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Mini-review

Beyond microRNA – Novel RNAs derived from small non-coding RNA and their implication in cancer

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ABSTRACT

Over the recent years, Next Generation Sequencing (NGS) technologies targeting the microRNA transcriptome revealed the existence of many different RNA fragments derived from small RNA species other than microRNA. Although initially discarded as RNA turnover artifacts, accumulating evidence suggests that RNA fragments derived from small nucleolar RNA (snoRNA) and transfer RNA (tRNA) are not just random degradation products but rather stable entities, which may have functional activity in the normal and malignant cell.

This review summarizes new findings describing the detection and alterations in expression of snoRNA-derived (sdRNA) and tRNA-derived (tRF) RNAs. We focus on the possible interactions of sdRNAs and tRFs with the canonical microRNA pathways in the cell and present current hypotheses on the function of these RNAs.

1. Introduction

Within less than a decade since the sequencing of the human genome it became clear that over ninety percent of our genes encode for RNA transcripts that never get translated to protein. The cellular pool of these so-called non-protein-coding RNAs (ncRNA) [1] is highly complex in terms of its diversity and function. Different structural and functional classes of ncRNA act as regulators of key cellular processes, many of which are associated with cancer.

Within the diverse group of ncRNA, small non-coding RNA and in particular microRNAs (miRNAs) have been the focus of much attention over the recent years because of their function as post-transcriptional modulators of gene expression during development and disease. The first small regulatory miRNA, *lin-4*, originally called small temporal RNA, was discovered in *Caenorhabditis elegans* in 1993 [2]. The development of next generation (deep) sequencing (NGS) technologies advanced the discovery of over 1900 distinct mature miRNAs originating from more than 1520 miRNA gene loci in human only (miRBase, Release 18, http://www.mirbase.org/, November 2011 and [3]). It is well established now that miRNAs have essential regulatory roles in cell proliferation, differentiation, apoptosis, and metabolism; and that alterations in miRNA function are involved in the pathogenesis of cancer and many other human diseases [4,5].

Alongside with miRNA, other types of small regulatory ncRNAs like exogenous and endogenous small interfering RNAs (siRNAs and endo-siRNAs) [6–8] and PiWi-interacting RNAs (piRNAs) [9] are also involved in gene regulation and genome defense and share components of the cellular pathways of RNA interference (RNAi) [10]. The cellular circuits by which small ncRNAs can control genetic programming are highly versatile and expand beyond post-transcriptional gene control. SiRNAs, miRNAs and their effector proteins can also guide processes like transcriptional gene silencing (TGS) [11–14] or activation (RNA activation, RNAa) [15–17] and even alternative splicing [18]. Many of these processes intertwine with nuclear epigenetic pathways that control DNA methylation status, histone modification, and chromatin remodeling [13,17,19].

While there is still a lot to learn about the versatility of pathways and the exact molecular mechanisms by which siRNAs and miRNAs perform their regulatory functions, recent reports demonstrate that other, long-known, small ncRNAs such as small nucleolar RNAs (snoRNA) and transfer RNAs (tRNA) expand beyond their canonical pathways and give rise to even smaller RNA species that operate in ways different from their precursor.

2. Types, general characteristics, biogenesis, and function of small non-coding RNA (ncRNA)

Within the diverse group of ncRNAs, the term small non-coding RNA is given to a rather diverse group of ncRNA products with a length of less than 400 nucleotides (nt) (Table 1).

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2.1. miRNA

MiRNAs are single stranded RNA molecules of ~22 nt that function as guide molecules for proteins of the AGO clade of the Argonaute (Ago) family during post-transcriptional gene regulation by the miRNA-induced silencing complex (miRISC). MiRNAs guide the miRISC by base-pairing of the short (7–8 nt) miRNA "seed" region with partially complementary target sites of messenger RNAs (mRNA), most commonly located in the 3′-untranslated region (UTR) [20].

Most miRNA are transcribed by RNA polymerase II (RNA Pol II) from either clustered miRNA genomic loci of several kilobases (kb), or co-transcriptionally from introns of other genes, generating hairpin-structured primary precursor transcription units (pri-miRNAs) [21] (Fig. 1). In the classical miRNA pathway, pri-miRNA are cropped at the hairpin stem to ~70 nt long precursor hairpins (pre-miRNAs) by the nuclear RNase III-type protein Drosha [6] assisted by the DiGeorge syndrome critical region gene 8 protein (DGCR8) [22]. Pre-miRNAs that originate from introns (mirtrons) are produced in Drosha/DGCR8 independent way by intron splicing and debranching, or via a splicing-independent, Drosha-dependent manner (simtrons) [23]. After nuclear processing, pre-miRNAs are exported to the cytoplasm by the nuclear transport receptor Exportin 5 (EXP5, XPO5) [24] and cleaved close to the terminal loop by the RNase III-type nuclease Dicer [25], releasing a ~22 nt long double stranded RNA duplex. One of the miRNA strands (referred to as a guide or major strand (miRNA), is loaded onto an AGO protein to generate the miRISC complex, while the other strand (passenger or minor strand, miRNA*) is degraded [21]. MiRNA strand selection and loading are determined by the relative thermodynamic strand-end stabilities of the miRNA duplex. The designated guide strand is usually characterized by relatively lower stability at its 5′-end caused by weaker base pairing (e.g. GU vs. GC pair or a mismatch) [26,27]. In some cases, passenger strands can also be loaded into RISC to function as miRNAs [28]. In mammals, AGO2, the only one of the four AGO proteins with preserved RNaseH-like endonuclease activity, can support Dicer processing by cleaving the 3′-arm of some pre-miRNAs [29]. The catalytically active AGO2 can also process the unusually short pre-miR-451 hairpin into mature miRNA independently of Dicer. In this case, AGO2 performs the endonucleolytic slicing of the 3′-arm of the 17 nt long pre-miRNA stem. This is followed by uridylation and exonucleolytic trimming of the 3′-end by unknown nuclease to finally produce the mature miR-451 [30–32].

