tRNA derived fragments (tRFs): origins, processing and functions.

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Key words: tRNA, small RNA, tRF, tRNA fragment, Dicer, Argonaute

Running title: small RNAs from tRNAs

Abstract

Deep sequencing approaches have revealed multiple types of small RNAs with known and unknown functions. In this review we focus on recently identified group of small RNAs that are derived from tRNAs, tRNA fragments (tRFs). We review the mechanism of their processing and their functions in mammalian cells, and highlight points of possible cross-talk between tRFs and the canonical small RNA pathway characterized by siRNAs, miRNAs and piRNAs. We also propose a nomenclature that is based on their processing characteristics.

Introduction

Gene expression studies have gone through a clear paradigm shift. The revolutionary advances in gene expression profiling techniques, such as the development of new generation sequencing, have revealed additional layers of gene regulatory mechanisms that are mediated by varieties of non-coding RNAs. By now it is clear that eukaryotic genomes produce a similar amount of non-coding RNAs as mRNA that can be translated into proteins. Non-coding RNAs (apart from those such as rRNAs, tRNA, snRNAs and snoRNAs) that are smaller than 200 nucleotides are categorized as small RNAs; however, this does not reflect similarities in their functions. Indeed, it defines a group of RNAs that have a wide range of both verified and suggested roles in gene regulation.

The still-expanding realm of small regulatory RNAs

The first non-structural regulatory small RNA was discovered in 1993. It was *lin-4,* a microRNA (miRNA, originally temporal RNA) identified in *C. elegans* using a genetic screen [1]. In the last two decades immense numbers of miRNAs have been identified in almost all eukaryotic organisms (the miRBase depository, version 16.0, contains more than 17000 entries) and it is now obvious that they have pivotal roles in regulating gene expression in almost all biological processes. Studies examining the mechanism of RNA interference led to

the discovery of the second class of small regulatory RNAs, called small interfering RNAs (siRNAs) [2]. Originally discovered as a small RNA species from external origins (transgenes, viruses), it is now clear that plants and animals generate a variety of siRNAs from endogenous sources to regulate different steps of gene expression (reviewed in reference 3). Another class of small RNAs was discovered in *D. melanogaster* and mouse germlines that seems to be responsible for germline integrity and fertility (piRNAs) [4 5 6]. The functions and biogenesis of these three small regulatory RNAs are usually reviewed together. The reason for this is that it is very likely that the machines that process and confer function to these three types of small regulatory RNAs have similar evolutionary origins. For instance, conserved RNase III enzymes are involved in the processing of miRNAs and siRNAs in every eukaryotic organism in which they have been described. But the key connection between all these small RNAs is that they are associated with a member of the Argonaute (Ago) protein family (with only one known exception) [7], which is required for their functionality and some cases for their processing.

The development of revolutionary deep sequencing technologies to identify small RNAs resulted in a rapid increase of catalogued miRNAs, siRNAs and piRNAs from many organisms. These approaches utilized the key structural characteristics of miRNAs and siRNAs, such as the terminal nucleotides having a 5´ monophosphate and 3´ hydroxyl as a result of RNase III cleavage. Also, the development of antibodies against the Argonaute protein family helped to sequence enriched small RNA fractions associated with these proteins and also contributed to the better understanding of the targeted transcriptome of these types of small regulatory RNAs.

The siRNA/miRNA pathway

siRNAs and miRNAs can be produced from a large variety of cellular sources of

double stranded RNA. siRNAs can be generated from sources such as overlapping sense-antisense transcription, inverted genomic repeats of transposons and natural antisense transcripts (reviewed in reference 3). miRNAs are produced from hairpin structures, that can be formed from miRNA "genes" after processing by the RNase III enzyme Drosha [8], or from intron lariats after debranching [9 10 11]. However, from this point all siRNAs and miRNAs are produced by similar mechanisms: the double stranded RNA is cleaved into 20-22 nt fragments by the RNase III enzyme Dicer [12 13], of which one strand (the "guide strand") is incorporated into an effector Argonaute protein[14 15].

