*Emerging roles of novel small non-coding regulatory RNAs in immunity and cancer*

Domenico Rosace[1], Judith López[1], Sandra Blanco[1,2]

[1] Centro de Investigación del Cáncer and Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas (CSIC) - University of Salamanca, 37007 Salamanca, Spain.

[2]Correspondence to: sandra.blanco@usal.es

*Abstract*

The term small non-coding RNAs (ncRNAs) refers to all those RNAs that even without encoding for a protein, can play important functional roles. Transfer RNA and ribosomal RNA derived fragments (tRFs and rRFs, respectively) are an emerging class of ncRNAs originally considered as simple degradation products, which though play important roles in stress responses, signalling or gene expression. They control all levels of gene expression regulating transcription and translation and affecting RNA processing and maturation. They have been linked to pivotal cellular processes such as self-renewal, differentiation and proliferation. For this reason, mis-regulation of this novel class of ncRNAs can lead to various pathological processes such as neurodegenerative and development diseases, metabolism and immune system disorders and cancer. In this review we summarize the classification, biogenesis and functions of tRFs and rRFs with a special focus on their role in immunity and cancer.

## Introduction

In the past decades the existence of functional regulatory small non-coding RNAs (ncRNAs) has been revealed in different species. Recent transcriptome wide studies and advances in high-throughput sequencing technologies have allowed to the discovery and sequence of the big plethora of these small ncRNAs. The term non-coding RNA refers to all the RNA molecules that do not encode for a protein, without however excluding that such RNAs do not contain information or play a specific function in a cell [1]. For instance, transfer RNAs (tRNAs) are ncRNAs with an important role in protein synthesis since they specifically recognize messenger RNA (mRNA) codons and transfer their charged amino acid into the growing peptide during translation [2]. Since the discovery in 1993 of small ncRNAs in *Caenorhabditis elegans* [3], many classes of small ncRNAs with various regulatory functions have been identified in a multitude of organisms of the eukaryotic domain [4]. The most well-known are the small-interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs). In this category are also included tRNA-derived small RNA fragments that have been recently discovered. tRNA fragments were first detected in the urine of cancer patients [5]. Since then, they have been described in all domains of life including bacteria, archaea and eukaryote and they are considered a new class of ncRNAs called tRNA-derived small RNAs [6-11].

Initially they were thought to be non-specific degradation products due to a defect in processing, folding and damaged functions of an RNA molecule. However, a growing body of evidence indicates that fragmentation could also represent a different way to generate truncated transcripts that will perform distinct functions compared to the original RNA molecules [12]. Considering their abundance inside the cell, the most common source for RNA fragments is represented by tRNAs and ribosomal RNAs (rRNAs). Small ncRNA-derived RNA fragments are usually 15-40 nucleotides (nt) long and are generated by endonucleolytic cleavage of fully mature transcripts mediated by different endoribonucleases [13]. These small RNA fragments have been implicated in specific biological functions such as suppression of gene expression, translational regulation, viral infections, regulation of survival, epigenetic inheritance, stem cell biology, cancer development, immune signalling or neurodevelopment disorders [14-23].

Recent research has revealed that tRNAs and rRNAs undergo stress-induced cleavage to produce stable RNA products that induce transient translational arrest [15, 16]. All the cells contain specific mechanisms to adapt and respond to stress conditions such as cell cycle arrest, translation repression, repair and in extreme cases cell death. Indeed, an important component of the stress response involves the control of

RNA metabolism. RNA surveillance mechanisms play a crucial role in the control of RNA expression, allowing for a rapid downregulation of specific RNA molecules or degradation of aberrant RNAs [24]. Recent findings have shown that a fraction of the small RNA fragment pool presents a characteristic size distribution and origin that is different from siRNAs and miRNAs. Despite this differences, some of them have been also found to associate with AGO family members suggesting a role in gene expression regulation similar to miRNAs [25]. Furthermore the RNA fragments show a specific response to the loss of components of the RNA interference (RNAi) pathway, suggesting the presence of an interplay between RNA silencing and RNA processing [26].

In this review we have analysed RNA fragments generated from tRNAs and rRNAs with a special insight regarding their classification, biogenesis, post-transcriptional modifications, their role in gene expression silencing and translation and their association with immune system and cancer development and progression.

### *tRNA fragments: classification and biogenesis*

*Types of tRNA fragments*

tRNA-derived fragments are generally classified according to their length, tRNA domain of origin and their function (Table 1). All are present in tissues and cells of different organisms. The class of tRNA regulatory fragments or tRFs, usually 13-30 nt in length, have a 3'-hydroxyl group, are similar to miRNAs at the 5'-phosphate and are produced by endonucleolytic cleavage of mature tRNAs or during pre-tRNA processing. They are classified into three mayor subclasses: tRF-1, tRF-3 or tRF-5, depending on their origin (Fig. 1A). tRF-3 and tRF-5 are generated from mature tRNAs by endonucleolytic cleavage. tRF-5s, also known as 5'tRFs, derive from 5' end cleavage of tRNAs near the D-loop. tRF-3s, or 3'tRFs, derive from the 3' end cleavage in the TψC-loop [6]. As 5' and 3' tRFs derive from mature tRNAs, they likewise lack introns, and in most cases contain the post-transcriptionally added 3'CCA sequence and may carry plenty of base modifications. In contrast to tRF-3 and tRF-5, tRF-1 are generated from pre-tRNA processing of the trailer sequences and contained a poly-U tail at the 3'end [11]. For instance, the tRF-1 called tRF1001 is generated by cleavage at the 3' end of the precursor of tRNA-Ser-TGA. Due to their origin, their number, compared to the abundance of the other two subclasses of tRFs, is extremely lower. The class or family of endogenous tRFs is more complex and derive from cleavage of internal domains of mature tRNAs, they are variable in length and domain origin and their production seem to be linked to disease states [27, 28]. Finally, the most known class is represented by the so-called tRNA halves are 30-40 bases long. 5'tRNA

halves start from the 5' end of a mature tRNAs and end in the anticodon loop, whereas 3' RNA halves start from the anticodon loop and terminate at the 3' end of a mature tRNA [29]. Thompson *et al*., using a model of oxidative stress, observed a stress response that was accompanied by tRNA cleavage at the anticodon loop and was conserved from yeasts to mammals [10]. For this reason, originally tRNA halves were called stress-induced tRNA fragments, tiRNAs or tsRNAs (Fig. 1A). Although tiRNAs are mainly found in cells undergoing stress, tRNA halves can be found under non-stress conditions [30-32].

## *Biogenesis*

tRNA fragments were initially considered to be debris of the tRNA surveillance and clearance pathways that degrade tRNAs with altered structural and functional integrity [2]. However, we know now that the generation of each of these classes of tRFs is extremely regulated and mediated by specific endonucleases (Fig. 1A). Indeed, although tRNAs are one of the most abundant RNA species in the cell (~10% of total cellular RNA), only a small fraction of tRNAs are cleaved to produce tRFs [33]. Stress-induced cleavage at the anticodon loop of mature tRNAs to originate tRNA halves are produced by Angiogenin (ANG), a RNase Type A ribonuclease, in high eukaryotes, and Rny1, a member of the RNase T2 family, in yeast 15, 33. ANG is induced by a large variety of stresses such as oxidative stress, UV irradiation, heat shock or viral infection 15, 30, 31. The primary targets of ANG *in vivo* in human HEK293T cells are exclusively tRNAs tRNA-Glu, tRNA-Gly, tRNA-Lys, tRNA-Val, tRNA-His, tRNA-Asp, and tRNA-SeC to produce tRNA halves and 5'tRFs that are 26–30 bases long [34]. Other endonucleases include endonucleases belonging to the RNase T2 family encoded by RNT2A, RNT2B and RNT2C genes in the protozoan *Tetrahymena thermophila* that are induced during amino acid starvation and contribute to the production of tRNA halves [7]. Ribonucleases of the RNase T2 family able to produce stress-induced tRNA fragments have not been found in mammals yet. Emerging data have also show that human cells may cleave tRNAs with RNase L in response to viral infections [35].

Concerning shorter tRFs, some studies have indicated that tRNAs show a secondary structure similar to a hairpin and this region is recognized and processed by DICER resulting in the generation of 5'tRFs and 3'tRFs 14, 36-38. Other reports have however indicated that biogenesis of most short tRFs is DICER independent 6, 39, and mostly nothing is known concerning the identification of the endonucleases responsible for the cleavage in the D or TψC loops of mature tRNAs (Fig. 1A). Those contradicting facts may indicate that DICER is essential for the biogenesis of a particular signature of tRFs which is dependent on the cell type, tissue, organism or condition, but it is not required for the vast number of tRFs. Several

other enzymes have been implicated in the generation of tRFs in vertebrates. For instance, RNase Z (ELAC2) cuts off the 3' trailer sequence from the tRNA precursors to produce mature 3' ends and the trailer sequence being liberated gives rise to tRF-1 series [11, 37].

Thus tRNA fragmentation, by generating a wide variety of tRNA microspecies, amplifies the complexity of the small ncRNA repertoire for binding to regulatory factors that will trigger the activation of pathways to respond to specific signals.

*RNA modifications in the regulation of tRNA cleavage*

tRFs production is not random but cell type, tissue, condition or disease associated. How endonucleases are activated and guided to the right tRNA domain or sequence motif is still beginning to be elucidated. tRNA base modifications seem to be some of the determinants of the biology associated with tRNA fragment biogenesis. Originally, it was thought that tRNA chemical modifications were a static process occurring on specific tRNA sites and tRNA modifications would contribute to secondary and tertiary structures preservation, thermodynamic stability, protection from degradation and rapid tRNA decay and translation control and fidelity (reviewed in [2]). However, recent evidences have shown that the population of covalent modifications dynamically fluctuate upon stress or along cell differentiation processes [40, 41]. Some of these modifications have been shown to be inhibitory and protect from stress-induced endonucleolytic cleavage, and thus indicating that tRNA fragmentation is a stress-regulated process and is tuned by tRNA modifications (Fig. 1B).

Cytosine-5 methylation at position C38 at the anticodon loop of tRNAs was shown by Schaefer and Lyko to protect tRNAs from cleavage in *Drosophila* upon stress [8]. Deposition of 5-methylcytosine ($m^5C$) at C38 is mediated by the methyltransferase DNMT2 and flies lacking *Dnmt2* showed an accumulation of tRNA halves derived from the 5' ends of DNMT2-tRNA targets [8]. While the fly ribonuclease responsible for the stress-induced cleavage of hypomethylated tRNAs was not found, Schaefer *et al.* showed that tRNAs lacking $m^5C38$ were susceptible to be cleaved by the human recombinant ANG *in vitro*. These and other studies have suggested a role for tRNA modifications at the anticodon loop protecting from ANG-induced tRNA cleavage [42, 43]. Later studies showed that lack of *Dnmt2* in mice led too to 5'tRNA halves accumulation [44], however mutant mice were not lethal. These studies suggested that tRNA fragmentation into tRNA halves is a well monitored process activated during stress response and tRNA modifications plays an important role impeding excessive loss of mature tRNAs, which would have lethal consequences.

Similar to DNMT2, the role of the cytosine-5 methyltransferase NSUN2 was later shown to be essential for tRNA halves biogenesis and stress responses. NSUN2 methylates cytosine residues mainly at the intersection of the variable loop and the T arm of most tRNAs [18] (Fig. 1B). In the absence of *NSun2* in mice and humans, tRNAs are not methylated at the variable loop and are more vulnerable to be sliced by ANG, leading to the accumulation of 5'-tiRNAs [18]. *NSUN2*-deficient human and mouse cells show reduced viability under stress conditions, which could be rescued upon treatment with ANG inhibitors [18, 19]. In Blanco *et al*., the study suggested that while tRNA fragmentation is an essential event for the initial activation of the stress response, prolonged tRNA fragmentation and excessive accumulation of 5'tiRNAs was detrimental and cells would need to protect from it [18]. Indeed, NSUN2 expression was shown to decrease sharply right after cell exposure to stress, but the expression would recover hours later [18, 45], suggesting that upon an initial period of decreased methylation activity, the subsequent increased expression of NSUN2 would fully methylate and protect tRNAs from ANG cleavage again. Deletion of *Nsun2* in mice leads to neurodevelopment problems and microcephaly [18, 46, 47]. In humans too, inactivating mutations of *NSUN2* lead to microcephaly and intellectual disability disorder [48]. These and other studies showed that lack of tRNA methylation, 5'tiRNAs and increased expression of ANG prompted cells into a state of stress hypersensitivity [15, 18, 19, 30]. In sum, the studies indicated that the levels of cytosine-5 methylation of tRNAs fine-tune the production of a subtype of tRNA halves that regulate stress responses, suggesting that cells may vary the levels of tRNA methylation to survive. Indeed, in a recent study, Gkatza *et al.* showed that the levels of $m^5C$ changes site-specifically and dynamically in response to stress, which in turn influence tRF biogenesis [45].