Several lines of evidence over the last years revealed that miRNAs do not operate exclusively in the cytoplasm. Mature miRNAs are also detected in the nucleus where they function in the processes of transcriptional gene regulation similarly to synthetic double stranded RNAs (reviewed in [16,17]). A prerequisite for these processes is the import of activated miRNA/protein complexes to the nucleus. It has been suggested that Exportin 1 (XPO1, CRM1) and Importin 8 (IPO8) are involved in the nuclear-cytoplasmic shuttling of cytoplasmically processed miRNAs complexed with AGO proteins [33,34]. In the nucleus, miRNAs can serve as TGS triggers by directing the association of AGO proteins with transcriptional repressors from the Polycomb Group, leading to the formation of silent-state heterochromatin at the promoter regions of target genes. MiRNA-directed TGS was described for the first time for miR-320 and the DNA-directed RNA polymerase III polypeptide D gene (POLR3D) [35]. Similar mechanisms were demonstrated also for miR-10a in human breast cancer cells [36], and for miR-223 in hematopoietic progenitor cells during cell fate decision (reviewed in [13]). Recently, a cellular senescence model based on miRNA-directed TGS was also proposed. In this model, let-7 in complex with AGO2 recognizes promoter-associated RNAs and cooperates with E2F/RB1 to induce or stabilize the repressed chromatin state of target genes via the recruitment of histone modifying enzymes [37].

Besides TGS, miRNAs are also involved in the process of RNAa, where they function as endogenous signals for transcriptional activation. miRNA-directed RNAa was reported for miR-373 that activates E-Cadherin (CDH1) in prostate cancer cells by associating

Table 1
Classes of small ncRNAs.

Class ncRNA	Common abbreviation	Approved Symbol (HUGO[a])	Size in nucleotides	Function
MicroRNA	miRNA	MIR	19–25	mRNA degradation and inhibition of mRNA translation
Transfer RNA	tRNA		~73	Transport specific amino acids to the ribosomes during protein translation
Spliceosomal RNA		RNU	100–188	Guide the formation of the major spliceosome; intron removal from mRNA
Small nucleolar RNA	snoRNA		60–300	Guide the site-specific post-transcriptional modifications to rRNAs, tRNAs and RNU
– H/ACA box		SNORA		
– CD box		SNORD		
– Small Cajal body RNA	scaRNA	SCARNA		
Endogenous small interfering RNA	endo-siRNA		20–25	RNA interference; post-transcriptional gene silencing [8]
PiWi interacting RNA	piRNA	PIRC	25–33	Transposon silencing in germ line cells [9]
U7 small nuclear RNA	U7	RNU7	63	3′-end maturation of histone pre-mRNA [146]
7SK RNA	7SK	RN7SK	332	Regulates activity of positive transcription elongation factor b (P-TEFb) [147]
7SL RNA	SRP 7SL	RN7SL	299	RNA component of the signal recognition particle (SRP) RNP that directs protein traffic and secretion; co-translational direction of secretory proteins to the ER [148]
Small ILF3/NF90-associated RNA		SNAR	~117	Unknown function; binds interleukin enhancer binding factor ILF3/NF90. May have role in cell growth and translational control [149]
Ro-associated Y RNA	Y RNA	RNY	84–185	Components of the Ro RNP; Proposed involvement formation of active chromosomal DNA replication forks *in vitro* [150]
Vault RNA		VTRNA	~100	Components of the valute RNP. Unknown function; possibly involved in multidrug resistance [151]
Ribonuclease P RNA component H1		RPPH1	341	Processing of tRNA precursors by cleaving the trailer sequence from the 5′-end [152]
RNA component of RNase MRP		RMRP	277	Maturation of precursor rRNAs by the splicing of the internal transcribed spacer; mitochondrial DNA replication [153]
Telomerase RNA	hTR	TERC	451	RNA component of human telomerase that serves as a template for synthesis of the telomere repeat 'TTAGGG' [154]

[a] HUGO Gene Nomenclature Committee (HGNC) (http://www.genenames.org/search); RNP, ribonucleoprotein particle; ER, endoplasmic reticulum.

