Phospho-RNA: THE HIDDEN LAYER OF TRANSCRIPTOMICS

RNA molecules bear a hydroxyl (3'-OH), a phosphate (3'-P) or a cyclic phosphate (2'-3'cP) group at their 3' end (Figure 1). 3'-P/2'-3'cP containing RNAs are generated by ribonucleases, toxin or ribozymes (*1*), but they are not mere side degradation products; rather they play a role in several biological processes, including RNA metabolism, rRNA and tRNA biogenesis (*2*), mRNA splicing, unfolding protein response and stress granules production.

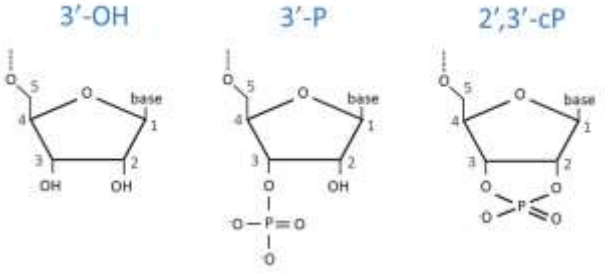

Figure 1. Chemical structure of RNA 3' end

Standard RNA-sequencing methods rely on 3'-OH ends, thus RNA molecules bearing a 3'-P/2'-3'cP are excluded or underestimated in sequencing outputs (*3*, *4*). To date, the repertoire of 3'-P/2'-3'cP containing RNAs includes mRNAs, rRNAs and snRNAs, but the best studied class is represented by specific tRNA fragments called tRNA halves (tiRNAs).

1) tRNA halves biogenesis

tRNAs are best known as adapter molecules essential for translation, but many biochemical and computational evidences have shown that tRNAs are not always final products but can further serve as a source of many other functional RNAs (*5*) . For example, upon different stress stimuli, tRNAs are cleaved by specific ribonucleases in the anticodon loop generating tRNA halves (tiRNA) (*6*). In mammalian cells, Angiogenin (ANG) is a well-known enzyme responsible for the generation of tiRNAs, which are 30- to 50-nucleotide-long fragments derived from either the 5'-(5 half) or 3'-part (3'-tRNA half) of mature tRNAs. 5'-(5 half) tRNAs have a 5' and 3' terminal phosphate.

tRNA halves can act as intracellular signalling molecules, or be released in biological fluids in complex with proteins (*7*) or inside exosomes (*8*). Since they are abundant in serum and in lymphoid organs (*7*), the question arised as to whether they are closely linked to pathological conditions or may alter the immune response. Dysregulation of tRNA halves have been shown in cancer, viral infection and Amyotrophic Lateral Sclerosis, designating them as potential biomarkers.