Another tRNA modification protects tRNA from cleavage is 1-methylguanosie ($m^1G$) at position G9 (Fig. 1B). This modification is catalysed by the tRNA methyltransferase TRMT10A [49]. *TRMT10A* deficiency leads to decreased of $m^1G$ deposition in tRNAs and to accumulation of 22-nt long 5'tRFs derived from tRNA-Gln, suggesting that $m^1G9$ protects tRNAs from endonucleolytic cleavage [49]. Both hypomethylated tRNAs in *TRMT10*A-deficient cells and transfection of cells with tRNA-Gln derived 5'tRFs increased β-cell apoptosis, leading to early-onset diabetes, microcephaly and intellectual disability [49, 50]. The enzymes generating tRNA fragments and the mechanism through which 5'tRFs trigger β-cell apoptosis remain to be elucidated.

There are, however, reports of tRNA modifications that are essential for endonucleolytic cleavage of tRNAs and tRFs biogenesis as is the case of wobble uridine (U34) modification catalysed by tRM9 in

*Saccharomyces cerevisiae* [51] (Fig. 1B). Zymocin (γ-subunit) of the dairy yeast *Kluyveromyces lactis* induces cleavage in the anticodon loop of *S. cerevisiae* tRNA-Lys and tRNA-Gln that carry a 5-methoxycarbonylmethyl-2-thiouridine (mcm$^5$s$^2$U) residue at position 34 [52], and the lack of mcm$^5$s$^2$U reduces Zymocin cleavage efficiency. In a recent report, Guzzi *et al.* observed that Pseudouridine Synthase 7 (PUS7)-mediated pseudouridylation was a critical regulator of the biogenesis of tRFs in human embryonic stem cells (hESCs) [17]. They found that Pseudouridine (ψ) at the eighth position of tRNAs regulated the production of 18-nt long tRNA fragments containing a 5' terminal oligoguanine (mTOGs). They could show that mTOGs were enriched in hESCs and *PUS7* deletion led to a significant depletion of mTOGs (Fig. 1B).

All these findings highlight the importance of tRNA modifications at any particular site in modulating the biogenesis of subsets of specific families of tRFs. Given the diversity of tRNA modifications within a tRNA and that any particular site can range from being unmodified to almost fully modified, modifications thus introduce an infinitive complexity that in different contexts may affect tRFs generation and function differently.

### *tRNA fragments' molecular activity*

It is now widely accepted that tRNA-derived small ncRNAs harbour biological activity and are not random fragmentation products due to increased cell death or stress. Since 2009 from their discovery, stress-induced tRNA fragments have been well characterized for having a conserved potent biological activity, which is repression of global protein synthesis and stress granule (SG) formation during stress [10, 15, 30, 31]. Several other biological activities have been described for tRNA fragments. For instance tRF-3 fragments can load into AGO complexes and have been involved in gene silencing, but also in retrotransposon silencing, ribosome biogenesis and protein translation inhibition [14-17, 22, 23, 25]. Other tRFs series have been shown to interact and participate in silencing activities.

#### *Role of tRNA fragments in gene expression silencing*

The discovery of stable tRFs-AGO complexes led to the hypothesis that tRFs could work as miRNA inhibiting gene expression. Initially it was found that tRF-5s were only poorly associated with AGO complexes [36], while tRF-1s were found to be preferentially associated with AGO3 and AGO4 in human cell lines [37]. In addition, Haussecker *et al.* also observed that while target inhibition by tRF-1s were not

sequence-dependent, they found an miRNA-like target inhibition for tRF-5s and tRF-3s. Similarly, Kumar *et al.* found too that tRF-3s and -5s, but not tRF-1s, were associated with the four human AGO proteins [6].

However only recently, mRNA targets for tRF-AGO complexes have been identified. In Maute *et al.* a tRF-3 derived from tRNA-Gly (called *CU1276*), was shown to bind to AGO and repress Replication Protein A1 (RPA1), a gene implicated in DNA replication and repair [14]. In addition, they showed that a tRF-3 fragment shared structural and functional characteristics with miRNAs, including a DICER-dependent biogenesis. This was the first evidence that a tRF can play the role of a miRNA in mammals. Other sub-class of tRF-3s have been shown to represses mRNAs in a AGO-dependent manner with sequence complementarity to the 3' untranslated region (UTR) [25].

tRF-5 fragments have been associated to post-transcriptional RNA silencing too. 12-nt long tRFs (5'tRFs and middle-derived tsRNAs) which are abundant in *Drosophila* were shown to repress expression of specific targets in an RNAi-like manner [53]. In Luo S. *et al.* it was also shown that tRFs inhibit the translational efficiency of specific mRNAs by conserved antisense pairing. tRF-mediated target silencing was further found to be dependent on AGO2, however, unlike miRNAs, tRF-5s were able to bind equally effective all over the mRNA [53]. Another miRNA gene targeting function was also observed in virus infected human cells, where tRF-5s would suppress anti-viral target genes by sequence-specific binding to the 3' UTR of viral mRNAs [54].

In sum, since AGO proteins are effector molecules of the RNA silencing complex, their association with tRFs suggests that tRFs are able to enter the RNAi pathway to regulate gene expression (Fig. 2A). However while some reports have demonstrated tRFs target complementary-dependent gene silencing, a general understanding of AGO-dependent tRFs-targeting is still lacking.

*Translation regulation by tRNA fragments*

The finding that oxidative stress inhibited protein translation in cells expressing a non-phosphorylatable form of eIF2$\alpha$ set Paul Anderson and co-workers to find the alternative translation control pathway [55]. Their discovery introduced stress-induced tRNAs fragments as previously unappreciated components of the mammalian stress response [15]. The finding that transfected 5' but not 3'tiRNAs inhibited protein synthesis revealed a functional difference between these tRNA fragments and excluded the possibility

that the observed translation repression was not due to depletion of the pool of cytoplasmic tRNA. In addition, Thompson *et al.* discovered that tRNA cleavage was a conserved and regulated response to several stress signals in eukaryotes and was not a mechanism for quality control of non-functional tRNAs [10], in fact the proportion of cleaved tRNAs was smaller than 1% of the pool of mature tRNAs [10, 15, 31]. To date, several mechanisms of translation inhibition have been discovered for these novel class of small ncRNAs.

In 2011, Ivanov *et al.* characterized one of the most studied translation inhibition mechanisms to date. They found that only the activity of 5'tiRNA-Ala and 5'tiRNA-Cys were able to trigger stress-induced translation repression [56]. Those 5'tiRNAs were characterized for binding to the translational repressor Y-box binding protein 1 (YB-1, YBX1), and containing a stretch of five guanosine residues at the 5'-end (TOG motif) [16]. Mechanistically, the authors showed that TOGs would assume G quadruplex (G4) structures and blocked translation initiation by specifically displacing 4eIF4G/eIF4A from capped mRNA preventing the assembly of the canonical translation pre-initiation complex [56]. This was shown to be partially due to the capacity of TOG-containing tiRNAs to bind to YB-1, since knocking down YB-1 only partially reversed translation repression, suggesting that other mechanisms were also involved in 5'tiRNA translation repression [16, 57] (Fig. 2B). YB-1 interaction was later shown to be critical for packaging of tiRNA-repressed mRNAs into stress granules (SGs) but it was dispensable for tiRNA-mediated translational repression [30, 58]. By contrast, in another recent study it was shown that tiRNAs interaction with YB-1 displaced YB-1 from specific mRNAs resulting in the downregulation of those transcripts in breast cancer and mammary epithelial cells [27]. The different mechanisms showed by both studies was most likely due to the type of tiRNAs involved. While P. Ivanov showed that TOG-containing 5'tiRNAs bound to YB-1, in Goodarzi *et al*. tiRNAs were characterized for mapping to the anticodon region and containing a common linear sequence motif (SCUBYC) that could bind to YB-1. Whether YB-1-tiRNAs interaction can mediate eIF4G displacement from capped mRNAs remains to be elucidated.

More recently, Guzzi *et al.* has shown that in hESCs a specific class of 18-nt TOG-containing 5'tRFs (mTOGs) were able to displace eIF4A/G from m$^7$G-coated beads. Remarkably they found that the presence of $\psi$ at position 8 was required for efficient mTOG-dependent displacement of eIF4A/G from capped-mRNAs. More importantly they discovered that mTOGs containing $\psi$8 (mTOG-$\psi$8) preferentially bound to polyadenylate-binding protein 1 (PABPC1), another factor which interacts with eIF4G to enhance eIF4F formation and stimulate translation, resulting in displacement of PABPC1 from capped mRNA and

repressing translation [17] (Fig. 2C). They also showed that YB-1 was instead mostly bound by $\psi-$unmodified mTOGs. These data demonstrate that tiRNAs can impair translation initiation complex formation and in addition, RNA modifications govern mTOG function by regulating their binding to specific proteins tuning the function of 5'tRFs in translation.

Other tRFs have been shown to inhibit protein synthesis by alternative mechanisms. Gebetsberger *et al.* found that 26-nt long 5'tiRNAs derived from tRNA-Val and produced upon stress (Val-tRF) in *Haloferax volcanii* were able to repress translation by directly binding to the small ribosome subunit [9]. More recently, the authors found that Val-tRF binds to the mRNA channel and competes with mRNA binding to the small ribosomal subunit and thus interfering with the formation of the translation initiation complex [59]. Other works demonstrated that both stress-dependent 5'and 3'tiRNAs in *S. cerevisiae* are able to block protein synthesis under stress conditions by binding to ribosomes [60]. Hutvagner's group presented similar data showing that 19-nt long 5'tRF derived from tRNA-Gln characterized for containing 3' terminal "GG" dinucleotide motifs that were able to repress translation in human cells by interacting with the Multisynthetase complex (MSC), a complex involved in translation [61, 62].

While most reports have shown that stress-induced tRFs interfere with initiation complex formation or the interaction of mRNA and the ribosomal subunits, other reports have described alternative mechanisms that either stimulate or inhibit global protein translation. ANG, besides the ability to cleave in the anticodon region of tRNAs, it cleaves 3'-CCA end of tRNAs [63]. While this report indicated an alternative role of ANG-induced tRNA cleavage in translation inhibition through a global depletion of translationally competent tRNAs, ANG endonuclease activity on the 3'-CCA ends has only been shown *in vitro* and *in vivo* data remains unclear. In contrast, other reports have indicated that tRF can affect translation of specific transcripts without affecting global translation. In Kim *et al.,* the authors discovered how a 22-nt long 3'tRF derived from tRNA-Leu-CAG (LeuCAG3' tsRNA) enhances specific transcript translation in hepatocellular cancer cells [64]. The authors established that there is a direct physical interaction between LeuCAG3' tsRNA and mRNAs of at least two ribosomal proteins, RPS28 and RPS15. They found that the inhibition of LeuCAG3' led to a decrease in RPS28 protein levels, which in turn blocked pre-18S rRNA processing, resulting in a reduction of 40S ribosomal subunits. More recently they have shown the functional target sites present in many vertebrates suggesting that LeuCAG3'-regulated translation is a conserved gene regulatory mechanism [65]. Further investigation will be required to establish whether other tsRNAs may regulate translational of specific transcripts in a similar fashion. tRFs can

enhance global translation too. In a recent study published by Polacek's group, the authors have identified 3'tRNA halves that when associated with ribosomes and polysomes unexpectedly stimulated global translation during the stress recovery phase particularly after prolonged starvation *Trypanosoma brucei* [666]. They further described that 3'tRNA halves derived from tRNA-Thr lacking the 3' CCA end produced during nutritional stress and during the stationary phase in the procyclic form of T. brucei. They concluded that mechanistically tRNA-Thr halves lacking the 3' CCA counteracted and inhibited the activity of inhibitory small RNAs in the same size range, including tRNA-derived RNAs, present in stressed cells.