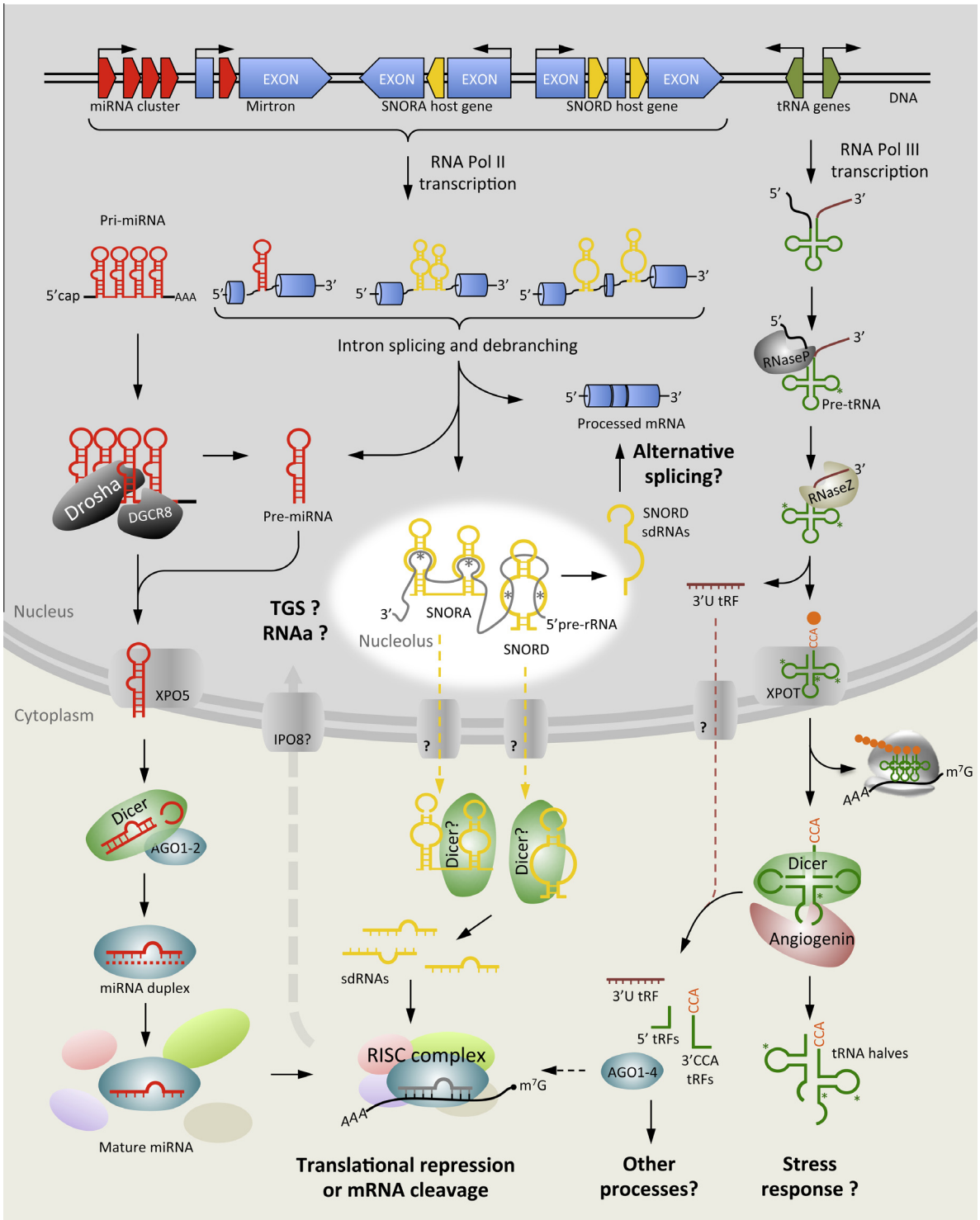

Fig. 1. Cross-talk between the pathways of biogenesis and function of miRNAs, snoRNAs, tRNAs, sdRNAs and tRFs. MiRNAs are encoded in clustered genomic loci or in the introns of other genes and are transcribed by RNA polymerase II (RNA Pol II). Pri-miRNA transcripts are processed to individual pre-miRNAs in the nucleus by Drosha. SnoRNAs and intron-encoded miRNAs are produced after splicing, debranching, and exonucleolytic trimming. Pre-miRNAs are exported to the cytoplasm by Exportin 5 (XPO5) and further processed by Dicer to mature miRNAs that enter the RNA-induced silencing complex (RISC). Cytoplasmically matured miRNAs in complex with proteins from the Ago family (AGO) may be imported back in the nucleus possibly by Importin 8 (IPO8) to participate in the processes of transcriptional gene silencing (TGS) or RNA activation (RNAa). SnoRNAs assemble with snoRNP-core proteins (not shown) and enter the nucleolus where they participate in the chemical modification of ribosomal RNA (rRNA) and other RNA species. SnoRNAs may be exported to the cytoplasm by unknown transporter proteins, where they are cleaved possibly by Dicer to short ~22 nt long sdRNAs and are loaded into RISC. Alternatively, snoRNAs may also be cleaved by unknown nucleases in the nucleus or nucleolus, to sdRNAs with a different size. Longer sdRNAs of ~27 nt do not exit the nucleus, but instead participate in the regulation of alternative splicing. tRNAs are transcribed from individual tRNA genes by RNA polymerase III (RNA Pol III). Pre-tRNA transcripts are processed by the endonucleases RNase P and RNase Z to remove 5′- and 3′-trailer sequences, and after chemical modification, CCA addition, and aminoacylation, are exported to the cytoplasm by Exportin-t (XPOT) to participate in protein synthesis. 3′U tRFs are produced by RNase Z after trimming of the 3′-trailer sequence. Stress factors may induce cleavage in the anticodon loop of mature tRNAs to tRNA halves performed by the endonuclease Angiogenin. Shorter 5′tRFs and 3′CCA tRFs may be produced from 5′- and 3′-ends of mature tRNAs by Dicer and associate with AGO proteins to participate in various processes of transcriptional and post-transcriptional regulation.

with complementary promoter sequences [38]; and for miR-744, -1186, and -466d-3p that interact with the promoter of the mouse Cyclin B1 gene to enhance cell proliferation [39]. The exact molecular mechanisms of activating miRNAs are yet to be elucidated but the involvement of components from the RNAi machinery and epigenetic events have been proposed [14,15,17,39].

Whether the involvement of miRNAs in transcriptional gene regulation is a common process or is adopted from other (possibly unknown) cellular mechanisms is to be investigated. However, the examples mentioned above illustrate a broader functional flexibility of small ncRNA and RNAi pathways in control of gene expression than previously thought.

2.2. snoRNA

Small nucleolar RNAs (snoRNA) are well-conserved, abundant, short non-coding RNA molecules, 60–300 nucleotides (nt) in length, which localize to a specific compartment of the cell nucleus – the nucleolus. The majority of snoRNAs in vertebrates are encoded in the introns of protein coding or non-coding genes and are transcribed simultaneously by RNA Pol II [40] (Fig. 1). Most snoRNA host genes encode for proteins or transcripts essential for ribosome biogenesis or function and often belong to the 5′-terminal oligopyrimidine (TOP) family that undergo growth-dependent translational regulation. The generation of most intronic snoRNAs involves splicing and debranching followed by a subsequent exonucleolytic trimming by the 5′- to 3′-exoribonuclease XRN2 and the exosome [41,42]. A second, splicing-independent pathway that generates intronic snoRNAs requires the endonucleolytic cleavage of the pre-mRNA host transcript by the RNase III-like enzyme Rnt1p in yeast [43]. Among higher eukaryotes this function is probably performed by homologues of the novel XendoU, endoribonuclease superfamily [44]. Interestingly, the human ortholog, ENDOU (human placental protein 11, PP11), is a tumor marker, expressed in different forms of cancer [45].

