) in the nuclei, where hnRNPF/H contributes to the formation of the Cajal body [84] (Figure 2B, bottom). Cajal bodies are known to regulate the production of nuclear small RNAs, including small nucleolar RNAs (snoRNAs), small Cajal body-specific RNAs (scaRNAs), and snRNAs. These nuclear small RNAs, along with tsRNAs, may form other nuclear RNP complexes that contribute to the processing [85] and modification [86] of rRNAs, resulting in **ribosome heterogeneity** that leads to translational specificity for a selected pool of mRNAs to direct the cell to a specific functional state [87]. To date, the possible nuclear function of tsRNAs remains mysterious but intriguing, especially in early embryo development. Recent evidence has shown that tsRNAs in mouse sperm are responsive to environmental stimuli [65,66], and tsRNAs are enriched at a higher level in the sperm head where the nucleus is condensed [14]. Importantly, pronuclear zygotic injection of tsRNA-containing sperm RNA fractions from high-fat diet-exposed males can induce metabolic phenotypes in the resulting offspring, suggesting that tsRNAs might function through nuclear events that drive butterfly effects throughout embryo development since the zygote stage, possibly by regulating the translational specificity of the early embryo [88].

Binding to Argonaute

tsRNA binding to Argonaute family proteins has been reported to mediate post-transcriptional silencing (either by RNA degradation or mRNA translational inhibition) in multiple cases [18,69,89–91], mostly in an RNAi-like fashion using complementary sequences binding to the targeted mRNAs (Figure 2C). However, the Argonaute-dependent RNAi systems only prevail in eukaryotes and are believed to have emerged primarily to defend against viruses, represented by either the AGO-centered antiviral machinery in the cytoplasm or the PIWI-centered function in the nuclei to suppress endogenous viruses (e.g., transposons) [20].

Unlike in eukaryotes, prokaryotic Argonaute proteins primarily use small DNA as a guide to cleave DNA, a process known as DNA interference [92], to inhibit the propagation of foreign plasmids and infection by phages [93] (functionally similar to the CRISPR/Cas system but mechanistically analogous to RNAi). We believe that this evidence from prokaryotic systems suggests an ancient origin of Argonaute proteins that pre-dates the emergence of RNAi. Since tsRNAs are widely present in prokaryotes [4,5,7], it is an intriguing hypothesis that tsRNAs might be used by prokaryotic Argonaute as an alternative substrate. Indeed, in a seminal study exploring the binding of bacterial Argonaute to RNAs, it was found that the 3' halves of two types of tsRNA^{Met} are abundantly found as the majority of 45 nt reads [94], although their biological functions remain unresolved. It is our hypothesis that other tsRNAs might also bind to the prokaryotic Argonaute, which awaits to be confirmed by improved library construction protocols that consider the specific tsRNA termini and modifications [14].

In fact, because tRNA/tsRNA sequences are conserved across species during evolution, tsRNAs might be harnessed under some circumstances to defend against foreign DNA/RNA invasions as a first-frontier responding molecules in a fashion similar to the innate immune system, given their rapid elevation upon active infection [95] and that tsRNAs activate Toll-like receptors (TLRs) [96]. This is in contrast to the siRNA/piRNA systems that more resemble the adaptive immune system in that they involve *de novo* generation of perfectly matched sncRNAs customized to the invading DNA/RNA [12].

Importantly, although the earlier mentioned tsRNA mechanisms of action have been discussed separately and from an evolutionary perspective, different mechanisms can coexist or function synergistically to achieve common outcomes. For example, the suppression of retrotransposons could be achieved by both tsRNA displacement of tRNA-mediated RT [74] and RNAi-like post-transcriptional suppression [74,97]. tsRNA-mediated translational regulation can be achieved by tsRNA-ribosome interaction, tsRNA-RNP-based eIF sequestration, and Argonaute-dependent RNAi effects [98]. In addition, it has been shown in *Tetrahymena* that tsRNA can bind to an Argonaute family protein to form nuclear RNPs, which may facilitate nuclear rRNA processing and cell growth [85,99].

tsRNAs on the move

In addition to functioning in the cytoplasm and nuclei, tsRNAs are also abundant in body fluids [100] and can be delivered between somatic cells or between somatic and germ cells [66], and even across species in which bacterial tsRNAs are essential for prokaryote-eukaryote communication to ensure microbiome-plant symbiosis for soybean growth [69] (Figure 3). Intercellular communication by tsRNAs could be either dependent or independent of extracellular vesicles (EVs) [101] (Figure 3A), which resonates with the wisdom of Charles Darwin – who proposed in 1868 that each cell emits small particles or molecules, called 'gemmules', that diffuse and move between both somatic cells and germ cells [102], a concept that is now gaining new momentum [103].