It is well established that stress-induced tRFs and ANG have been reported to mediate a stress response, which results in SG assembly and inhibition of protein synthesis [30]. However the exact mechanism underlying the regulation of stress response processes remains still unclear. SGs formation upon tRNAs cleavage induction promote cell survival by sequestering proapoptotic signalling proteins, translationally stalled ribonucleoprotein complexes and promoting selective recruitment of ribosomes to mRNAs whose protein products are required for responding to stress (reviewed in [67]). For instance, ribosome profiling data from *Nsun2* deficient mice or human cells derived from patients carrying inactivating mutations in *NSUN2* showed an increased production of 5'tiRNAs and increased translation of genes associated to stress response pathways [19]. While the exact mechanism and whether 5'tiRNAs directly favoured translation of the specific set of transcripts is still unknown, mechanistically the data showed an increased ribosomal density on 5' UTRs. Importantly the data also showed decreased ribosomal densities on genes associated to development, differentiation and migration, indicating that 5'tiRNAs could regulate too cell motility and differentiation processes. Indeed, accumulation of 5'tiRNAs in *Nsun2*-deficient cells were associated to decreased development, defective differentiation and reduced cell migration in mouse embryos and adult tissues [18, 19, 46, 47]. In a similar study, lack of *Dnmt2* in mice led too to 5'tRNA halves accumulation and deficient haematopoietic progenitor cell differentiation [44]. Similarly in Krishna *et al.* the authors observed that a specific set of 5'tRFs was enriched during differentiation of mouse ESCs, which sequestered the ribosomal binding protein IGF2BP1 from *cMyc* mRNA , a potent regulator of pluripotency, which in turn is no longer stabilized and gets degraded perpetuating differentiation processes [20]. Similar results were found in Guzzi *et al.*[17]. They observed that PUS7 was preferentially enriched in hESCs and its loss led to decreased pseudouridylated mTOGs production, increased protein translation rates, defective mesoderm specification and hematopoietic stem cell commitment. While the authors could show that translation initiation was regulated by pseudouridylated mTOGs, it is still undetermined the exact mechanism by which those tiRNAs regulate differentiation processes. In addition, given that extensive

base modifications in tRNAs are crucial for their function, future studies should address the potential role of these modifications in tRNA fragments as well as whether they can cooperate to redirect the translation machinery to specific sets of mRNAs.

In sum, all these mechanisms indicate an adaptive translation control by which cells quickly modulate their biological processes in response to environmental changes. This processes may lead to the generation of proteins with distinct functions that may help the cells to adapt to a new environment or stress condition.

### tRNA fragments and Immunity

The connection between small ncRNAs and immune system has already been well documented, given the role that miRNA and siRNA play in suppressing gene expression. miRNA and siRNA affect all facets of immune system development, from haematopoiesis to activation in response to infection and host–virus interaction [68]. For instance upon viral infections, host cells alter their small ncRNA expression profiles as a defence mechanism. On the other hand, viruses can circumvent host defence and promote their propagation by altering cellular small ncRNA expression or by expressing their own small ncRNAs [69]. Most of the information about this mechanisms is related to RNAi pathways while other kind of small ncRNAs remain largely unexplored. For this reason, and for the previous analysed association with AGO proteins, there are strong indications that also tRNA fragments could influence the immune system responses.

In 2013 in Wang *et al.* the authors analysed the effect that the respiratory syncytial virus (RSV) had on the immune system response. They showed that in human airway epithelial cells, RSV was able to induce tRNA cleavage in the anticodon site, resulting in the induction of tRNA halves formation [70]. They found that RSV-induced tRFs derived mainly from 5' ends and resulted to be important molecules for viral replication. In addition, one of the identified tRFs, tRF-5-GluCTC, exhibited trans-silencing capability against target genes and therefore had gene silencing activity that was, in any case, different from that of miRNAs/siRNAs. They concluded that the positive effects of tRFs on viral replication were most likely due to the suppression of host defence gene expression.

In order to identify the molecular mechanism by which RSV-induced tRNA fragments can inhibit host immune system, in Deng *et al.,* the authors used an unbiased approach combining biochemical screenings, gene expression data analysis and *in silico* sequence complementary prediction to identify tRF targets [54].

In particular, they studied the tRF5-GluCTC previously described in Wang *et al.* [70] because of its trans-silencing capability and found different tRF5-GluCTC sequence-complementary targets. One of the targets under study was the apolipoprotein E receptor-2 (APOER2), a cell surface receptor involved in many processes, such as neuronal development and immunity. They found that tRF5-GluCTC was able to suppress *APOER2* mRNA expression using its 3'-portion to recognize a target site in the *APOER2* 3'-UTR (Fig. 3A). Among all the different functions of the receptor, APOER2 can inhibit RSV replication. The inhibition promoted by tRF5-GluCTC on the receptor had, therefore, a positive effect on virus replication [54].

Considering the connection between tRFs and RSV infection, other studies have aimed to analyse the process and prove whether tRNA cleavage is one of the mechanisms carried out by viruses to escape from host immune system. In contrast with RSV, increased accumulation of tRFs was not observed in cells infected with human metapneumovirus (hMPV), despite considerable alterations in the miRNA pool [71]. Nevertheless, in hepatocytes of patients affected by B and C hepatitis, a considerable accumulation of tRNA fragments was found. Specifically, the levels of tRNA halves resulted significantly increased in non-malignant liver tissue of patients with chronic viral infections, while *ANG* mRNA levels were reduced in both B and C associated cancer compared to non-malignant tissue [72]. Even if the reason for such accumulation of tRFs and the biological function are still undescribed, evidences support that infection-induced tRF biogenesis is a mechanism that enhances viral replication, as observed for RSV, as well as a response mechanisms to specific types of cellular stress (Fig. 3B). Another function assigned to tRFs is priming activity for reverse transcriptase. A tRF-3 derived from tRNA-Pro was shown to be capable of priming HTLV-1 (Human T-cell leukemia/lymphoma virus type 1) reverse transcriptase, thus suggesting an important role of these tRFs in HTLV-1 viral infection [22].

In addition, there are strong evidences supporting the idea that tRNA fragments could regulate immune cells. For instance deep sequencing studies revealed that tRFs derived from the 5' and 3' ends of mature tRNAs were abundant in the cytoplasm of immune cells and small RNAs derived mostly from the 5' ends were selectively enriched in vesicles derived from these immune cells [73]. In Maute *et al.*, a significative down-regulation of CU1276, a DICER1-dependent tRF-3, was observed in lymphoma cell lines and biopsies [14]. CU1276 was shown to repress endogenous *RPA1*, a specific gene involved in genome replication. The reduced expression of CU1276 in mature B cells and the lack of *RPA1* repression could confer a growth advantage to B cells. Regarding the role of tRFs in T cell activation during an immune response, in Chiou

*et al*., they documented an enrichment of tRFs in T cell-derived extracellular vesicles (EVs) in comparison to all the other cellular small RNA species during host defence activation [74]. Furthermore, the authors found that the number of tRFs was higher in EVs derived from activated T cells compared to non-activated lymphocytes, and the inhibition of EV biogenesis specifically led to the accumulation of these activation-induced EV-enriched tRFs within multivesicular bodies and the consequent inhibition of T cell activation. In contrast, the use of antisense oligonucleotides to inhibit these tRFs enhanced T cell activation [74]. Furthermore, if used as adjuvant of hepatitis B antigens in mice, tRNA fragments induced Th1 response, characterized by the production of Interferons (IFNs) through recognition of TLR3 [75]. Considering that the Protein Kinase Receptor (PKR) is IFN-effector protein and its action in the presence of double stranded RNAs (dsRNAs) modulates translational inhibition [76], the activation of the PKR pathway could be another alternative way for tRNA fragments of blocking protein translation. Even if no data referring to this hypothesis are present in the literature, recently, Ivanov *et al.,* showed as tRNA fragments can inhibit translation, independently from the eIF-2 pathway, by displacing the elongation factor eIF-4 from the initiation translational complex [16].

All this information indicates a strong connection between tRNA fragments and immune system. This observation is also supported by the discovery of a higher presence of tRNA fragments both in human and mice hematopoietic and lymphoid tissues compared to other tissues [77]. Furthermore, these fragments have been found in high presence in circulating mouse and human bloodstream [78]. The presence of tRNA fragments in the extracellular environment and their role in the modulation of different biological function including the immune response, suggests a possible role of cell-cell communication immune signalling molecules. To support this hypothesis, another interesting observation is that the largest human tRNA gene cluster is located in the major histocompatibility complex (MHC)[79], the set of genes that encode for cell surface proteins that are essential both in the innate and adaptatively immune response indicating a closed transcriptional association between immunity and tRNA fragments.

In summary, these observations provide support that circulating 5'tRFs are highly expressed in hematopoietic and lymphoid tissues, which strongly suggest that tRFs may act as immune signalling molecules. Future studies addressing their biogenesis regulation, secretion and uptake, and functional role in immune cells and associated tissues will provide insights into the proposed role of tRFs in the immune and hematopoietic systems or other as yet undetermined functions as well as in hematopoietic disorders

***tRNA fragments associated to cancer progression***

Early studies detected tRFs in the urine of cancer patients raising the possibility that tRFs could be used as biomarkers for early cancer detection and that they may have potential oncogenic activity [5]. Nonetheless, the investigation of tRFs as potential biomarkers and therapeutic targets in cancer did not begin till recently. In 2009, tRF-1001 was the first tRF to be found highly expressed in several cancer cell lines [11]. Because tRF-1001 was produced by tRNase Z (ELAC2) activity and since ELAC2 is a known prostate cancer susceptibility gene, tRF-1001 was thought to be oncogenic, and indeed its biogenesis inhibition impaired cell proliferation [11] (Fig. 4A). Later, other studies have identified other rare tRF-1s in cancer samples confirming an association between cancer and these potential regulatory tRFs [37, 80, 81].

Since then the expression of other tRFs have been found to be associated to cancer are being explored as alternative biomarkers of disease [37, 80, 81]. Sex hormone-dependent tRNA-derived RNAs (also known as SHOT-RNAs) were identified to be highly and constitutively expressed in breast and prostate cancer cell lines but were absent in other cancer tissues [21]. Other type of 5' stress-derived tRFs were identified to be specifically altered in serum of breast cancer and in head and neck squamous cell carcinoma patients [82, 83]. In clear cell renal cell carcinoma, the expression of a 5' tRNA half derived from tRNA-Val was found downregulated and its expression was correlated with the stage and grade of the disease [84]. In myelodysplastic syndrome patients, differential expression of six tRFs were correlated with treatment response [85]. In other studies, downregulation of specific tRNA-derived ncRNAs was associated with chronic lymphocytic leukaemia [86]. In *Balatti V et al.,* by analysing the data from chronic lymphocytic leukemia, lung, colon, breast, and ovarian cancer samples, the authors found a cancer specific signature of 31 differently expressed tRFs that was able to discriminate the cancer type as well as the cancer development and staging [87].

In a recent attempt to explore the whole transcriptome of tRFs in cancer, Pliatsika *et al*. processed all data of The Cancer Genome Atlas datasets (TCGA) and identified the expression of 23 413 tRFs associated to cancer [88, 89]. While differential expression of tRFs have been associated to several cancer types, prostate cancer shows the highest repertoire of tRFs. In *Olvedy M et al.,* the expression of tRFs transcripts was analyzed in patient cohorts of different stages of prostate cancer [80]. The analysis identified 598 differentially expressed tRFs whose deregulation was correlated with cancer stage, highlighting their

potential as candidate biomarkers for detection of prostate cancer. Most of the upregulated tRFs belong to the tRF-5 series and the majority of the downregulated tRFs belong to the tRF-3 series.