SnoRNAs associate with small nucleolar ribonucleoprotein particles (snoRNP) and serve as guide molecules for the snoRNPs in the post-transcriptional modification of ribosomal RNA (rRNA) and small nuclear RNA (snRNA). According to the presence of conserved sequence motifs snoRNAs are sub-categorized into H/ACA-box (SNORA) that guide pseudouridylation, and C/D-box (SNORD) that guide 2′O-ribose methylation of targeted RNA. The two types of snoRNAs associate with distinct sets of proteins, necessary for the proper formation and enzymatic function of the snoRNP.

SNORAs adopt a conserved double hairpin structure, where the two irregular stems are linked by a hinge region containing the conserved sequence motif 5′-ANANNA-3′ (H-box), and are followed by a small tail containing the 5′-ACA-3′ motif (ACA-box). The hairpin stems contain short antisense elements complementary to the target RNA positioned within an internal loop called the pseudouridylation pocket [46]. The core H/ACA RNP includes the pseudouridine synthase dyskerin (Cbf5p in yeast), and the proteins Nop10, Nhp2, and Gar1 [47,48].

Interestingly, hTR, the RNA component of mammalian telomerase resembles SNORA RNA and its association with the core H/ACA proteins is essential for the processing, stability, and trafficking of telomerase RNA *in vivo* [49,50].

SNORDs are characterized by the conserved structural motifs 5′-RUGAUGA-3′ (C-box) and 5′-CUGA-3′ (D-box). The C-box and D-box form an unusual kink-turn (K-turn) structure, which is essential for the folding, assembly, function, and nucleolar localization of the snoRNP. A second internal C′/D′-box pair forms a K-loop and is often degenerated in eukaryotes [51]. Substrate recognition is achieved via the formation of a short (11–12 bp) double stranded RNA helix between the targeted RNA and a short complementary sequence stretch in the SNORD (antisense box) with the nucleotide targeted for modification positioned exactly 5 nt upstream of the D or D′ box [46,52]. The formation of an active SNORD-RNP in eukaryotes requires the association of Nop58/56 proteins with the K-turn motif, which serves as a matrix for the binding and proper positioning of the methyltransferase fibrillarin [46].

A combination type of snoRNAs that comprises features of both SNORAs and SNORDs localizes specifically to the Cajal bodies, small sub-organelles in the nuclei of proliferative or metabolically active cells involved in the biogenesis of small nuclear RNPs. Cajal-body-specific RNAs (SCARNAs) contain a specific element, termed the Cajal body box (CAB box) that is necessary for their retention within the Cajal bodies [46]. SCARNAs can guide both the methylation and pseudouridylation of the RNA Pol II transcribed spliceosomal RNAs.

To date, over 400 different snoRNA species have been identified in the human genome [53]. However, target sites have been identified or predicted for only half of them. The remaining and increasing number of snoRNAs that lack complementarity to other RNA molecules, and therefore, have no known function, is referred to as "orphan" snoRNAs [54].

2.3. tRNA

tRNAs function as carriers that transport amino acids to the growing polypeptide chain during the translation of mRNA. Mature tRNAs are about 73 nt long and contain an amino acid attached to their 3′-ends. The secondary fold of a tRNA resembles a cloverleaf, with three internal stem loops (D loop, T loop, and anticodon loop) that interact with each other via conserved nucleotides to give a compact L-shaped tertiary structure.

Up to 506 genes encoding a set of 49 different tRNAs are found in the human genome [55]. tRNA genes are transcribed by RNA polymerase III (RNA Pol III) (Fig. 1). Pre-tRNA transcripts have the typical cloverleaf structure with additional 5′-leader and 3′-trailer sequences of various lengths. The 5′-leader sequence is removed by the endoribonuclease P (RNase P), which has to recognize the tRNA fold in order to perform the cleavage [56]. Endonucleolytic removal of the 3′-trailer sequence is performed by the endonuclease Z (RNase Z, ELAC2) [57]. After removal of the 3′-trailer, the terminal trinucleotide 5′-CCA-3′, obligatory for the aminoacylation of tRNAs, is added at the 3′-acceptor stem by the CCA-adding tRNA nucleotidyl transferase, TRNT1 [58,59].

Some pre-tRNAs contain intronic sequences (14–60 nt in length) positioned 3′ to the anticodon that are removed by the evolutionarily conserved tRNA splicing endonuclease [60] and the tRNA splicing ligase complex [61]. During the process of maturation, tRNAs also acquire many site-specific post-transcriptional modifications that can influence the tRNA shape, structure, stability, and function [62,63]. Finally, tRNAs are aminoacylated by twenty different highly conserved aminoacyl-tRNA synthetases (aaRSs), one for each canonical amino acid, and exported from the nucleus by the nuclear transport receptor Exportin-t (XPOT, XPO3) to participate in translation.

Although incredibly stable, tRNAs are subject to turnover and degradation. Two quality control pathways monitor integrity of tRNA during and after biogenesis. Nuclear surveillance of hypomodified pre-tRNAs and degradation from the 3′-end of 3′-unprocessed pre-tRNAs is performed by the TRAMP complex and the nuclear exosome. Mature tRNAs turnover is controlled by the Rapid tRNA Decay (RTD) pathway from the 5′-end (reviewed in [64,65]).