In fact, the phenomenon of membrane vesicle release in Bacteria, Archaea, and Eukaryotes is an evolutionarily conserved mechanism [104] that might enable robust horizontal information exchange and facilitate the selection and propagation of traits under pressure. Whether and how tsRNAs are selectively or randomly encapsulated into EVs as cargo and specifically delivered to another cell or organism to exert their function remains largely unknown and certainly deserves intense investigation.

Importantly, tsRNAs have been observed outside the cell without being encapsulated into EVs [95,105,106]. In these cases, tsRNA could travel with extracellular RNPs or depend on specific

RNA structures (e.g., dimers, RNA G4 tetraplexes [107,108]) or modifications, which increase their stability. Interestingly, engineered mRNAs harboring TLSs with predicted stem–bulge–stem–loop structures are sufficient to mediate systemic mRNA transport in plants and move through graft junctions [109]. Thus, specific tRNA-related and tsRNA-mediated structures may

Box 3. Harnessing the 'tsRNA code' for precision medicine

tsRNAs are relatively stable in body fluids, and this may be due in part to the fact that they are heavily modified. RNA modifications also provide additional layers of information compared with other RNA-based biomarkers (such as mRNAs and miRNAs). More specifically, a complete tsRNA profile should include not only the expression level of each tsRNA sequence but also the cleavage site/pattern from which they are derived, the overall spectrum of RNA modifications, and the site-specific RNA modifications on each tsRNA. This complex nature of tsRNA profiles, in theory, would generate astronomical permutations when considering each layer of information [2], making the following data analyses high-dimensional, sparse, and possibly nonlinear and thus difficult to precisely describe using traditional statistical approaches. Furthermore, we believe that, given the emerging application of artificial intelligence in biomedical research, the complex layers of information embedded in tsRNA profiles provides an excellent research/clinical opportunity to analyze tsRNA signatures with **deep learning** algorithms such as **artificial neural networks** (Figure 1) [139]. In our opinion, the level of complexity buried in the tsRNA signature, once fully deciphered with deep learning programs, holds great promise to provide superior biomarkers with better resolution for the diagnosis and prognosis of complex diseases that were previously difficult to distinguish at the molecular level, facilitating the future development of precision medicine [140].