Generation of tRNA fragments has been shown to be involved in regulating tumorigenesis too. Similar to stress-dependent tiRNAs, SHOT-RNAs were found to be produced by endonucleolytic cleavage at the anticodon loop, however their biogenesis was not triggered by stress [21]. Instead their expression was hormone-dependent and knockdown of ER and AR resulted in their decreased abundance. The authors also found that tRFs expression induced proliferation in cancer cells, and concluded that tRFs could represent a mechanism of tumour progression in response to oncogenic stress (Fig. 4A). Regardless their expression not been triggered under stress, the authors suggested that SHOT-RNAs were too produced by ANG [21]. ANG was discovered and characterized by its ability to promote angiogenesis in tumours, and is widely established to be consistently up-regulated in different cancers making it a potential anti-cancer therapeutic target [90]. While the mechanisms by which ANG promotes angiogenesis is still not fully established, it is dependent upon its catalytic activity, suggesting that ANG-induced tRFs may be involved in carcinogenesis.

Other tRFs have been reported to have oncogenic activity. For instance, a tRNA-Leu-CAG-derived tRF-3 was recently reported to increase cell viability and its inhibition induced apoptosis in rapidly dividing cells *in vitro* as well as in a hepatocellular carcinoma by regulating the formation of 80S ribosomal complexes [64, 65] (Fig. 4A). These studies showed that inhibition of tRFs could reduce proliferation or induce apoptosis of tumorigenic cells thus indicating that blocking or repressing tRFs expression could be a novel therapeutic strategy.

While some studies have revealed tRNA fragments to have pro-oncogenic effects on cancer cells, in contrast recent studies have reported that tRNA-derived fragments can too supress proliferation and metastasis [27]. So far, the best mechanistic explanation for a role of tRFs in cancer progression was found for a specific set of hypoxia-induced tRFs [27]. As hypoxia is a major stress encountered during at cancer onset and early progression, Goodarzi *et al.* analysed the pool of tRFs induced under low-oxygen conditions and found a group of tRFs that were specifically induced in breast cancer cell lines as well as in normal mammary epithelial. Mechanistically, endogenous tRFs were found associated with the oncoprotein YB-1 [27]. YB-1 is an oncogene up-regulated in a wide variety of cancers which binds to and stabilizes mRNA targets encoding for genes that promotes tumorigenesis [91]. Mechanistically, tRFs

complexed to YB-1 were able to destabilize oncogenic transcripts inhibiting tumour progression [27] (Fig. 4B). Consistently, they found that highly metastatic breast cancer cells evaded this mechanism by blocking the induction of these tumour suppressive tRFs and thus fostering metastatic properties. The authors concluded that tRF expression may represent a mechanism of tumour suppression in response to oncogenic stress. However, such tumour suppression mechanism is likely inactivated during metastatic progression by decreased expression of tRFs and YB-1 up-regulation.

Similar results were found in other cancer types. In prostate cancer, while samples from primary tumours showed upregulation of a set of tRFs, samples from metastatic tumours expressed other set of tRFs, indicating that specific tRF populations may play different roles during tumour onset and progression [92]. In colorectal cancer, the tRNA-Leu-derived miR-1280 suppressed cancer stem cell functions and metastasis [93]. Mechanistically miR-1280 inhibited Notch signalling pathway by directly binding to the 3' UTR of Jagged Canonical Notch Ligand 2 (JAG2) and inducing its degradation (Fig. 4C). Thus, its downregulated expression in colon cancer correlated with its tumour suppressive role. Likewise, in B cell lymphoma cell lines, several tRNA-derived fragments were shown to suppress cell proliferation and modulate DNA damage respond by regulating the expression of DNA damage response genes [14,94]. In lung cancer cell lines, tRFs ts-46 and ts-47 were shown to inhibit cell growth and colony formation suggesting that their deficiency could favour cell proliferation and cancer onset and progression [87]. ts-53 (previously designated as "miR-3676") was found to be down-regulated in CLL and mechanistically was shown to target the 3' UTR of *TCL1* mRNA, a key oncogene in the development of aggressive CLL, and thus its down-regulation inversely correlated with TCL1 expression in leukemic cells [95].

While further work is needed to better understand the role of tRNA fragments in cancer, it can be concluded that tRNA fragments are broadly associated with cancer and have oncogenic or tumour suppressor functions, depending on the tRNA and tumour type. These studies further suggest the use of tRFs as novel therapeutic targets to repress tumour progression.

### *Small Ribosomal RNA-derived fragments*

Compared to mRNA and tRNA, rRNA is the most common form of RNA in cells, and similar to tRNAs, rRNA can be cleaved into small ribosomal RNA-derived fragments (rRFs). Eukaryotic ribosomes are constituted by four different rRNAs: 5S, 5.8S, 18S and 28S (higher eukaryotes)/25S (yeast) [96]. Ribosome production is initiated in the nucleolus, where a single rRNA precursor (47S rRNA) containing 5.8S, 18S and 28S rRNAs

and spacer regions known as external and internal transcribed spacers (EST, ITS1 and ITS2), is transcribed by RNA polymerase I, while 5S rRNA is transcribed by RNA polymerase III [97] (Fig. 5A). Traditionally, reads corresponding to rRNA were removed in RNA sequencing analysis and rRFs casually identified during the analysis were considered as by-products of rRNA degradation [98, 99]. In addition, rDNA is not usually included in reference genome assemblies and, even when included, information can be missed since reference transcriptomes do not include all rDNA possible variations [97]. Despite these limitations, interest for studying these rRNA-related fragments is steadily growing and there is increasingly evidence that short reads mapping rRNA are stable and may play a functional role in the cell.

*Types and biogenesis of rRFs*

Similar to tRFs, rRFs have been identified in several species and are classified depending on the domain of rRNA where they derive from (Table 2). The first report documenting the existence of rRFs was Lee H *et al.* in 2009 [100]. The authors found that DNA damage in *Neurospora crassa* induced the accumulation of a novel class of small ncRNAs, named QDE-2-interacting small RNAs (qiRNAs), which were mainly originated from 28S rRNA and the external and internal transcribed spacers of 47S rRNA ETS, ITS1 and ITS2 (Fig. 5A). It was proposed that rRFs were subsequently matured by consecutive actions of a RNA polymerase (RdRP or QDE-1), a helicase (QDE-3) and DICER [100]. Since then, many studies have reported the existence of rRFs in other species.

In humans and mice, analysis of small RNA high-throughput sequencing datasets has revealed a significant percentage of reads that mapped to rDNA. Among those rRFs, about 9.6-15.1%, 8.1-15.8% and 67.6-80.4% were distributed in regions coding 18S, 5.8S and 28S rRNA, respectively [101] (Fig. 5A). rRFs have been found to be produced from either end (3' or 5') of the three rRNAs, with preference for the 3' end in human cell lines, yeast and the tick *Amblyomma testudinarium* [10, 39, 102]. This differs from *Danio rerio* and plants in which 5' end rRFs are more abundant [103, 104]. In zebrafish both maternal- and somatic-type 5.8S, 18S and 28S rRNAs produce several fragments, with a strong bias for 3' and 5' ends [103]. Interestingly, 5.8S also yield 2 fragments roughly corresponding to 3' and 5' halves, which are most likely generated by a single cut of the molecule, similarly to what happens with tRNAs [31], although the endonuclease responsible of this cleavage is still unknown.

Several reports have documented the existence of rRFs produced under distinct conditions and in some cases the responsible endonuclease has been identified. In *Saccharomyces*, similar to tRFs, Rny1p

19

produces rRFs mainly derived from 25S rRNA and under oxidative stress conditions [33]. Yet, Rny1p knock-out strains still present rRFs, suggesting that other endonucleases might participate in rRFs formation. *Caenorhabditis elegans* possesses a type of DICER-independent rRNA fragments called antisense ribosomal RNAs (risiRNAs) with sequence complementarity to 18S and 26S rRNA [105]. risiRNAs have been recently identified in *Arabidopsis thaliana* too [106]. In this recent study, Cui and Mo's group found that risiRNAs are generated from both rDNA strands and from ETS/ITS regions of the rDNA loci in the absence of the dephosphorylating enzyme FIERY1 involved in several biological processes such as stress signalling or plant immunity, suggesting a role of risiRNAs in stress and infections regulation [106]. In addition, the authors identified that rRNA production was dependent on both nuclear exoribonucleases including EXORIBONUCLEASE2 (XRN2) and XRN3, and cytoplasmic exonucleases including RNA-dependent direct polymerase 1 (RDR1) and RDR16 and DICER-like 2 (DCL2) and DCL4, complicating predictions about the subcellular localization of risiRNA biogenesis. In a similarly study in plants, other family of rRFs called phased small interfering RNAs (phasiRNAs) were found to be generated from 28S and 5.8S rRNAs by the cytoplasmic exonuclease DCL [107]. In mice, endogenous small interference RNAs (endo-siRNAs) are generated from loci that mapped to rDNA loci and are capable of forming dsRNA in a DICER-dependent manner, and are specially abundant in spermatozoa [108]. Interestingly, several miRNAs and piRNAs have been found to map to rDNA loci too, demonstrating that they are actually rRFs. For instance, mmu-miR-5102, mmu-miR-5105, mmu-miR-5109 and mmu-miR 5115 are produced from 28S rRNA in mice, while mmu-miR-712 in mice, mir-10404/mir-ITS1 in *Drosophila* and has-miR-663 in humans derive from spacer regions [109, 110].

Similar to tRFs, rRFs production seems to be linked to post-transcriptional modifications. Indeed, defects of post-transcriptional N1-methyladenosine ($m^1A$), N7-methylguanosine ($m^7G$) and N6-dimethyladenosine ($m^6_2Am^6_2A$) modifications in *C. elegans* 18S rRNA have been shown to induce the accumulation of risiRNAs [111].

In summary, the different rRNA domains of origin of rRFs, together with the differential size distribution, and their expression under distinct cell conditions support the hypothesis that the formation of rRFs is not a random degradation process, instead it shows that rRFs biogenesis is highly specific and regulated endonucleolytic process, and thus suggesting that similar to tRFs, they are regulatory molecules with capacity to fine tune cell processes.

*Functional role of rRFs*

Similar to tRFs, several rRFs have shown to interact with AGO proteins suggesting a role in gene silencing. In human fibroblasts, around 7% of the rRFs were found to co-immunoprecipitate with AGO proteins (Fig. 5B). Interestingly,  those rRFs differed in distribution and size patterns compared to the pool of total rRFs, suggesting a specific role for some rRFs in silencing [101]. Indeed, overexpression or inhibition of several rRFs in mouse hepatoma cells showed to alter the expression of several key metabolic enzymes, suggesting that rRFs can regulate metabolic processes in a similar manner to miRNAs or siRNAs [101]. In *Drosophila,* rRFs were also shown to co-immunoprecipitate with AGO1 and AGO2 complexes, in a distinct distribution patterns, further confirming to have a defined role [101]. In *Neurospora*, qiRNAs are associated with QDE-2, an AGO protein required for quelling, a RNA-mediated gene silencing phenomenon [100] (Fig. 5C). qiRNAs were found to be involved in the protein synthesis inhibition process occurred after DNA damage in *Neurospora* [100] and required for the DNA damage response and repair pathways in plants [112], thus qiRNAs production was suggested to be a conserved mechanism that contributes to DNA damage checkpoints. In *C. elegans,* risiRNAs have been found to act, at least in part, through the nucleolar RNAi pathway by associating with the AGO protein NRDE-3 to downregulate pre-rRNA [105, 111] (Fig. 5D). Apart from AGO proteins, other complexes can bind rRFs. For instance, some 28S rRNA-related rRFs co-immunoprecipitate with tRNase $Z^L$ in human kidney 293 cells and may function as a small guide RNAs to downregulate the levels of specific mRNAs [113] (Fig. 5E).

rRFs have been shown to be precursors for the production of miRNAs.  In zebrafish, rRFs derived from the 5' end of 18S rRNA form a secondary structure containing a stem which is thought to act as a non-canonical precursor for the biogenesis of miRNAs [103]. In addition, analysis of published AGO-complexed small-RNA pool datasets in humans and mice confirmed that both guide and passenger strand of the homologous stem are detected in the small RNA pool that co-immunoprecipitates with AGO proteins, further confirming their role as miRNAs precursors [103]. Those findings, together with several piRNA sequences matching with rRFs [101, 108, 109], further support an important functional role of rRFs in gene expression regulation.