3. snoRNA-derived RNAs and tRNA-derived fragments

For a long time, snoRNAs and tRNAs have been considered as cellular housekeeping molecules that maintain proper ribosomal

maturation and protein translation. Nevertheless, over the last years several independent NGS studies have demonstrated the existence of small RNA species originating from snoRNAs and tRNAs [66–73]. These small RNA molecules are often referred to as snoRNA-derived RNAs (sdRNAs) [68] and tRNA-derived fragments (tRFs) [69].

In experiments originally designed to profile miRNAs, reads derived from snoRNAs and tRNAs constitute only a minor fraction of the total library (1.5–10% depending on the NGS platform and the size-range of the original RNA fraction used for sequencing). Therefore, sdRNAs and tRFs are often ignored from further analysis as sequencing artifacts or degradation products of the much longer mature snoRNAs and tRNAs. However, targeted and comparative analysis of these small RNA populations and reanalysis of existing sequencing data demonstrated that sdRNAs and tRFs are evolutionary conserved across distantly related species and ubiquitous in eukaryotes [66,68,74–78].

Most importantly, independent evidence from deep sequencing studies, genetic screens, transcriptional, and molecular analyses indicates that alterations in the expression of these small RNAs, their precursors, or their genomic loci are associated with genetic disorders [79–81] and malignancies such as prostate [69,82,83], liver [66], lung [84,85] or breast cancer [86–88], B-cell lymphoma [89], and acute leukemia [90,91], as well as with cancer related processes like aging [77], oxidative stress [92–95], and embryonic development [96,97].

4. sdRNAs - functional evidence, evolutionary characteristics, and proposed mechanisms of action

4.1. sdRNAs can function as miRNAs

The first functional evidence demonstrating miRNA-like activity for an sdRNA was obtained by RNA deep sequencing of immunoprecipitated AGO1 and AGO2 complexes from Human Embryonic Kidney 293 cells (HEK293). This sdRNA, originating from SCARNA15 (ACA45), was produced in a Drosha/DGCR8-independent, Dicer-dependent manner. Luciferase reporter assays targeting its predicted target cyclin-dependent kinase 11B (CDK11B), demonstrated its miRNA-like activity [67]. The same authors identified seven additional sdRNAs with miRNA-like processing features originating from two SCARNAs and five SNORAs in follow-up experiments with newly designed AGO1-4 libraries. Parallel NGS studies of the small transcriptome in mice revealed the presence of SNORA-originating miRNAs in embryonic stem cells and demonstrated that sdRNAs exhibit tissue specific expression [98,99].

At the same time, SNORD-originating miRNAs were described in the protozoan *Giardia lamblia*, a unicellular parasite whose genome does not encode Drosha and XPO5. These miRNA-like sdRNAs are produced in a Drosha/DGCR8-independent, Dicer-dependent manner [100,101]. The same pathway was described one year later in cells infected with the Epstein-Bar virus (EBV). In this system, EBV expresses a miRNA-like precursor endogenously encoded by a viral SNORD (v-snoRNA1) to suppress the viral DNA polymerase upon induction of the lytic cycle [102]. Several small RNA deep sequencing studies in the last few years extended the catalogue of sdRNAs further by detecting sdRNA species originating from C/D box snoRNAs in different normal and cancerous human cells and tissues [73,78,83,103]. Efficient gene silencing *in vitro* by SNORD-sdRNA was also reported, indicating the existence of cytoplasmic processing and recruitment to the RNA silencing machinery for this type of sdRNAs [73].

The obvious similarities in the generation of sdRNA and mirtrons, as well as the accumulating functional evidence of silencing capacities for sdRNAs, led to investigation of the possible evolutionary relationship between miRNAs and snoRNAs. It was shown that the genomic loci of some *bona fide* miRNAs overlap with those of snoRNA, and that many of these miRNAs resemble the structures of known snoRNAs. Furthermore, such miRNAs still contain functional snoRNA elements and can bind protein components of snoRNP complexes. Together, this led to the proposition that miRNAs and snoRNAs share ancestral origins and that miRNAs may have evolved from ancient snoRNAs via retrotransposition ([103–105] and reviewed in [106]).

4.2. SNORD-sdRNAs as regulators of alternative splicing

The above described repression of complementary reporter-targets by sdRNA, their association with Dicer and AGO complexes, and their apparently common evolutionary origin support microRNA-like processing and activity for sdRNAs. However, while SNORA-sdRNAs have the typical size of Dicer products and are ~22 nt long, most SNORD-sdRNAs detected in deep sequencing data display a bimodal size distribution at ~18 and ~27 nt [68,73,83], suggesting the involvement of other nuclease(s) in SNORD-sdRNAs generation. A microRNA-biogenesis protein that could be involved also in the biogenesis of SNORD-sdRNAs is AGO2. AGO2 can perform the Dicer-independent cleavage of pre-miRNA-451 (described above). Nonetheless, the mature miRNA-451 is predominantly 3′-uridilated, while most of the SNORD-sdRNAs are not, pointing out to a still unknown mechanism that generates longer SNORD-sdRNAs.