Most organisms have multiple Argonaute proteins that can load siRNAs/miRNAs; there are four in humans (reviewed in reference 16). Argonaute proteins have two main roles: some have endonuclease ("slicer") activity and can catalyze the sequence-specific cleavage of complementary targets. Of the human proteins, Ago2 and Ago3 have conserved catalytic residues but only Ago2 has been shown to have slicer activity. Argonaute proteins without slicer activity act as sequence specific scaffolds, whereby they exert their effects by recruiting other proteins to targeted mRNA to cause responses such as translational inhibition, mRNA degradation and transcriptional silencing (reviewed in reference 17).

Non-coding RNAs can be processed and enter the miRNA/siRNA pathway

Recent studies showed that abundant non-coding RNAs with known functions can be processed by enzymes that generate miRNAs/siRNAs and/or can be incorporated into the effector Argonaute protein. snoRNAs were the first example demonstrated to be associated with the miRNA pathway. So far, one snoRNA has been shown to produce a small RNA that functions as a canonical miRNA and regulates endogenous gene expression [18]. However, the relationship between snoRNAs and miRNAs may be more general since many other small RNAs derived from snoRNA have been identified, their production dependent on the miRNA

machinery, and are awaiting the identification of cellular targets [19]. Also, evolutionary connections between miRNAs and snoRNA have been proposed based on the suggestion that the structures of many pri-miRNAs also contain well-defined snoRNA elements [20].

rRNAs also can be associated with the miRNA/siRNA pathways, mainly in stressed cells. In *N. crassa*, UV damage initiates the generation of small RNAs derived from rRNAs (qiRNAs) that are processed by the siRNA machinery and loaded into QDE-2, an Argonaute homologue [21]. These RNAs interfere with the ribosome and decrease translation as a response to UV damage. In *S. pombe* small RNAs generated from rRNAs are loaded to Ago1, without processing by the normal siRNA machinery, when the TRAMP/exosome complex is compromised [22].

tRNAs

A properly folded tRNA has three stem loops: the D loop, anticodon loop, and T loop (Figure 1). tRNAs go through several stages of maturation before becoming competent for translation (reviewed in reference 23). The order of these events is not well defined, and likely varies between organisms and tRNA isotypes. A primary transcript (pre-tRNA) is spliced if necessary, and the 5' and 3' ends are trimmed by the endonucleases RNase P and RNase Z respectively [24 25]. The 3' 'CCA' acceptor stem must be added as this is not encoded in eukaryotes [26]. Also, the tRNA undergoes many modification events to generate non-canonical bases. Modification is involved in ensuring the correct secondary and tertiary structures, and modifications have been variously described to have structural functions in both loosening secondary structure where extensive base-pairing would otherwise occur, in order to ensure cloverleaf formation, and in forming tertiary interactions. Overall, correctly modified tRNAs show lower conformational flexibility and higher thermal stability than their unmodified forms (reviewed in reference 27). Finally, tRNAs undergo aminoacylation and

are exported from the nucleus to become a competent substrate for translation [28].

Whilst full-length tRNA has a well-defined role in protein translation, multiple groups have cloned and sequenced shorter tRNA fragments (reviewed in reference 29). The fragments identified so far can be classified into those that were generated by cleavage at the anticodon loop to produce longer RNA species of around 35 nt, and those that are around 20 nt (often corresponding to a cleavage in the D or T loops), which is conspicuously similar to the size of siRNAs and miRNAs. Of the former category, both 5' and 3' halves have been cloned. Of the latter category, three types have been cloned: those from the 5' end of tRNAs, those from the 3' end of mature tRNAs, and those from the 3' end of pre-tRNAs (Figure 1).

tRNA halves

Accumulation of RNAs of 30-35 nucleotides that correspond to tRNA halves were first reported in *Tetrahymena thermophila* [30], and subsequently shown to be a conserved response to oxidative stress in a wide variety of eukaryotes [31]. The nuclease responsible for tRNA half formation was discovered to be angiogenin [32]. Fascinatingly, a number of tRNAs (including $tRNA^{Asp(GTC)}$, $tRNA^{Val(AAC)}$ and $tRNA^{Gly(GCC)}$) can be methylated by Dnmt2 and this protects these tRNAs from cleavage in stress conditions [33]. This layer of specificity to which tRNAs are cleaved may cause different tRNAs to be cleaved in different situations, raising the possibility that the spectrum of cleaved tRNA fragments can contain information.