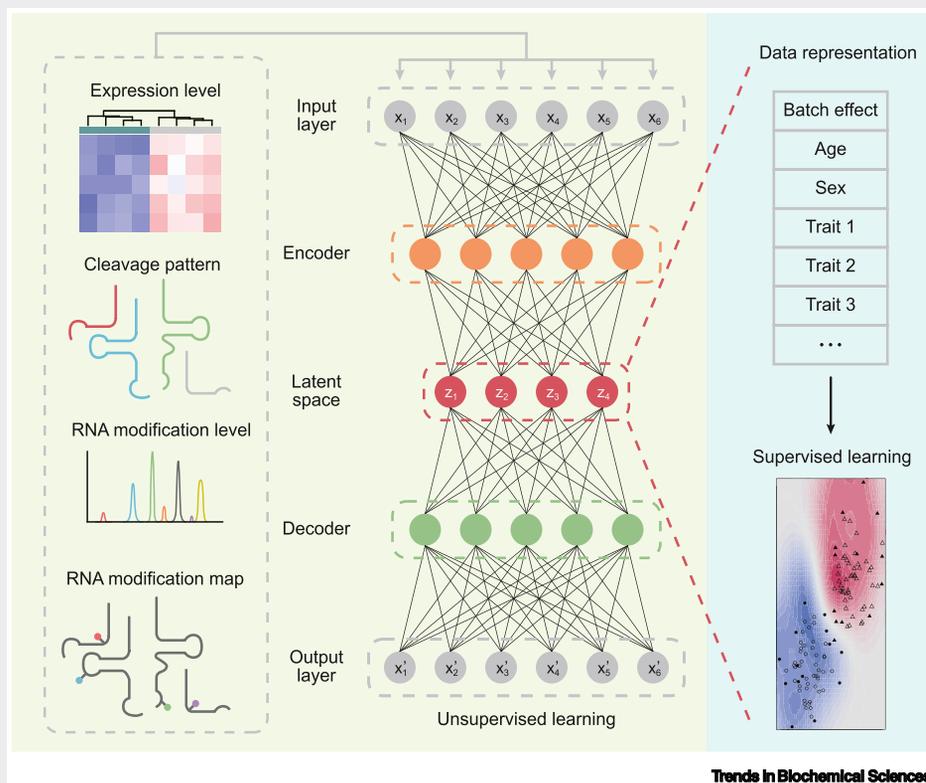


Figure 1. Schematics of the use of artificial neural networks to analyze multiple layers of tRNA-derived small RNA (tsRNA) profiles. The input tsRNA data are processed by an unsupervised deep learning algorithm, Autoencoder. With multiple hidden layers, the encoder maps the input tsRNA data (\mathbf{X}) into a lower-dimensional latent space (\mathbf{Z}) with fewer nodes, which captures a compressed knowledge representation (supposedly containing biological/clinical features such as age, sex, disease status, batch effect, etc.) from the original input and forms the basis for reconstructing the input in the output layer (\mathbf{X}') via the multiple hidden layers in the decoder. In turn, the latent representation can be fed into a supervised machine learning algorithm that will aid diagnosis, prognosis, and preventive and therapeutic interventions in precision medicine.

represent RNA mobility motifs that facilitate EV-independent RNA transfer, and their entry into a target cell may or may not rely on specific cell-membrane receptors. This type of extracellular tsRNA transfer could have significant implications because it might be involved in crosstalk between food-derived RNAs and the gut microbiome, or enable the direct transport of food-derived small RNAs into animal cells [110] (Figure 3B).

Concluding remarks

As summarized here, the studies on the biogenesis and functions of tsRNAs have advanced greatly and now constitute an emerging field in RNA biology; as a result, the association and causative roles of tsRNAs in various disease conditions are also being revealed. In particular, tsRNAs are found in all types of body fluids [100] and are dynamically regulated under pathophysiological conditions [95,106], which we argue potentially makes tsRNAs excellent candidates as liquid biopsy biomarkers for clinical translational applications, especially when augmented with the power of machine learning to reveal the multifaceted nature of the 'tsRNA code' (Box 3).

Looking forward, reports of other noncanonical small RNAs, such as rRNA-derived small RNAs (rsRNAs) and YRNA-derived small RNAs (ysRNAs), are also emerging as new analytical tools become available [14,111]. The biogenesis and function of rsRNAs and ysRNAs are less understood but may follow the same general principles as tsRNAs because they are similarly fragmented and processed from ancient RNA species (i.e., rRNA, YRNA) that exist in all domains of life. For example, RNase T2 can cleave both rRNA and tRNA to generate rsRNAs/tsRNAs [28,29], and both tsRNA and rsRNAs are sensitive to environmental exposures such as oxidative stresses, diet, and inflammation [42,112–115]. YRNAs/tRNAs can also be cleaved inside EVs by RNases (e.g., RNase L, RNase 1) to generate ysRNAs/tsRNAs, and may contribute to circulating tsRNAs/ysRNAs in the serum and other biofluids [26,116]. Interestingly, recent data have shown that tsRNAs/rsRNAs/ysRNAs can together form a 'disease RNA code' that can distinguish human diseases such as lung cancer and tuberculosis, and this can outperform miRNA-based signatures [117], suggesting that their complex forms in nature (e.g., versatile cleavage patterns) enabled more permutations and superior information capacity and specificity that can be harnessed to distinguish between and subcategorize complex diseases for translational medicine.

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Declaration of interests

The authors declare no conflicts of interest.

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Outstanding questions

How many more (modified) tsRNAs can be discovered with improved RNA sequencing methods?

Do RNA modifications regulate tsRNA intracellular sorting, compartmentalization, and functionality in conjunction with associated RNA-binding proteins?

Is the principle of the functionality of each tsRNA universal or context-dependent?

Could tsRNAs mediate cross-species regulation between food and the microbiome, or between the microbiome and the host?

To what extent do RNA modifications and 3D RNA structures affect the functions of tsRNAs, in addition to their sequences?

How can tsRNA levels be experimentally manipulated without affecting their tRNA precursors?

Can multiple layers of information be extracted and integrated from tsRNAs (e.g., expression level, cleavage patterns, RNA modifications) for deep learning and clinical translational applications?

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