Interestingly, the highly abundant "orphan" SNORD115 (H/MBII-52) and SNORD116 (N/MBII-85) produce even larger sdRNAs of 34–73 nt in length originally termed psnoRNAs [79,107]. Both snoRNAs are encoded in long gene clusters at the human 15q11q13 locus. SNORD116 has been implicated in the imprinted Prader-Willi Syndrome [108], while SNORD115 was proposed to regulate the alternative splicing of serotonin receptor 2C (HTR2C) mRNA [109]. Surprisingly, follow-up research on SNORD115 demonstrated that not the full-length SNORD115 but sdRNAs derived from this "orphan" snoRNA recruit spliceosomal factors and regulate the alternative splicing of HTR2C [79]. Recently, the function of SNORD115-originating sdRNAs in alternative splicing was challenged by another study which confirmed the processing of SNORD115 and SNORD116 to smaller RNAs but proposed a *bona fide* snoRNA function for these molecules [110]. Nonetheless, a more detailed analysis of the conservation of human SNORDs processing across multiple cell types demonstrated that a region of SNORD88C (HBII-180C) can influence the alternative splicing of the fibroblast growth factor receptor 3 pre-mRNA (FGFR3), supporting the role of some sdRNAs in regulation of splicing [78]. Furthermore, over hundred alternative splicing target sites for SNORD115–116 and five other "orphan" SNORDs from the 15q11q13 locus were computationally predicted to have significant association with alternatively spliced genes [111].

So far, no biogenesis mechanisms have been proposed for sdRNAs originating from SNORD88C, SNORD115, or SNORD116 and their involvement in alternative splicing has to be investigated further. Nevertheless, regulation of exon-skipping or exon-inclusion is an attractive hypothesis for the function of sdRNAs originating from "orphan" SNORDs and it is supported by data obtained from experimental use of synthetic short RNAs mimicking snoRNA (reviewed in [112]). Furthermore, regulation of alternative splicing through siRNA-mediated TGS was also reported, demonstrating the existence of an ncRNA controlled point of intersect between the processes of gene transcription and splicing [18].

4.3. snoRNAs and sdRNAs as tumor suppressors and oncogenes

One of the first implications of snoRNAs in carcinogenesis came from a study in human B-cell lymphoma which identified the

chromosome translocation breakpoint t(3;6)(q27;q15) as the genetic locus of intronically encoded SNORD50 (U50) [89]. Eight years later, a 2 bp homozygous deletion in the same snoRNA was found in 30 prostate cancer cell lines/xenografts and nine out of 89 localized prostate cancers but not in the 104 normal controls. Ectopic expression of SNORD50 significantly reduced colony formation in prostate cancer cells. Further analysis of 1371 prostate cancer cases and matched controls showed that, the homozygosity of this deletion was significantly associated with clinically significant prostate cancer and allowed the authors to propose U50 as a candidate tumor-suppressor gene in prostate cancer [82] and one year later in breast cancer [86].

Recently, four snoRNAs (SNORD44, SNORD43, SNORD48 and RNU6B) often used as normalization controls in real-time PCR experiments were profiled in 219 breast cancers and 46 head and neck squamous cell carcinomas [88]. Interestingly, low snoRNA expression correlated with markers of aggressive pathology, while decreased levels of SNORD44 (RNU44) were also associated with a poor prognosis. The full length SNORD44 is encoded in one of the introns of the protein non-coding growth arrest specific transcript (GAS5) shown to be down-regulated in breast tumors compared to normal adjacent controls [113]. Interestingly, sdRNAs derived from SNORD44, SNORD78 and other snoRNAs encoded in GAS5 are up-regulated in prostate cancer [83], which suggests that separate mechanisms may control the post-transcriptional levels of snoRNA products derived from the same precursor transcript. Similarly, divergent expression between a snoRNA host gene transcript and the snoRNAs encoded in its introns has been described for the conserved mouse ortholog of human ZFAS1 gene known to be down-regulated in breast cancer [87].

Another example of a snoRNA that is positively associated with carcinogenesis and thought to be a putative oncogene is SNORA42. SNORA42 is located at 1q22, a genomic region frequently subjected to amplification in lung carcinomas [114]. This snoRNA is also commonly overexpressed in lung tumors [93]. For SNORA42 it has been shown that a knockdown in non-small-cell-lung carcinoma (NSCLC) cells inhibits *in vitro* and *in vivo* tumorigenicity. Enforced SNORA42 expression in bronchial epithelium increases cell growth and colony formation. It was also demonstrated that SNORA42 expression in lung tumor tissue specimens is inversely correlated with survival of NSCLC patients [85].

A specific snoRNA down-regulation pattern allowing the differentiation between tumor and control samples was also recently observed in acute leukemia [90]. The same study reported snoRNA expression signature specific for a subset of patients with acute promyelocytic leukemia. This signature corresponds to transcriptional activation of restricted chromosomal regions encoding the "orphan" intronic SNORD112–114 cluster. *In vitro* experiments demonstrated that transcription of these snoRNAs was lost under retinoic acid-mediated differentiation and induced by enforced expression of the PML–RAR alpha fusion protein in negative leukemic cell lines. These results led to the hypothesis that snoRNAs might be specifically regulated in a background of chromosome instability and thus may have an important role in oncogenesis, similarly to miRNAs.

Finally, an indication that snoRNAs are also targeted by epigenetic inactivation in human cancer came from the observed hypermethylation and inactivation of snoRNA host gene-associated 5′-CpG islands. This led to transcriptional silencing of the snoRNAs SNORD123, SNORA70C and SNORA59B in cancer cells and primary malignancies, particularly in leukemia [91].

At present, it remains to be discovered whether the altered expression of sdRNAs and/or their snoRNA precursors in cancer is the result of a specific process or of more general defects in cellular metabolism caused by malignant transformation and cancer progression. The exact molecular mechanisms by which snoRNAs and sdRNAs may provoke cell survival and proliferation and inhibit cell death *in vivo* remain unknown and should be the subject of further investigations.

5. tRNA fragments – types, functional evidence, and proposed action mechanisms

Already in the early 1970s, the expression of a fragment derived from $tRNA^{leu}$, was observed in *Escherichia coli* shortly after bacteriophage T4 infection [115]. Almost 30 years later, a tRF was isolated from human urinary bladder carcinoma and for the first time also functionally studied [116]. By now, the generation of NGS data led to the identification of small tRNA fragments of different size and origin on a genome wide scale and is starting to address the questions surrounding their biogenesis and function [66,69,70,72,75,117,118].