What is the biological effect of half-tRNA fragments? The Anderson group report that isolated 5' half-tRNA fragments can cause eIF2α-independent translational repression when transfected into cells [34]; however, these experiments were done with size-fractionated whole cell RNA and may contain species that are not tRNA-derived. They also reported that 5' half-tRNA fragments cause stress granule formation [35] and this result was obtained both with size-fractionated RNA and synthetic oligos. Half-tRNAs have also been shown to accumulate in

cytoplasmic granules in trypanosomes [36].

When angiogenin cleaves tRNA, it is likely that the immediate *in vivo* product is a base paired tRNA-like molecule with a nick in the anticodon loop. Some interesting experiments have been done in plants that show that nicked tRNA (produced *in vitro* by incubation with low concentrations of RNase A) inhibits translation in wheat germ extract, but tRNA fragments (produced by denaturation after RNase A treatment but before adding to the in vitro reaction) do not [37]. The fact that the Anderson group did their experiments with transfected single strands, and that they only saw an effect with 5' halves and not 3' halves, suggests that half-tRNAs can also have a biological effect when separated from their cognate partner.

Small tRNA fragments (tRFs)

A number of reports have been published in the last few years detailing the sequencing and analysis of tRNA-derived small RNAs of approximately 20 nt in size, and these are summarised in Table 1. The small tRNA-derived molecules reported lend themselves to a natural classification scheme, which is based on what part of tRNA or pre-tRNA from which they are derived (see also Figure 1).

The first class of short tRNA fragment (tRF) are derived from the 5' end of the molecule and are formed by a cleavage in the D loop. These have been reported in mammalian cells, plants and fission yeast [22 38 39 40 41]. The second class of tRFs are those derived from the 3' end of a mature tRNA by cleavage in the T loop [38 42 43 44 45]. As mature tRNA end in the acceptor trinucleotide CCA, these RNAs also characteristically end CCA. The final class of tRFs are those derived from the 3' end of a pre-tRNA [38 44 46 47]. In most cases these begin directly after the 3' terminus of the mature tRNA, and end in a series of U residues that are residual from RNA polymerase III transcription run-off.

The nomenclature used to refer to different types of tRF varies in the literature. We propose, and will use in this review, a scheme based on the part of the molecule from which they are derived: those from the 5' end of a tRNA are 5' tRFs, those from the 3' end of a mature tRNA are 3' CCA tRFs (as they end -CCA), and those from the 3' end of a pre-tRNA are 3' U tRFs (as they end in a series of uridine residues) (Figure 1). This scheme is sufficiently descriptive to be expanded if necessary. 3' CCA tRFs were previously called Type I and 3' U tRFs were previously called Type II[44].

Table 1. Instances of tRFs reported in the literature

Reference	**Cell line/organism**	**Length**	**tRF type**	**Nuclease**
42	HepG2 (liver carcinoma)	22 nt	3' CCA	Not investigated
22	*S. pombe*	23 nt	5'	Dicer independent
46	Mouse ES	21 nt	3' U	Dicer
43	HIV-1 infected MT4 cells	18 nt	3' CCA	Dicer
38	LNCaP and C4-2 (prostate carcinoma)	18 – 22 nt	5'	Not investigated
			3' CCA	
			3' U	RNase Z
39	HeLa	19 nt	5'	Dicer
40	Phosphate starved *A. thaliana* roots	19 nt	5'	Not investigated
47	5-8F	19 nt	3' U	Not

	(nasopharyngeal carcinoma)			investigated
44	HEK293 and HCT116	20 – 22 nt	3’ CCA	Dicer
			3’ U	RNase Z
45	*Tetrahymena*	~ 23 nt	3’ CCA	Not investigated