The function of rRFs is not limited to gene silencing, in fact their structure is also thought to play a crucial functional distinct role in rRNA stability. For instance, structural analysis of zebrafish 28s rRNA-related fragments revealed that their 3' ends can bind 3' ends of complete 28S rRNAs and may play a role in their degradation [103] (Fig. 5F). Similarly, 5.8S halves maintain a strong secondary structure with long stable

stems, which could prevent 28S to interact to 5.8S rRNA [103]. Thus, 5.8S halves are thought to participate in 28S and 5.8S rRNAs separation during ribosome degradation (Fig. 5G). However, their specific role remains to be elucidated.

*Physiological relevance of rRFs*

Similar to tRFs, rRFs production is highly linked to stress responses. For instance, in *C. elegans* risiRNAs are upregulated after UV radiation and cold shock to silence pre-rRNA expression to avoid the accumulation of erroneous rRNA [105]. In yeast, levels of some rRFs also increase during oxidative stress and entry into stationary phase [10].

rRFs function has been linked to other physiological processes. Overexpression or inhibition of several rRFs in mouse hepatoma cells showed to alter the expression of several key metabolic enzymes, suggesting that rRFs can regulate metabolic processes in a similar manner to miRNAs or siRNAs [101]. In addition, miRNAs mapping to rDNA such as mmu-mir-712 in mice and hsa-miR-663 in humans have been reported to play a role in inflammatory responses [110, 114]. In human kidney 293 cells, tRNase Z$^L$ guided by rRFs downregulate the levels of DYNC1H1 (Dynein Cytoplasmic 1 Heavy Chain 1), suggesting a role in mitosis regulation [113]. All these evidences suggest that rRFs play significant roles in stress responses, proliferation, metabolic and inflammatory processes and thus suggesting implications in disease development or progression as it has been described for tRNA-derived fragments.

Altogether, the data reveal that several fragments can be generated from rRNA precursors and from mature molecules. rRNA degradation products are nearly undetectable in normal conditions, since they are rapidly degraded [115]. This suggest that detected rRFs are likely stable products generated by highly regulated processes rather than random degradation, as it has been previously considered [98, 99, 103]. Among these fragments, a large subgroup has been found to interact with ribonucleoprotein complexes, and might play a role in rRNA processing and gene expression regulation [100, 101, 103, 105, 111, 113]. In addition, while defective accumulation or loss of rRFs have not been associated to disease yet, their physiological implications in stress responses, mitosis, inflammation or metabolic regulation strongly indicate an association to diseased processes. Further studies will shed light on the functional role and pathological relevance of these abundant rRNA-related fragments.

**Concluding remarks**

With advances in technology and experimental approaches, we are beginning to appreciate the variety of small ncRNAs derived from tRNA and other long ncRNAs that can tune gene expression and protein biogenesis in a cell- and tissue-specific manner, and the precision by which stress and other signals are coordinated to induce their biogenesis. With the development of next generation sequencing technologies, the repertoire of regulatory small ncRNAs derived from other RNAs is growing [116, 117] and it will be fully uncovered in the near future. Improved understanding of their functions will provide valuable insights into human physiology and pathology.

The biogenesis and biological functions of most tRFs and other RNA-derived fragments are still unclear. Nonetheless, to date research into RNA fragments biology has shed light on the existence of another layer of gene expression regulation. tRNA and rRNA fragments are pieces of a big puzzle that adjust and tune posttranscriptional gene expression regulation coordinating the silencing and translation machinery. For instance, novel research has shown that 5'tRFs can suppress the expression of transcripts that are driven by the long terminal repeat (LTR)-retrotransposon MERVL in the developing mouse embryo [23]. Similar observations were made for other LTR-retrotransposons, where 3' tRNA fragments were shown to block their reverse transcription by binding to the primer binding site (PBS) of LTR-retrotransposon transcripts or reduce their transcript levels in a RNAi-mediated manner [118]. In addition, recent findings have established tRNA fragments as " non-genetic" inheritance factors of metabolic traits that drive epigenetic remodelling of gametes [119]. This is a key area of research that will enable us to understand more clearly the complexity behind the transcriptome regulation.

The availability of genome sequencing data from patients with diverse conditions has already resulted in the identification of tRNA-derived fragments signatures and to some extend they have been shown to have oncogenic or tumour suppressive activity. The mechanisms that give rise to those fragments that can either suppress or promote tumorigenesis need to be uncovered. tRFs are also induced by viral infections establishing a posttranscriptional mechanism that can fine-tune gene expression during other pathological states and provides a potential new target for treating infection diseases or immune-related disorders including cancer . The real challenge now is to ascertain whether these tRNA fragments are the core of the molecular pathology of the disease and whether they are best diagnostic tools of druggable targets to develop potent and efficient therapeutic strategies. In the past year, several preclinical formulations of RNA-based therapies have shown clinical benefits for treating cancer and other diseases [120, 121]. The data shown in this review indicates a promising therapeutic potential of targeting these small

ncRNAs derived from RNA fragments disrupting their functions to treat cancer, immune-associated diseases and other disorders. In the near future, tRFs- or rRFs-based therapies may become valuable to provide treatment for previously untreatable diseases.

Secondary products originating from mature RNAs that were considered to be metabolically stable might be functionally relevant in cancer. For instance, several recent studies have demonstrated a link between tRNA modifications and tumour cell survival in response to chemotherapy. While, the modification status of these RNAs fragments and whether or not RNA modifications contribute to tRF functions remains still unknown, however some are already known to contribute to their biogenesis. The coordinated depositions of $m^5C$ by NSUN2 and $m^7G$ by METTL1 to tRNAs were implicated in mediating sensitivity of yeasts and HeLa cells towards the cytotoxic agent 5-fluorouracil (5-FU) [122, 123]. Deletion of *Nsun2* in mouse skin tumours increased the number of ANG-induced 5'tRFs and *Nsun2*-lacking tumour initiating cells were highly sensitive towards treatment with 5-FU and cisplatin [19]. Removal of similar RNA modifications in yeast also induced sensitivity to cytotoxic stress [123]. These findings highlight the importance of the dynamic deposition of modifications into tRNAs and the possible use as therapeutic targets to increase cancer cells sensitivity to chemotherapeutic agents. Thus, it will be important to decipher the tRFs epigenetic RNA ''code'' directing tRFs biogenesis and activity.

The challenge will be separating the functional impact of RNA-derived fragments from their molecules of origin. Recent advances in targeted gene disruption in mammalian cells offer a useful tool to assess the contribution of single genes, including tRNA genes, to pathology. In addition knowledge of the tRNA and rRNA fragments biology in different tissues will allow us to gain a more accurate understanding of the tissue-specific aspects that modulate disease severity and progression.

*Acknowledgements*

*Declaration of interest statement*

The authors declare no conflict of interest.

## References

1.      Mattick JS, Makunin IV. Non-coding RNA. Hum Mol Genet 2006; 15 Spec No 1:R17-29.
2.      Phizicky EM, Hopper AK. tRNA biology charges to the front. Genes Dev 2010; 24:1832-60.
3.      Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75:843-54.
4.      Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell 2014; 157:77-94.
5.      Borek E, Baliga BS, Gehrke CW, Kuo CW, Belman S, Troll W, et al. High turnover rate of transfer RNA in tumor tissue. Cancer Res 1977; 37:3362-6.
6.      Kumar P, Anaya J, Mudunuri SB, Dutta A. Meta-analysis of tRNA derived RNA fragments reveals that they are evolutionarily conserved and associate with AGO proteins to recognize specific RNA targets. BMC Biol 2014; 12:78.
7.      Andersen KL, Collins K. Several RNase T2 enzymes function in induced tRNA and rRNA turnover in the ciliate Tetrahymena. Mol Biol Cell 2012; 23:36-44.
8.      Schaefer M, Pollex T, Hanna K, Tuorto F, Meusburger M, Helm M, et al. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. Genes Dev 2010; 24:1590-5.
9.      Gebetsberger J, Zywicki M, Kunzi A, Polacek N. tRNA-derived fragments target the ribosome and function as regulatory non-coding RNA in Haloferax volcanii. Archaea 2012; 2012:260909.
10.     Thompson DM, Lu C, Green PJ, Parker R. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. RNA 2008; 14:2095-103.
11.     Lee YS, Shibata Y, Malhotra A, Dutta A. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). Genes Dev 2009; 23:2639-49.
12.     Tuck AC, Tollervey D. RNA in pieces. Trends Genet 2011; 27:422-32.
13.     Tomecki R, Dziembowski A. Novel endoribonucleases as central players in various pathways of eukaryotic RNA metabolism. RNA 2010; 16:1692-724.
14.     Maute RL, Schneider C, Sumazin P, Holmes A, Califano A, Basso K, et al. tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. Proc Natl Acad Sci U S A 2013; 110:1404-9.
15.     Yamasaki S, Ivanov P, Hu GF, Anderson P. Angiogenin cleaves tRNA and promotes stress-induced translational repression. J Cell Biol 2009; 185:35-42.
16.     Ivanov P, Emara MM, Villen J, Gygi SP, Anderson P. Angiogenin-induced tRNA fragments inhibit translation initiation. Mol Cell 2011; 43:613-23.
17.     Guzzi N, Ciesla M, Ngoc PCT, Lang S, Arora S, Dimitriou M, et al. Pseudouridylation of tRNA-Derived Fragments Steers Translational Control in Stem Cells. Cell 2018; 173:1204-16 e26.
18.     Blanco S, Dietmann S, Flores JV, Hussain S, Kutter C, Humphreys P, et al. Aberrant methylation of tRNAs links cellular stress to neuro-developmental disorders. EMBO J 2014; 33:2020-39.
19.     Blanco S, Bandiera R, Popis M, Hussain S, Lombard P, Aleksic J, et al. Stem cell function and stress response are controlled by protein synthesis. Nature 2016; 534:335-40.
20.     Krishna S, Yim DG, Lakshmanan V, Tirumalai V, Koh JL, Park JE, et al. Dynamic expression of tRNA-derived small RNAs define cellular states. EMBO Rep 2019; 20:e47789.
21.     Honda S, Loher P, Shigematsu M, Palazzo JP, Suzuki R, Imoto I, et al. Sex hormone-dependent tRNA halves enhance cell proliferation in breast and prostate cancers. Proc Natl Acad Sci U S A 2015; 112:E3816-25.
22.     Ruggero K, Guffanti A, Corradin A, Sharma VK, De Bellis G, Corti G, et al. Small noncoding RNAs in cells transformed by human T-cell leukemia virus type 1: a role for a tRNA fragment as a primer for reverse transcriptase. J Virol 2014; 88:3612-22.

23.      Sharma U, Conine CC, Shea JM, Boskovic A, Derr AG, Bing XY, et al. Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. Science 2016; 351:391-6.

24.      van Hoof A, Wagner EJ. A brief survey of mRNA surveillance. Trends Biochem Sci 2011; 36:585-92.

25.      Kuscu C, Kumar P, Kiran M, Su Z, Malik A, Dutta A. tRNA fragments (tRFs) guide Ago to regulate gene expression post-transcriptionally in a Dicer-independent manner. RNA 2018; 24:1093-105.

26.      Taft RJ, Glazov EA, Lassmann T, Hayashizaki Y, Carninci P, Mattick JS. Small RNAs derived from snoRNAs. RNA 2009; 15:1233-40.