5.1. Types of tRNA fragments

Since their discovery, the terminology regarding small ncRNAs derived from tRNAs has been highly inconsistent. Names such as tRNA halves [74,94], tRNA-derived halves [119], tRNA-derived RNA fragments (tRFs) [69] stress-induced small RNAs (tiRNAs) [95,120], tRNA-derived small RNAs (tsRNAs) [117] or urinary bladder carcinoma RNAs (ubcRNAs) [116] are used by various research groups and refer to similar entities. Recently, a nomenclature based on tRNA fragment size and the part of the tRNA molecule from which fragments are derived was proposed [121]. According to this nomenclature, tRNA fragments can be separated in two major classes: tRNA halves and small tRNA fragments (tRFs). tRNA halves have a size of 30–35 nt and are produced by a cleavage in or near the anticodon loop in response to hypoxia, oxidative stress, or apoptotic inducers [74,93,94]. Interestingly, Angiogenin, the endonuclease responsible for this cleavage in mammals [94,95] is a potent vascularization agent in normal and malignant cells and a potential oncogene.

Small tRFs of approximately 20 nt in length are derived from either the 5′- or 3′-end of mature tRNAs (5′tRF and 3′CCA tRFs). tRFs are also produced from 3′-pre-tRNA trailers (3′U tRFs) during processing of pre-tRNAs by RNase Z, a nuclease also known as the prostate cancer susceptibility gene ELAC2 [69] (Fig. 1). The mechanisms by which 5′- and 3′CAA tRFs are generated in the cell are not yet completely understood but it has been proposed that Dicer may be involved in tRFs production [70].

5.2. Are tRNA fragments degradation products of tRNA or novel biological entities?

The abundance of tRNAs, their ubiquitous expression and their central role in cellular metabolism impose the question whether tRNA fragments often found in sequencing experiments are functional entities or "just" products of tRNA turnover and cellular stress response.

Several lines of evidence demonstrate that tRNA fragments are produced in a controlled fashion suggesting that these small RNA molecules are novel biological entities. For example, levels of mature tRNA do not change significantly during stress induced tRNA cleavage and tRNA halves production [74,93,95]. Furthermore, the process seems to be controlled by RNA methylation [122], and it is induced only by specific instead of all stress stimuli [123]. Next, cleavage often occurs at specific positions in tRNA suggesting that fragments are derived from fully mature tRNAs, after intron excision and 5′-CCA-3′ addition (reviewed by [121]). Finally, a recent detailed analysis of human healthy, cancerous, or

transformed cells and tissues showed that despite the significant increase in absolute tRNA gene levels in cancerous compared to healthy cells, the relative composition of full length tRNAs remains almost identical and it is not associated with tRF abundance [124]. This study confirms the original findings by Kawaji et al. [66] that tRF frequencies have no correlation with tRNA gene copy number or codon usage distribution. It also rejects the hypothesis that tRF abundance reflects tRNA gene expression and provides additional evidence that tRFs are independent entities and not a product of tRNA turnover and degradation.

5.3. Biological role and action mechanisms of tRNA-derived small RNAs

Although, the exact roles of tRNA halves and tRFs are yet to be elucidated, accumulating evidence suggests that tRNA-derived small RNAs participate in two main types of biological processes.

5.3.1. tRNA-derived fragments as signal molecules in stress induced response

By now, several investigations have shown that tRNA halves are up-regulated by stress conditions and particularly under starvation-induced and oxidative stress [93,95,123]. tRNA halves can promote the assembly of stress granules [120], a type of cytoplasmic RNPs, that play an important role during stress-induced translational inhibition and translational induction of cell-repair and cell-survival proteins [125]. In this context, promotion of stress granule assembly by tRNA-derived fragments may play an important signaling role for hypoxia and starvation-induced stress in tumor cells [126]. Interestingly, there is also evidence that tRNAs can inhibit apoptosis via association with cytosolic cytochrome-C, preventing its interaction with Apaf-1 and blocking subsequent caspase-9 activation. At the same time, treatment with tRNA-specific RNase enhances caspase-9 activation and apoptosis [127]. On the contrary, it has been shown that tRFs are up-regulated in prostate cancer [83]. Furthermore, in prostate cancer cell lines, 3′U tRFs expression positively correlates with the cellular proliferation rate and siRNA mediated 3′U tRF knockdown results in impaired cell proliferation [69].

Taken together, tRFs seem to participate in cellular stress response and to influence cell proliferation. Stress conditions such as nutritional starvation or oxidative stress, often precede apoptosis but are also common in the tumor environment. In most of these conditions the expression of tRNA-derived fragments raises proportionally [93,123]. It is possible that in normal cells tRNA-derived fragments function as intrinsic apoptotic signals or cause apoptosis indirectly, by e.g., inhibition of protein translation. It also can be assumed that the pathways of stress-related tRNA cleavage interact or intercept with major cancer pathways that lead to apoptosis escape and induce proliferation of malignant cells. Further research is needed to elucidate the exact pathways of generation and function of stress-induced tRNA fragments and their role in cancer biology.

5.3.2. tRNA-derived fragments as regulators of gene expression

One of the first reports suggesting involvement of tRNA-derived fragments in gene regulation and silencing, addressed the characterization of small RNAs in human immunodeficiency virus (HIV) infected cells [128]. In this study, Yeung et al., reported the identification of a highly abundant, 18 nt long, small RNA, originating from the double-stranded RNA hybrid formed by the HIV primer-binding site and the 3′-end of the human cellular $tRNA^{lys}$. $tRNA^{lys}$ is one of the three tRNAs ($tRNA^{lys}$, $tRNA^{pro}$, or $tRNA^{trp}$) used by viral reverse transcriptases as primers for the initiation of reverse transcription and DNA synthesis [129,130]. The association of this $tRNA^{lys}$-derived 3′tRF with Dicer and AGO2, and its silencing activity in luciferase reporter assays, led to a proposed function in the cellular RNA interference (RNAi) machinery for targeting HIV and viral defense.