In some studies the nuclease involved in producing tRFs was investigated. The production of 5’ tRFs is reported as Dicer dependent in mammals, although not in yeast; differences in length support the conclusion that different biogenesis mechanisms are at work (in mammals, the tRFs are 19 nt long, whereas in yeast the one that has been reported is 23 nt long) [22] [39]. 3’ CCA tRFs have also been reported to be generated by Dicer [43] [44]. In *Tetrahymena*, although the nuclease identity was not proven, the 3’ CCA tRFs have 5’ monophosphates that are characteristic of Dicer processing, and this contrasts with the 5’ hydroxyls found on longer half-tRNAs produced by starvation-induced cleavage [45]. 3’ U tRFs are normally Dicer-independent and produced by RNase Z cleavage of a pre-tRNA transcript [38] [44], although one 3’ U tRF has been reported that is produced when RNase Z does not act on the transcript, but instead the pre-tRNA is predicted to fold into a bulged hairpin and a 3’ U tRF is produced by a Dicer dependent mechanism [46].

The tRNA source of tRFs

tRNAs could be diverted from their normal processing pathway at a number of points and undergo cleavage to form a tRF (Figure 2). Clearly 3’ U tRFs are formed from the pre-tRNA as they contain the polyuridine tract left over from transcription, but it has been pointed out that as 3’ U tRFs (and, indeed, all described tRFs) are almost exclusively

cytoplasmic they may be formed by a cytoplasmic pool of RNase Z[38 44 48]. Otherwise, they may undergo rapid nuclear export after formation.

On the other hand, 3' CCA tRFs must be generated from tRNA after CCA addition and 5' tRFs may be generated at any stage after 5' end formation by RNase P. In one case, 5' tRFs were identified to be produced after the base-pairing of $tRNA^{Lys}$ with the HIV-1 primer binding site (PBS), raising the possibility that, at least in certain cases, tRFs may be produced when a tRNA basepairs with another RNA molecule [43]. However, this is probably an exceptional case caused by the involvement of $tRNA^{Lys}$ in making double stranded RNA in HIV replication; it is likely that in most cases tRFs are produced by the cleavage of a single tRNA molecule.

As one tRF has been reported to be generated by Dicer from a potentially misfolded pre-tRNA substrate, where the pre-tRNA effectively folds into a hairpin [46], it is intriguing to speculate if this is a general mechanism. In this case, incorrectly formed tRNAs would be cleaved into tRFs. If this process is efficient, it would effectively destroy the aberrant tRNAs and even if inefficient, would still produce tRFs which may act as intracellular signaling molecules. These incorrectly formed tRNAs could be pre-tRNAs that undergo premature nuclear export, or hypomodified tRNAs. In both cases, they would be unlikely to fold into a correct cloverleaf structure.

Biological function of tRFs

Do tRFs have *bona fide* biological functions, or are they merely the products of tRNA degradation or nuclease off-target effects? If they do have functions, how diverse are these functions given their diverse processing mechanisms? Several lines of evidence point towards regulated production, suggesting that they may be functional RNA species. First, the sequencing abundance of different types of tRF does not correlate with the number of parent

tRNA gene copies [39 40 42] with the exception of those found in *Tetrahymena* [45]. Second, they are all produced by cleavage at specific bases, and third, whilst tRFs corresponding to the 5' and 3' ends of tRNA have been reported, those corresponding to the middle (incorporating the anticodon loop) have not.

In one case, a tRF has been shown to target RNAs similarly to siRNAs. HIV-PBS tRF associates with Ago2, and causes RNA cleavage of complementary sequences [43]. As the HIV RNA genome contains a PBS sequence, this is an intriguing example of a mammalian cell employing RNAi and tRFs in virus defence.

Other tRFs have also been shown to be associated with Argonautes therefore they have a potential to function as siRNAs or miRNAs, although endogenous targets have not been demonstrated yet. Haussecker and colleagues[44] investigated the propensity of 3' tRFs (both types) to associate with Argonaute proteins and have an effect on luciferase reporter genes; their results were surprising. They found that both types of 3' tRF associated with Argonaute proteins, but often more effectively with Ago3 and Ago4 than Ago1 or Ago2. They found that 3' CCA tRFs had a moderate effect on reporter transgene silencing, but 3' U tRFs did not. However, upon cotransfection of a small RNA complementary to a 3' U tRF, they found that the tRF preferentially associated with Ago2 and silenced a reporter transgene, a phenomenon they termed Sense-Induced Transgene Silencing (SITS). This is in stark contrast with results normally obtained in the miRNA field where sequences complementary to miRNAs relieve repression. Haussecker and colleagues suggest that the double stranded perfect RNA helix produced when a sense strand is present causes the more efficient loading into Ago2, which is consistent with *in vitro* studies showing that Ago2's slicer activity causes more efficient loading of perfect duplexes [49].