27.      Goodarzi H, Liu X, Nguyen HC, Zhang S, Fish L, Tavazoie SF. Endogenous tRNA-Derived Fragments Suppress Breast Cancer Progression via YBX1 Displacement. Cell 2015; 161:790-802.

28.      Telonis AG, Loher P, Honda S, Jing Y, Palazzo J, Kirino Y, et al. Dissecting tRNA-derived fragment complexities using personalized transcriptomes reveals novel fragment classes and unexpected dependencies. Oncotarget 2015; 6:24797-822.

29.      Saikia M, Krokowski D, Guan BJ, Ivanov P, Parisien M, Hu GF, et al. Genome-wide identification and quantitative analysis of cleaved tRNA fragments induced by cellular stress. J Biol Chem 2012; 287:42708-25.

30.      Emara MM, Ivanov P, Hickman T, Dawra N, Tisdale S, Kedersha N, et al. Angiogenin-induced tRNA-derived stress-induced RNAs promote stress-induced stress granule assembly. J Biol Chem 2010; 285:10959-68.

31.      Fu H, Feng J, Liu Q, Sun F, Tie Y, Zhu J, et al. Stress induces tRNA cleavage by angiogenin in mammalian cells. FEBS Lett 2009; 583:437-42.

32.      Lee SR, Collins K. Starvation-induced cleavage of the tRNA anticodon loop in Tetrahymena thermophila. J Biol Chem 2005; 280:42744-9.

33.      Thompson DM, Parker R. The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in Saccharomyces cerevisiae. J Cell Biol 2009; 185:43-50.

34.      Su Z, Kuscu C, Malik A, Shibata E, Dutta A. Angiogenin generates specific stress-induced tRNA halves and is not involved in tRF-3-mediated gene silencing. J Biol Chem 2019; 294:16930-41.

35.      Donovan J, Rath S, Kolet-Mandrikov D, Korennykh A. Rapid RNase L-driven arrest of protein synthesis in the dsRNA response without degradation of translation machinery. RNA 2017; 23:1660-71.

36.      Cole C, Sobala A, Lu C, Thatcher SR, Bowman A, Brown JW, et al. Filtering of deep sequencing data reveals the existence of abundant Dicer-dependent small RNAs derived from tRNAs. RNA 2009; 15:2147-60.

37.      Haussecker D, Huang Y, Lau A, Parameswaran P, Fire AZ, Kay MA. Human tRNA-derived small RNAs in the global regulation of RNA silencing. RNA 2010; 16:673-95.

38.      Babiarz JE, Ruby JG, Wang Y, Bartel DP, Blelloch R. Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. Genes Dev 2008; 22:2773-85.

39.      Li Z, Ender C, Meister G, Moore PS, Chang Y, John B. Extensive terminal and asymmetric processing of small RNAs from rRNAs, snoRNAs, snRNAs, and tRNAs. Nucleic Acids Res 2012; 40:6787-99.

40.      Chan CT, Dyavaiah M, DeMott MS, Taghizadeh K, Dedon PC, Begley TJ. A quantitative systems approach reveals dynamic control of tRNA modifications during cellular stress. PLoS Genet 2010; 6:e1001247.

41.      Basanta-Sanchez M, Temple S, Ansari SA, D'Amico A, Agris PF. Attomole quantification and global profile of RNA modifications: Epitranscriptome of human neural stem cells. Nucleic Acids Res 2016; 44:e26.

42.      Wang X, Matuszek Z, Huang Y, Parisien M, Dai Q, Clark W, et al. Queuosine modification protects cognate tRNAs against ribonuclease cleavage. RNA 2018; 24:1305-13.

43.		Vitali P, Kiss T. Cooperative 2'-O-methylation of the wobble cytidine of human elongator tRNA(Met)(CAT) by a nucleolar and a Cajal body-specific box C/D RNP. Genes Dev 2019; 33:741-6.

44.		Tuorto F, Herbst F, Alerasool N, Bender S, Popp O, Federico G, et al. The tRNA methyltransferase Dnmt2 is required for accurate polypeptide synthesis during haematopoiesis. EMBO J 2015.

45.		Gkatza NA, Castro C, Harvey RF, Heiss M, Popis MC, Blanco S, et al. Cytosine-5 RNA methylation links protein synthesis to cell metabolism. PLoS Biol 2019; 17:e3000297.

46.		Flores JV, Cordero-Espinoza L, Oeztuerk-Winder F, Andersson-Rolf A, Selmi T, Blanco S, et al. Cytosine-5 RNA Methylation Regulates Neural Stem Cell Differentiation and Motility. Stem Cell Reports 2017; 8:112-24.

47.		Blanco S, Kurowski A, Nichols J, Watt FM, Benitah SA, Frye M. The RNA-methyltransferase Misu (NSun2) poises epidermal stem cells to differentiate. PLoS Genet 2011; 7:e1002403.

48.		Martinez FJ, Lee JH, Lee JE, Blanco S, Nickerson E, Gabriel S, et al. Whole exome sequencing identifies a splicing mutation in NSUN2 as a cause of a Dubowitz-like syndrome. J Med Genet 2012; 49:380-5.

49.		Cosentino C, Toivonen S, Diaz Villamil E, Atta M, Ravanat JL, Demine S, et al. Pancreatic beta-cell tRNA hypomethylation and fragmentation link TRMT10A deficiency with diabetes. Nucleic Acids Res 2018; 46:10302-18.

50.		Igoillo-Esteve M, Genin A, Lambert N, Desir J, Pirson I, Abdulkarim B, et al. tRNA methyltransferase homolog gene TRMT10A mutation in young onset diabetes and primary microcephaly in humans. PLoS Genet 2013; 9:e1003888.

51.		Studte P, Zink S, Jablonowski D, Bar C, von der Haar T, Tuite MF, et al. tRNA and protein methylase complexes mediate zymocin toxicity in yeast. Mol Microbiol 2008; 69:1266-77.

52.		Lu J, Esberg A, Huang B, Bystrom AS. Kluyveromyces lactis gamma-toxin, a ribonuclease that recognizes the anticodon stem loop of tRNA. Nucleic Acids Res 2008; 36:1072-80.

53.		Luo S, He F, Luo J, Dou S, Wang Y, Guo A, et al. Drosophila tsRNAs preferentially suppress general translation machinery via antisense pairing and participate in cellular starvation response. Nucleic Acids Res 2018; 46:5250-68.

54.		Deng J, Ptashkin RN, Chen Y, Cheng Z, Liu G, Phan T, et al. Respiratory Syncytial Virus Utilizes a tRNA Fragment to Suppress Antiviral Responses Through a Novel Targeting Mechanism. Mol Ther 2015; 23:1622-9.

55.		McEwen E, Kedersha N, Song B, Scheuner D, Gilks N, Han A, et al. Heme-regulated inhibitor kinase-mediated phosphorylation of eukaryotic translation initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure. J Biol Chem 2005; 280:16925-33.

56.		Lyons SM, Gudanis D, Coyne SM, Gdaniec Z, Ivanov P. Identification of functional tetramolecular RNA G-quadruplexes derived from transfer RNAs. Nat Commun 2017; 8:1127.

57.		Ivanov P, O'Day E, Emara MM, Wagner G, Lieberman J, Anderson P. G-quadruplex structures contribute to the neuroprotective effects of angiogenin-induced tRNA fragments. Proc Natl Acad Sci U S A 2014; 111:18201-6.

58.		Lyons SM, Achorn C, Kedersha NL, Anderson PJ, Ivanov P. YB-1 regulates tiRNA-induced Stress Granule formation but not translational repression. Nucleic Acids Res 2016; 44:6949-60.

59.		Gebetsberger J, Wyss L, Mleczko AM, Reuther J, Polacek N. A tRNA-derived fragment competes with mRNA for ribosome binding and regulates translation during stress. RNA Biol 2017; 14:1364-73.

60.		Bakowska-Zywicka K, Kasprzyk M, Twardowski T. tRNA-derived short RNAs bind to Saccharomyces cerevisiae ribosomes in a stress-dependent manner and inhibit protein synthesis in vitro. FEMS Yeast Res 2016; 16.

61.		Sobala A, Hutvagner G. Small RNAs derived from the 5' end of tRNA can inhibit protein translation in human cells. RNA Biol 2013; 10:553-63.

62.      Keam SP, Sobala A, Ten Have S, Hutvagner G. tRNA-Derived RNA Fragments Associate with Human Multisynthetase Complex (MSC) and Modulate Ribosomal Protein Translation. J Proteome Res 2017; 16:413-20.

63.      Czech A, Wende S, Morl M, Pan T, Ignatova Z. Reversible and rapid transfer-RNA deactivation as a mechanism of translational repression in stress. PLoS Genet 2013; 9:e1003767.

64.      Kim HK, Fuchs G, Wang S, Wei W, Zhang Y, Park H, et al. A transfer-RNA-derived small RNA regulates ribosome biogenesis. Nature 2017; 552:57-62.

65.      Kim HK, Xu J, Chu K, Park H, Jang H, Li P, et al. A tRNA-Derived Small RNA Regulates Ribosomal Protein S28 Protein Levels after Translation Initiation in Humans and Mice. Cell Rep 2019; 29:3816-24 e4.

66.      Fricker R, Brogli R, Luidalepp H, Wyss L, Fasnacht M, Joss O, et al. A tRNA half modulates translation as stress response in Trypanosoma brucei. Nat Commun 2019; 10:118.

67.      Panas MD, Ivanov P, Anderson P. Mechanistic insights into mammalian stress granule dynamics. J Cell Biol 2016; 215:313-23.

68.      Davidson-Moncada J, Papavasiliou FN, Tam W. MicroRNAs of the immune system: roles in inflammation and cancer. Ann N Y Acad Sci 2010; 1183:183-94.

69.      Boss IW, Renne R. Viral miRNAs: tools for immune evasion. Curr Opin Microbiol 2010; 13:540-5.

70.      Wang Q, Lee I, Ren J, Ajay SS, Lee YS, Bao X. Identification and functional characterization of tRNA-derived RNA fragments (tRFs) in respiratory syncytial virus infection. Mol Ther 2013; 21:368-79.

71.      Deng J, Ptashkin RN, Wang Q, Liu G, Zhang G, Lee I, et al. Human metapneumovirus infection induces significant changes in small noncoding RNA expression in airway epithelial cells. Mol Ther Nucleic Acids 2014; 3:e163.

72.      Jackowiak P, Hojka-Osinska A, Philips A, Zmienko A, Budzko L, Maillard P, et al. Small RNA fragments derived from multiple RNA classes - the missing element of multi-omics characteristics of the hepatitis C virus cell culture model. BMC Genomics 2017; 18:1-21.

73.      Nolte-'t Hoen EN, Buermans HP, Waasdorp M, Stoorvogel W, Wauben MH, t Hoen PA. Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions. Nucleic Acids Res 2012; 40:9272-85.

74.      Chiou NT, Kageyama R, Ansel KM. Selective Export into Extracellular Vesicles and Function of tRNA Fragments during T Cell Activation. Cell Rep 2018; 25:3356-70 e4.

75.      Wang Z, Xiang L, Shao J, Yuan Z. The 3' CCACCA sequence of tRNAAla(UGC) is the motif that is important in inducing Th1-like immune response, and this motif can be recognized by Toll-like receptor 3. Clin Vaccine Immunol 2006; 13:733-9.

76.      Pindel A, Sadler A. The role of protein kinase R in the interferon response. J Interferon Cytokine Res 2011; 31:59-70.

77.      Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Boffelli D, Mote P, et al. 5' tRNA halves are present as abundant complexes in serum, concentrated in blood cells, and modulated by aging and calorie restriction. BMC Genomics 2013; 14:298.

78.      Meiri E, Levy A, Benjamin H, Ben-David M, Cohen L, Dov A, et al. Discovery of microRNAs and other small RNAs in solid tumors. Nucleic Acids Res 2010; 38:6234-46.

79.      Horton R, Wilming L, Rand V, Lovering RC, Bruford EA, Khodiyar VK, et al. Gene map of the extended human MHC. Nat Rev Genet 2004; 5:889-99.