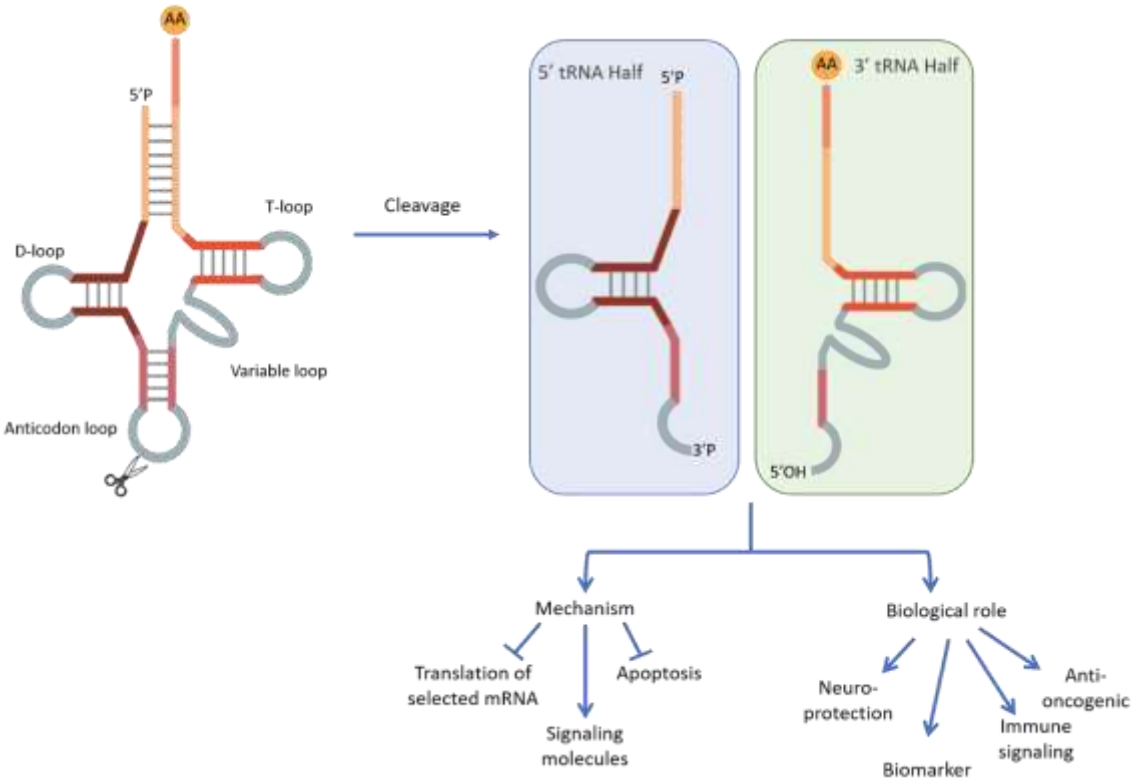

Figure 2. Production and biological roles of tRNA halves.

tiRNA halves and cancer:

In the field of cancer research and care, one of the most important medical need is the profiling of new biomarkers for early diagnosis, patient stratification and monitoring therapy.

Although tRNA halves are constitutively generated in human cells and tissues, their accumulation increases by ANG-induced cleavage. Because ANG has a key role in several kinds of cancer and its level is found to be up-regulated, it is reasonable to suppose that ANG-induced tRNA halves might be enriched in these scenarios.

Preliminary results from Honda et al. showed a correlation between cell proliferation and expression of 5'tRNA-Asp^{GUC} and 5'tRNA-His^{GUG} halves in estrogen receptor (ER)-positive breast cancer and androgen receptor (AR)-positive prostate cancer cell lines and patient tissues (*9*). They further found different expression patterns of 5'tRNA halves in several tumour cell lines (i.e. liver, pancreas, stomach, colon, esophagus, oral, lung, breast, and prostate cancers), suggesting that production of tRNA halves is specifically regulated. Further studies demonstrated that inflammatory breast cancer was associated with increases in circulating 5'tRNA-Ala in comparison to non-inflammatory breast cancer (*10*). In summary, the variable expression and function of tRNA halves are just now being elucidated. It will take some time to identify clinical applicability and determine if these small RNAs have potential as biomarkers of cancer disease.

tiRNA and viral infection:

ANG-induced tRNA halves have been also shown to implicated in the cellular response to virus infection. In particular, the first report of tiRNA induction upon infection was assessed in respiratory

Syncytial virus (RSV) (*11*). The induced cleavage of a specific tRNA subset (e.g $tRNAGlu^{CTC}$) seems to promote RSV replication through a gene-silencing mechanism, different from miRNA/siRNA mediated silencing. In animal model of LPS-induced acute inflammation, serum tRNA half showed a rapid increase during first days, suggesting an active involvement in the acute phase of body inflammation (*12*). Further, initial screening of human samples from patients under active hepatitis B virus (HBV) infection also revealed a significant upregulation of tiRNA in serum when compared with healthy donors or patients during HBV quiescent phase (*12*, *13*).

tiRNA and Neurodegenerative disorders:

The neuroprotective actions of ANG have been already extensively studied. ANG protects motor neurons against excitotoxic injury (*14*), hypoxic injury and endoplasmic reticulum stress-induced cell death. An early report by Greenway *et al.* (*15*) identified ANG missense mutations in amyotrophic lateral sclerosis (ALS) patients, leading to loss of ANG RNase activity. Later, a subset of ALS-associated ANG mutants have also been found in Parkinson disease (PD) patients (*16*). Loss of its RNase activity will bring a reduction of ANG-induced tiRNAs level. As tiRNA have been implicated in inhibition of translation (*2*), formation of stress granules (*17*), and inhibition of apoptosis (*18*), it is possible to deduce that ANG-induced tiRNAs could be essential for neuron survival during stress. In fact, even if the mechanisms for neurodegeneration in ALS are not completely understood it has been reported that motor neuron degeneration structurally resembles apoptosis (*19*). The anti-apoptotic role of tiRNAs might be a key aspect in preventing neuronal death, paving the way for their future application as therapeutic agents.

Disease	tRNA half	Regulation	Mode of action	Reference
Breast cancer	tiRNA-Asp	Up	Enhance cell proliferation	(*9*)
	tiRNA-His	Up	Enhance cell proliferation	(*9*)
	tiRNA-Ala	Up	ND	
	tiRNA-Val	Down	Inhibit the FZD3/Wnt/β-Catenin signalling pathway	(*20*)
Prostate cancer	tiRNA-Asp	Up	Enhance cell proliferation	(*9*)
	tiRNA-His	Up	Enhance cell proliferation	(*9*)
Colorectal cancer	tiRNA-Val	Up	Regulate colorectal cancer metastasis	(*21*)
HBV infection	tiRNA-Gly	Up	ND	14
	tiRNA-Glu	Up		
RSV infection	tiRNA-Glu	Up	Repress target mRNA Promote RSV replication	12

Table 1. tRNA halves in diseases. ND, not defined.

Methods for tiRNA detection:

Since there is an increasing interest in the role and mechanism of action of tiRNA, researchers have used various bioinformatics tools and molecular biology methods to analyse tRNA halves. Currently, the main technologies used are:

- single gene analysis (e.g northern blot and qRT-PCR),

- High Throughput analysis (e.g microarray analysis and small RNA-sequencing)

Single-gene analysis
Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Northern blot allow to study the expression of target tRNA halves by designing specific tiRNA amplification primers or probes. However, these methods are not the best suited for the discovery and analysis of the entire repertoire and complexity of tRNA halves.

High-throughput analysis
Standard RNA-seq methods include an adapter ligation step that requires a 3'-OH group and cannot capture tiRNA because their 3'-P group inhibits adapter ligation. However, there are two alternatives that enable the genome-wide profiling of tRNA halves:

- tiRNA qPCR Arrays combines qPCR performance with the ability of microarrays to detect the expression of many tiRNA simultaneously. Microarrays are designed to detect known tRNA halves.
- circAID is a technology that takes advantage of the ability of a ligase called RtcB to catalyze the ligation of an adaptor to 3'-P/2'-3'cP containing RNAs. Therefore, circAID is able to selectively sequence 3'-P/2'-3'cP containing RNAs and is suitable for the discovery of new tiRNA, in addition to the expression analysis of known tiRNAs. (to see correlated products click here)

However, it is important to underline that tRNA half are molecules with a high modification density, including methyhlation, 3' aminoacetylation, presence of 3' or 2'-3' phosphate group and these chemical modifications may affect their detection and quantification. tRNA pre-treatment with specific demethylases (DM-seq and ARM-seq) (*22*, *23*), or deacylation reagent could help to overcome these obstacles, while the removal of 3' phosphate could result in loss of useful information since it modification is a clear signature of ribonuclease activity (such ANG).

It is also worth to consider that base resolution information from small RNA-Seq has its own advantages for tiRNA detection over hybridization methods. For instance, small RNAseq offer a better sensitivity compared to Northern Blotting and other hybridization based methods. In fact, since tiRNA sequences are highly conserved, this might not be distinguishable by hybridization based methods, resulting in less accurate analysis.

In conclusion, phospho RNAs are a complex galaxy of under evaluated RNA molecules. IMMAGINA provides uniquely enabling technologies to break down walls on 3'P RNAseq studies.

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