Serendipitously, Lee and colleagues[38] and Haussecker and colleagues [44] chose to characterize the same tRF, called cand45 by Haussecker et al. and tRF-1001 by Lee et al.

Whereas Lee et al. did not look at Argonaute association of tRF-1001, they did find that its knockdown by siRNA decreased cell proliferation and re-addition increased proliferation. Hence Lee et al. found a function for 3' U tRFs in a situation where Haussecker et al. showed them to be primarily associated with Ago3 and Ago4. Could tRF association with Ago3/4 be necessary for cell proliferation? In these experiments, the level of mature and pre-tRNA does not change, suggesting a role of tRFs independent of modulating the level of their parental tRNA.

It has also been shown that 3' CCA tRFs associate with the PIWI protein Twi12 in *Tetrahymena* [45], which does not contain the residues necessary for RNA cleavage. The function of this is unclear, as is whether tRF fragments associate with PIWI proteins in mammalian cells; it is possible that some proteins, such as Twi12 and human Ago3 and Ago4, have evolved to mop up tRF fragments when they are not needed. Alternatively their association with these types of small RNA may be a clue to a function which, in the case of mammalian Ago1, 3 and 4 for instance, is not clear yet.

In contrast to 3' tRFs, 5' tRFs show only inefficient association with Argonautes [39] and do not exhibit detectable silencing effects (AS and GH, unpublished observations). Their functions remain to be fully elucidated.

Regulation of tRF production

To date, no systematic quantitative analysis of tRF abundance across different cell types has been done. Most of the studies on tRFs have been done on cancer-derived cell lines or highly proliferative ES cells. Interestingly, various researchers have noticed that the abundance of tRFs is correlated to cell proliferation; increasing media serum concentration elevates the amount of 3' and 5' tRFs [38 44] and overexpressing the tRNA transcription factor Brf1, a situation known to lead to increased cell proliferation [50], also increases tRF levels [44].

Future directions

In the almost 20 years since its inception, the small RNA field has concentrated on the siRNA, miRNA and piRNA small RNA classes. In recent years the unbiased sequencing of small RNA fractions has further widened the horizon of small RNA research revealing the existence of many novel small RNA species such as 5´-capped promoter-associated small RNAs (PASRs) [51], transcription initiation RNAs (tiRNAs) [52], transcription start site-associated RNAs (TSSa RNAs) [53], splice site RNAs (spliRNAs) [54], and short RNA products from rRNA [22], snoRNA [18] and tRNA as covered in this review. It is very unlikely that the databases of verified small non coding RNAs are complete. Bioinformatic studies still predict many more miRNAs and other small RNAs [55 56] that may so far have avoided identification due to either not being present in cells that have been subjected to deep sequencing or not been sequenced due to technical issues [57]. However, the most important task in the small regulatory RNA field is to go beyond the cataloguing of this huge arsenal of small RNAs and to associate them with functions, as is already being carried out in the case of siRNAs, miRNAs, and piRNAs.

tRFs could have a variety of functions. Where they are incorporated into Argonaute proteins, they would be expected to inhibit gene expression of specific mRNAs by either translation inhibition and mRNA destabilization, or by endonucleolytic cleavage. If this is the case, targets for these RNAs can be found by the use of bioinformatic or systemic approaches such as transcription profiling and proteomics. Alternatively, could an Argonaute protein loaded with a tRF be directed to interact with mature tRNAs directly? If so, it could conceivably affect tRNA maturation or result in Argonaute proteins being brought to the ribosome in a general, non-messenger specific way. This would be extremely interesting since Ago2 originally was described as a co-factor for eIF2 and its activity was shown to stimulate ternary complex formation [58 59 60]. In accordance with this full length mitochondrial

$tRNA^{Met}$ has been described to associate with Ago2 [61].