80.      Olvedy M, Scaravilli M, Hoogstrate Y, Visakorpi T, Jenster G, Martens-Uzunova ES. A comprehensive repertoire of tRNA-derived fragments in prostate cancer. Oncotarget 2016; 7:24766-77.

81.      Kumar P, Mudunuri SB, Anaya J, Dutta A. tRFdb: a database for transfer RNA fragments. Nucleic Acids Res 2015; 43:D141-5.

82.     Victoria Martinez B, Dhahbi JM, Nunez Lopez YO, Lamperska K, Golusinski P, Luczewski L, et al. Circulating small non-coding RNA signature in head and neck squamous cell carcinoma. Oncotarget 2015; 6:19246-63.

83.     Dhahbi JM, Spindler SR, Atamna H, Boffelli D, Martin DI. Deep Sequencing of Serum Small RNAs Identifies Patterns of 5' tRNA Half and YRNA Fragment Expression Associated with Breast Cancer. Biomark Cancer 2014; 6:37-47.

84.     Nientiedt M, Deng M, Schmidt D, Perner S, Muller SC, Ellinger J. Identification of aberrant tRNA-halves expression patterns in clear cell renal cell carcinoma. Sci Rep 2016; 6:37158.

85.     Guo Y, Bosompem A, Mohan S, Erdogan B, Ye F, Vickers KC, et al. Transfer RNA detection by small RNA deep sequencing and disease association with myelodysplastic syndromes. BMC Genomics 2015; 16:727.

86.     Pekarsky Y, Balatti V, Palamarchuk A, Rizzotto L, Veneziano D, Nigita G, et al. Dysregulation of a family of short noncoding RNAs, tsRNAs, in human cancer. Proc Natl Acad Sci U S A 2016; 113:5071-6.

87.     Balatti V, Nigita G, Veneziano D, Drusco A, Stein GS, Messier TL, et al. tsRNA signatures in cancer. Proc Natl Acad Sci U S A 2017; 114:8071-6.

88.     Pliatsika V, Loher P, Magee R, Telonis AG, Londin E, Shigematsu M, et al. MINTbase v2.0: a comprehensive database for tRNA-derived fragments that includes nuclear and mitochondrial fragments from all The Cancer Genome Atlas projects. Nucleic Acids Res 2018; 46:D152-D9.

89.     Pliatsika V, Loher P, Telonis AG, Rigoutsos I. MINTbase: a framework for the interactive exploration of mitochondrial and nuclear tRNA fragments. Bioinformatics 2016; 32:2481-9.

90.     Yu D, Cai Y, Zhou W, Sheng J, Xu Z. The Potential of Angiogenin as a Serum Biomarker for Diseases: Systematic Review and Meta-Analysis. Dis Markers 2018; 2018:1984718.

91.     Maurya PK, Mishra A, Yadav BS, Singh S, Kumar P, Chaudhary A, et al. Role of Y Box Protein-1 in cancer: As potential biomarker and novel therapeutic target. J Cancer 2017; 8:1900-7.

92.     Martens-Uzunova ES, Jalava SE, Dits NF, van Leenders GJ, Moller S, Trapman J, et al. Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. Oncogene 2012; 31:978-91.

93.     Huang B, Yang H, Cheng X, Wang D, Fu S, Shen W, et al. tRF/miR-1280 Suppresses Stem Cell-like Cells and Metastasis in Colorectal Cancer. Cancer Res 2017; 77:3194-206.

94.     Martens-Uzunova ES, Olvedy M, Jenster G. Beyond microRNA--novel RNAs derived from small non-coding RNA and their implication in cancer. Cancer Lett 2013; 340:201-11.

95.     Balatti V, Rizzotto L, Miller C, Palamarchuk A, Fadda P, Pandolfo R, et al. TCL1 targeting miR-3676 is codeleted with tumor protein p53 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2015; 112:2169-74.

96.     Ben-Shem A, Garreau de Loubresse N, Melnikov S, Jenner L, Yusupova G, Yusupov M. The structure of the eukaryotic ribosome at 3.0 A resolution. Science 2011; 334:1524-9.

97.     Zentner GE, Saiakhova A, Manaenkov P, Adams MD, Scacheri PC. Integrative genomic analysis of human ribosomal DNA. Nucleic Acids Res 2011; 39:4949-60.

98.     Choy JY, Boon PL, Bertin N, Fullwood MJ. A resource of ribosomal RNA-depleted RNA-Seq data from different normal adult and fetal human tissues. Sci Data 2015; 2:150063.

99.     Fowler EK, Mohorianu I, Smith DT, Dalmay T, Chapman T. Small RNA populations revealed by blocking rRNA fragments in Drosophila melanogaster reproductive tissues. PLoS One 2018; 13:e0191966.

100.    Lee HC, Chang SS, Choudhary S, Aalto AP, Maiti M, Bamford DH, et al. qiRNA is a new type of small interfering RNA induced by DNA damage. Nature 2009; 459:274-7.

101.    Wei H, Zhou B, Zhang F, Tu Y, Hu Y, Zhang B, et al. Profiling and Identification of Small rDNA-Derived RNAs and Their Potential Biological Functions. PLOS ONE 2013; 8:e56842.

102.    Chen Z, Sun Y, Yang X, Wu Z, Guo K, Niu X, et al. Two featured series of rRNA-derived RNA fragments (rRFs) constitute a novel class of small RNAs. PLoS One 2017; 12:e0176458.

103.    Locati MD, Pagano JFB, Abdullah F, Ensink WA, van Olst M, van Leeuwen S, et al. Identifying small RNAs derived from maternal- and somatic-type rRNAs in zebrafish development. Genome 2018; 61:371-8.

104.    Asha S, Soniya EV. The sRNAome mining revealed existence of unique signature small RNAs derived from 5.8SrRNA from Piper nigrum and other plant lineages. Sci Rep 2017; 7:41052.

105.    Zhou X, Feng X, Mao H, Li M, Xu F, Hu K, et al. RdRP-synthesized antisense ribosomal siRNAs silence pre-rRNA via the nuclear RNAi pathway. Nat Struct Mol Biol 2017; 24:258-69.

106.    You C, He W, Hang R, Zhang C, Cao X, Guo H, et al. FIERY1 promotes microRNA accumulation by suppressing rRNA-derived small interfering RNAs in Arabidopsis. Nat Commun 2019; 10:4424.

107.    Zheng Y, Wang S, Sunkar R. Genome-Wide Discovery and Analysis of Phased Small Interfering RNAs in Chinese Sacred Lotus. PLOS ONE 2014; 9:e113790.

108.    Garcia-Lopez J, Hourcade Jde D, Alonso L, Cardenas DB, del Mazo J. Global characterization and target identification of piRNAs and endo-siRNAs in mouse gametes and zygotes. Biochim Biophys Acta 2014; 1839:463-75.

109.    Chak LL, Mohammed J, Lai EC, Tucker-Kellogg G, Okamura K. A deeply conserved, noncanonical miRNA hosted by ribosomal DNA. RNA 2015; 21:375-84.

110.    Son DJ, Kumar S, Takabe W, Kim CW, Ni CW, Alberts-Grill N, et al. The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. Nat Commun 2013; 4:3000.

111.    Zhu C, Yan Q, Weng C, Hou X, Mao H, Liu D, et al. Erroneous ribosomal RNAs promote the generation of antisense ribosomal siRNA. Proc Natl Acad Sci U S A 2018; 115:10082-7.

112.    Chen H, Kobayashi K, Miyao A, Hirochika H, Yamaoka N, Nishiguchi M. Both OsRecQ1 and OsRDR1 Are Required for the Production of Small RNA in Response to DNA-Damage in Rice. PLOS ONE 2013; 8:e55252.

113.    Elbarbary RA, Takaku H, Uchiumi N, Tamiya H, Abe M, Takahashi M, et al. Modulation of gene expression by human cytosolic tRNase Z(L) through 5'-half-tRNA. PLoS One 2009; 4:e5908.

114.    Ni CW, Qiu H, Jo H. MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells. Am J Physiol Heart Circ Physiol 2011; 300:H1762-9.

115.    Houseley J, Tollervey D. The Many Pathways of RNA Degradation. Cell 2009; 136:763-76.

116.    Pircher A, Bakowska-Zywicka K, Schneider L, Zywicki M, Polacek N. An mRNA-derived noncoding RNA targets and regulates the ribosome. Mol Cell 2014; 54:147-55.

117.    Fish L, Zhang S, Yu JX, Culbertson B, Zhou AY, Goga A, et al. Cancer cells exploit an orphan RNA to drive metastatic progression. Nat Med 2018; 24:1743-51.

118.    Schorn AJ, Gutbrod MJ, LeBlanc C, Martienssen R. LTR-Retrotransposon Control by tRNA-Derived Small RNAs. Cell 2017; 170:61-71 e11.

119.    Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, et al. Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. Science 2016; 351:397-400.

120.    Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16:203-22.

121.    Lieberman J. Tapping the RNA world for therapeutics. Nat Struct Mol Biol 2018; 25:357-64.

122.    Okamoto M, Fujiwara M, Hori M, Okada K, Yazama F, Konishi H, et al. tRNA modifying enzymes, NSUN2 and METTL1, determine sensitivity to 5-fluorouracil in HeLa cells. PLoS Genet 2014; 10:e1004639.

123.    Gustavsson M, Ronne H. Evidence that tRNA modifying enzymes are important in vivo targets for 5-fluorouracil in yeast. RNA 2008; 14:666-74.

124.	Lee H-C, Chang S-S, Choudhary S, Aalto AP, Maiti M, Bamford DH, et al. qiRNA is a new type of small interfering RNA induced by DNA damage. Nature 2009; 459:274-7.
125.	Zhou X, Feng X, Mao H, Li M, Xu F, Hu K, et al. RdRP-synthesized antisense ribosomal siRNAs silence pre-rRNA via the nuclear RNAi pathway. Nature Structural & Molecular Biology 2017; 24:258-69.
126.	You C, He W, Hang R, Zhang C, Cao X, Guo H, et al. FIERY1 promotes microRNA accumulation by suppressing rRNA-derived small interfering RNAs in Arabidopsis. Nature Communications 2019; 10:4424.
127.	García-López J, Hourcade JdD, Alonso L, Cárdenas DB, del Mazo J. Global characterization and target identification of piRNAs and endo-siRNAs in mouse gametes and zygotes. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 2014; 1839:463-75.
128.	Castellano L, Stebbing J. Deep sequencing of small RNAs identifies canonical and non-canonical miRNA and endogenous siRNAs in mammalian somatic tissues. Nucleic Acids Research 2013; 41:3339-51.
129.	Zhu C, Yan Q, Weng C, Hou X, Mao H, Liu D, et al. Erroneous ribosomal RNAs promote the generation of antisense ribosomal siRNA. Proceedings of the National Academy of Sciences 2018; 115:10082.
130.	Thompson DM, Lu C, Green PJ, Parker R. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. RNA (New York, NY) 2008; 14:2095-103.
131.	Li Z, Ender C, Meister G, Moore PS, Chang Y, John B. Extensive terminal and asymmetric processing of small RNAs from rRNAs, snoRNAs, snRNAs, and tRNAs. Nucleic Acids Research 2012; 40:6787-99.
132.	Asha S, Soniya EV. The sRNAome mining revealed existence of unique signature small RNAs derived from 5.8SrRNA from Piper nigrum and other plant lineages. Scientific Reports 2017; 7:41052.
133.	Elbarbary RA, Takaku H, Uchiumi N, Tamiya H, Abe M, Takahashi M, et al. Modulation of Gene Expression by Human Cytosolic tRNase ZL through 5'-Half-tRNA. PLOS ONE 2009; 4:e5908.
134.	Thompson DM, Parker R. The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in Saccharomyces cerevisiae. The Journal of Cell Biology 2009; 185:43-50.
135.	Son DJ, Kumar S, Takabe W, Woo Kim C, Ni C-W, Alberts-Grill N, et al. The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. Nature Communications 2013; 4:3000.
136.	Chak LL, Mohammed J, Lai EC, Tucker-Kellogg G, Okamura K. A deeply conserved, noncanonical miRNA hosted by ribosomal DNA. RNA 2015; 21:375-84.
137.	Ni C-W, Qiu H, Jo H. MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells. American Journal of Physiology-Heart and Circulatory Physiology 2011; 300:H1762-H9.

*Figure legends*

**Figure 1. Biogenesis and diversity of transfer RNA-derived fragments (tRFs). A)** Different types of tRFs are produced from pre-tRNAs or mature tRNAs. RNase Z removes the 3' trailer of pre-tRNAs and produces tRF-1 (brown). Several tRFs are produced by endonucleolytic cleavage of the mature tRNAs. 3' tRF (or tRF-3s and 5' tRFs (or tRF-5s) (purple) are produced by DICER and other unknown endonucleases (?) cleaving at the D- and T-loop of mature tRNAs. 5' and 3' tRNA halves or tiRNAs (light blue) are produced by endonucleolytic cleavage mediated by ANG and RNase L (in vertebrates), Ryn1 (yeast), and other unknown enzymes (?). **B)** The mature tRNA contains multiple modified nucleosides which regulate its cleavage into tRNA fragments. Modifications that effect tRNA cleavage are indicated in red as well as the modifying enzyme (in brackets) and the enzyme responsible for cleavage (when known) and the tRNA fragment that is produced by the modification (indicated by -->) or inhibited (indicated by –l).

**Figure 2. Mechanisms of tRFs-mediated sequence-specific gene silencing or repression of translation initiation. A)** tRF-5s and tRF-3s associate with AGO proteins and sequence-specifically silence mRNAs. Target sites are found typically at the 3' UTR, but also distributed all over the respective mRNAs. **B)** 5' tiRNAs with a 5' TOG motif form a R4G structure that is required to displace the translation initiation complex eIF4A/G/E from capped mRNAs repressing translation initiation in cells under stress or in stem cells (SCs). **C)** In human embryonic stem cells (hESCs), 5' tiRNAs with a 5' TOG and $\Psi$ at position 8 displace PABPC1 from the translation initiation complex repressing translation of capped mRNAs.

**Figure 3. tRFs facilitate viral infections. A)** 5'tRNA half biogenesis increases upon respiratory syncytial virus (RSV) infection to silence the expression of host genes (APOER2) and facilitate viral replication in epithelial cells. **B)** Hepatitis B and C virus (HV (B, C)) trigger tRNA half biogenesis which in turn facilitate viral replication via an unknown molecular mechanism in hepatocytes.

**Figure 4. Association of tRFs with cancer initiation and progression. A)** tRFs and tRNA halves can foster cancer cell proliferation. **B)** tRNA fragments mapping to the anticodon loop of tRNAs are induced upon hypoxic stress in normal mammary epithelial and breast cancer cells to sequester YB-1 from oncogenic transcripts and induce their degradation supressing cancer progression. As metastatic breast cancer cells inhibit generation of these tRNA fragments, YB-1 binds and stabilizes tumour-promoting mRNAs (lower

panel). **C)** In colon cancer cells, tRF-3s promote *JAG2* mRNA degradation inactivating the cancer promoting Notch signalling pathway.

**Figure 5. Ribosomal RNA-derived fragments. A)** Eukaryotic ribosomes are constituted by four different rRNAs: 5S, 5.8S, 18S and 28S (higher eukaryotes). Ribosome production is initiated in the nucleolus, where a single rRNA precursor (47S rRNA) containing 5.8S, 18S and 28S rRNAs and spacer regions known as external and internal transcribed spacers (EST, ITS1 and ITS2), is transcribed. **B)** rRFs derived from rRNAs and transcribed spacers in humans, flies and mice interact with AGO proteins and downregulate several mRNAs by sequence complementary. **C)** qiRNAs derived from 3' ends of 28S rRNA and spacers ITS1 and ITS2 interact with AGO protein QDE-2 to contribute to mRNA degradation in *N. Crassa*. **D)** risiRNAs derive from 28S and 18S rRNA and act through the RNAi pathway by association with the AGO protein NRDE-3 to downregulate pre-rRNA in *C. elegans*. **E)** Some 28S rRNA-related fragments may function as small guide RNAs (sgRNA) to guide the endonuclease tRNase $Z^L$ in human cells to downregulate the levels of specific mRNAs. **F)** rRFs derived from the 3' end of 28S rRNA can reverse complement a mature 28S rRNA molecule and induce its degradation. **G)** Mature 5.8S and 28S rRNAs interact in 3 regions by sequence complementarity. The cleavage of 5.8S rRNA in halves leads to the formation of strong stem loop structures between 28S rRNA and 5.8S rRNA halves preventing the interaction with 5.8S mature rRNA and thus participating in rRNA separation during ribosome degradation.

**Table 1.** *Definition of several features of the  most common tRNA-derived fragments*

| Name and type | Length (nt) | Domain of origin | Endonucleases | Known Biological Functions |
|---|---|---|---|---|
| tRNA fragment 1 (tRF-1) | 13-30 | Generated from pre-tRNA processing of trailer sequence | RNase Z (ELAC2) | • Gene silencing not sequence-dependent<br>• Inhibition of T cell activation<br>• Cancer biomarker<br>• Associated to cancer development<br>• Tumor suppression in response to oncogenic stress |
| tRNA fragment 3 (tRF-3) | 13-30 | Generated from mature tRNA by endonucleolytic 3'-end cleavage in the TΨC loop | • DICER<br>• No identified endonucleases responsible of TΨC loop cleavage | • Gene and retrotransposon silencing<br>• Ribosome biogenesis<br>• Translation inhibition<br>• Enhance specific transcripts related to cancer<br>• Associated to cancer development and lymphoma progression<br>• Inhibition of T cell activation<br>• Increase cell viability<br>• Tumour suppression in response to oncogenic stress |
| tRNA fragment 5 (tRF-5) | 13-30 | Generated from mature tRNA by endonucleolytic 5'-end cleavage close to the D loop | • DICER<br>• No identified endonucleases responsible of D loop cleavage | • Gene silencing<br>• miRNA-like target inhibition<br>• Translation inhibition<br>• Enhance virus replication<br>• Associated to cancer development and lymphoma progression<br>• Inhibition of T cell activation<br>• May act as immune signalling molecule<br>• Tumour suppression in response to oncogenic stress |
| 3' tRNA halves | 30-40 | Generated from mature tRNA by endonucleolytic cleavage in the anticodon loop. Start from the anticodon loop and | Angiogenin and Rny1 | • Translation inhibition<br>• Stress-induced granule formation<br>• Enhance virus replication<br>• Tumor suppression in response to oncogenic stress |

| | | | | |
|---|---|---|---|---|
| | | terminate in the 3'-end of mature tRNA | | |
| **5' tRNA halves** | 30-40 | Generated from mature tRNA by endonucleolytic cleavage in the anticodon loop. Start from the 5'-end and terminate in the anticodon loop of mature tRNA | Angiogenin and Rny1 | <ul><li>Translation inhibition</li><li>Enhance transcripts involved in stress response</li><li>Stress-induced granule formation</li><li>Associated to cell motility decrement</li><li>Associated to cancer progression</li></ul> |

**Table 2.** *Definition of several features of the most common rRNA-related fragments*

| Name and type | Origin | Length (nt) | Strand | Function | Biogenesis | Organism | Reference |
|---|---|---|---|---|---|---|---|
| qiRNA | 28S, ETS and ITS | 20-21 | Both strands | RNA-mediated gene silencing (quelling) by interaction with the AGO protein QDE-2 in fungus | QDE-1, QDE-3 and DICER-dependent in fungus. OsRecQ1, OsRDR1 and DICER-dependent in plants | Filamentous fungus and plants | [112, 124] |
| risiRNA | 18S and 26S | 22 | Antisense | pre-rRNA downregulation by interaction with que AGO protein NRDE-3 | RdRP-dependent, DICER-independent | *C. elegans* | [125] |
| risiRNA | rDNA, ETS and ITS | 21-22 | Both strands | Gene silencing by interaction with AGO1 complexes | XRN2, XRN3, RDR1/6, DCL2/4 -dependent | Plants | [126] |
| phasiRNA | 28S and 5.8S rRNA | 21 or 24 | Sense | Gene silencing by interaction with AGO proteins | AGO, RDR6, SGS3 and DCL4-dependent | Plants | [107] |
| Endo-siRNAs | 5.8S | 18-24 | Both strands | Gene silencing by interaction with AGO2 complexes | DICER-dependent | Mouse | [127-129] |
| 28S rRFs | 28S | Multiple lengths. Peak at 21 nt in humans and 80 nt in zebrafish | Mainly sense orientation | Co-immunoprecipitated with AGO complexes in human, plants and *Drosophila.* co-immunoprecipitated with tRNase Z$^L$ in human Predicted to participate in 28S rRNA degradation in zebrafish | Not reported | Human, mouse, Drosophila, yeast, thick, zebrafish and plants | [101-103, 130-133] |
| 18S rRFs | 18S | Multiple lengths | Mainly sense orientation | Predicted to participate in gene silencing by interaction with AGO complex in zebrafish | Not reported | Human, mouse, Drosophila, | [101, 103, 132] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 21 nt peak (maternal-type rRNA) and 130 nt (Somatic-type rRNA) in zebrafish | | Function not reported in other organisms | | yeast, thick and plants | |
| 5.8S rRFs | 5.8S | Multiple lengths | Mainly sense orientation | Co-immunoprecipitated with AGO complexes in plants Function not reported in other organisms | Not reported | Human, mouse, Drosophila, yeast, thick, zebrafish and plants | 101, 103, 132 |
| 5.8S halves | 5.8S | 75-76 and 83 nt (maternal-type rRNA). 74 and 81 nt (Somatic-type rRNA) | Sense | Predicted to participate in 28S and 5.8S separation | Endonuclease not known | Zebrafish | 103 |
| 25S rRFs | 25S | Multiple lengths | Not reported | Not reported | Rny1p- and other endonucleases-dependent | Yeast | 130, 134 |
| mmu-miR-5102 | 28S | 24 | Both strands | Gene silencing by interaction with AGO proteins | DICER-independent | Mouse | 128 |
| mmu-miR-5105 | 28S | 20 | Antisense | Gene silencing by interaction with AGO proteins | DICER-independent | Mouse | 128 |
| mmu-miR-5109 | 28S | 23 | Antisense | Gene silencing by interaction with AGO proteins | DICER-independent | Mouse | 128 |
| mmu-miR 5115 | 28S | 19 | Sense | Gene silencing by interaction with AGO proteins | DICER-independent | Mouse | 128 |

| mmu-miR-712 | ITS2 | 21 nt | Not reported | Gene silencing by a mechanism not reported. Plays a role in inflammation and atherosclerosis | XRN1 and DICER-dependent | Mouse | [135] |
|---|---|---|---|---|---|---|---|
| mir-10404/mir-ITS1 | ITS1 | 22 nt | Sense | Gene silencing by interaction with AGO1 complexes | DICER-dependent, DROSHA-independent | Drosophila | [136] |
| has-miR-663 | ITS1 | 22 nt | Antisense | Gene silencing by interaction with AGO proteins | Not reported | Human | [137] |

Figure 1

Figure 2

a

RISC comlpex

AGO1-4

tRFs

mRNA

mRNA silencing

mRNA degradation

b

5'tiRNA-TOGs

R4G

AAAAAAAA

eIF4A/G/E

40S

Active translation

AAAAAAAA

eIF4A/G/E

40S

Repressed translation in
stressed cells or SCs

c

5'mTOGψ8

AAAAAAAA

PABPC1

eIF4A/G/E

40S

Active translation

PABPC1

AAAAAAAA

eIF4A/G/E

40S

Repressed translation in hESCs

**Figure 3**

a

APOER2

5'tRNA halves

*APOER2 mRNA silencing*

RSV

Host epithelial cell

APOER2

b

HV(B,C)

tRNA halves

*Unknown mechanism*

Host liver cell

Figure 4

Figure 5