Where tRFs are not Argonaute-associated they may have other functions outside of the canonical small RNA pathways that still need to be elucidated. tRNAs have already been shown to have diverse functions outside translation; for example, they act anti-apoptotically by directly binding cytochrome c [62]. Possibly tRFs can act as RNA aptamers for as-yet uncharacterized ligands. A recent review insightfully points out that the very 5' and 3' ends of tRNAs that are represented as tRFs specify aminoacylation specificity in the full length molecule, and presumably coevolved with tRNA aminoacyl synthetases and the anticodon sequence. It also notes that the 5' and 3' CCA tRFs are to some degree complementary, and may form double stranded RNA *in vivo* if the local concentration is high enough [29].

Accumulation of small tRNAs and rRNAs and their association with the siRNA/miRNA machinery seems to be characteristic of highly proliferative cells and cells with aberrant cell division characteristics. Indeed, in *S. pombe* an active mechanism was identified in which rRNAs and tRFs were excluded from entering the siRNA pathway by the TRAMP complex [22]. The impairment of this complex resulted in replacing centromere associated short RNAs with tRFs and short rRNAs in Ago1 that eventually led to cell death. Does this mechanism exist in higher eukaryotes? If yes, how do cancer cells accumulate this type of RNA and overcome their potential cell toxicity? One possibility is that Argonautes other than Ago2 mop up these RNA fragments providing a buffering capacity that help to avoid cell death in organisms that encode multiple Argonaute proteins.

Accurately profiling tRFs could be technically challenging. It is known that current deep sequencing methods show some degree of sequence bias depending on the method used for library generation [63]. Additionally, any tRFs derived from mature tRNAs would be expected to be base modified. Some tRNA base modifications, such as N^1-methyl-A, significantly inhibit reverse transcriptases [64] and this would have to be addressed in any

sequencing technology used for quantification. Mapping modified nucleotides in tRFs is also essential to understand their function, as any base-pairing function will be significantly altered by the presence of altered nucleotides. It has also been shown that modifications of the terminal nucleotides of small RNAs could be characteristic of what Argonaute they are loaded onto [65] [66], so where tRFs are derived from mature tRNA sequence the different preferences of tRFs for different Argonautes may be a consequence of the possibly diverse modifications of the different types of tRFs.

Whatever the answer to these questions, it seems possible that tRFs could have very different roles to those small RNAs discovered so far. Defining their effects on gene regulation and other cellular processes will no doubt lead to a better understanding of the highly diverse, still expanding universe of small RNAs.

Acknowledgements

This work was supported by the Wellcome Trust Career Development Program, the Wellcome Trust GRE Centre, and the European FP6 consortium SIROCCO. A.S is funded by a BBSRC Doctoral Training Fund.

Figure Legends

Figure 1. Proposed nomenclature of small (less than 30nt) tRNA fragments (tRFs). 3´U tRFs are processed from pre-tRNAs and consist of the sequence between the RNAse Z cleavage site and the PolIII run-off poly(U) tract. Mature tRNAs can generate two types of tRFs: one is processed from the 5´end (5´tRFs) and one from the 3´end that contains the added CCA sequence (3´CCA tRFs).

Figure 2. Possible processing and maturation of tRFs. 3´ U tRFs could be processed from pre-tRNAs by RNase Z and promptly exported into the cytoplasm where they incorporate into Argonaute complexes. Alternatively, pre-tRNAs may go through premature rapid nuclear export and be processed by a cytoplasmic RNase. tRNA processed by RNAse P and Z with the 3' terminal CCA added may be prematurely exported into the cytoplasm and a lack of base modifications may cause them to form a Dicer accessible structure. Alternatively, mature aminoacylated tRNAs may enter the small RNA pathway via Dicer processing. Dicer could produce two types of tRFs from these tRNAs: 5´tRFs and 3´CCA tRFs. 3´ CCA tRFs can incorporate into Argonaute proteins. Open triangle: RNAse Z cleavage site, filled triangle: RNase P cleavage site. Red arrows denote possible premature nuclear export.

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