Interestingly, mammalian herpes viruses have adopted the tRNA biogenesis pathway to generate fully functional viral miRNAs. In this system, short transcripts containing a 5′ tRNA-like molecule flanked by a 3′-pre-miRNA hairpin are produced by RNA Pol III and subsequently cleaved by the cellular RNase Z to liberate the pre-miRNA hairpins, which are further processed to mature viral miRNAs by Dicer [118,131,132]. Similar structures, also giving rise to small RNAs, have been found in the mouse host genome, indicating a convergent usage of RNA Pol III transcripts for the generation of small RNAs from both mammalian and viral genomes [118]. In the human genome, a highly conserved tRNA-like structure (mascRNA) is processed from the 3′-region of MALAT1, a long non-coding RNA known to be deregulated in many human cancers. MascRNA is processed by RNase P and RNase Z and is subjected to CCA addition to generate the mature 61 nt mascRNA transcript, which is subsequently exported to the cytoplasm [133]. Although the function of mascRNA is still unknown, its existence in the human genome is probably not a solitary event. Recently, a tRNA-like cloverleaf structure similar to $tRNA^{gly}$ was discovered in the intron of the POP1 gene encoding one of the protein subunits of RNase P that interact with the 5′-end of tRNAs. For this POP1-ncRNA a function in auto-regulation of the POP1 transcripts was proposed, with the RNase P complex binding and potentially cleaving the hybrid [134].

The miRNA generation system adopted by murine herpes viruses strikingly resembles the production of a 3′U tRF (tRF-1001, cand45) described in prostate cancer cell lines [69] and in human hepatitis delta transformed embryonic kidney cells [117]. However, in contrast to viruses, no silencing activity could be demonstrated for this tRF in luciferase reporter assays, suggesting that 3′U tRFs operate in pathways other than miRNAs [69,117]. Furthermore, predominant association of 3′U tRFs was observed with AGO3 and AGO4 [117] whose function at present is largely unknown. The same study reported a similar association with AGO proteins also for 3′CCA tRFs, which however did demonstrate modest silencing effect in reporter assays. Interestingly, strikingly similar 3′CCA tRFs from *Tetrahymena* associate with the growth-essential PIWI protein Twi12 and may represent an ancient pathway of gene regulation or heterochromatin formation [135].

Similarly to 3′tRNA-derived fragments, small 5′tRFs do not seem to associate strongly with AGO2 but instead preferentially associate with AGO1 [70,71]. Interestingly AGO1 has been implicated in transcriptional gene silencing via heterochromatin formation mediated by siRNAs and miRNAs targeting promoter regions in human cells [14,35,136]. This proposes a participation in the TGS pathways (discussed in Section 2.1) also for tRNA-derived fragments.

Last year, Rudinger-Thirion et al. demonstrated that the poorly aminoacylated human $tRNA^{asp}$ isodecoder could adopt an alternative hairpin secondary structure. In this isoform, $tRNA^{asp}$ binds to an embedded Alu element in the 3′UTR of its aspartyl-tRNA synthase mRNA and stabilizes the mRNA for efficient translation [137]. Interestingly, $tRNA^{asp}$ binds the Alu element via extensive base pairing from positions 21 trough 55, leaving the 20 nt long 5′-terminal portion of the tRNA (that in size and position resembles a 5′tRF) exposed and accessible for nuclease cleavage. The results of this study demonstrate not only a possible function for the many tRNA isodecoders and pseudogenes, but also hint to an unanticipated way of tRF generation.

Finally, a recent deep sequencing study and a computational comparison of human cancer cells and mouse embryonic cells by Li et al. [72] demonstrated the presence of 16–18 nt long 3′CCA tRFs that do not undergo Dicer processing but are endogenously

associated with AGO2 and are able to guide down-regulation of target RNAs *in vitro*. Interestingly, these small tRFs are highly complementary to endogenous retroviral primer binding sites in the human genome. Therefore a role in regulating the unwarranted expression of endogenous viruses through the RNA interference pathway was proposed for these tRFs.

Taken together, although tRNA-derived fragments seem to be highly abundant, currently, their actual functions remain unknown. Nevertheless, a growing body of evidence suggests that tRFs can act as signaling molecules under stress conditions and in gene regulation by alternative mechanisms possibly evolved from ancient regulatory pathways. Additional research addressing the molecular partners of these tiny RNAs and development of computational tools for the prediction of their targets is warranted and necessary in order to elucidate the exact pathways in which they operate.

6. Future perspectives

At present, the interest in small RNAs other than miRNAs is rapidly growing. Their involvement in cancer and other human diseases has provided fresh perspectives for the exploration and development of new biomarkers and novel therapeutic strategies for the detection, monitoring, and treatment of human disease.

6.1. Biomarker potential

The high stability and abundance of miRNAs in body fluids and their power to discriminate cancer patients from healthy controls [138], opened the prospect for development of minimally invasive, ncRNA-based biomarker tests for the detection and monitoring of solid tumors. Although current research is primarily focused on miRNAs as candidate fluid-based biomarkers, the involvement of sdRNAs, tRFs, and their precursor molecules in pathological processes and the demonstrated differential expression in solid tumors [83–85,88] and hematological malignancies [89,90] suggest yet unexploited clinical potential also for these ncRNAs. Specific cancer-related snoRNA signatures in blood plasma were recently described for non-small-cell lung cancer [84]. Already in the late 1970s, elevated levels of tRNA breakdown products were found in the urine of humans and mice with different malignancies [139] and earlier this year, fragments of the spliceosomal small nuclear RNA, RNU2 were found in human serum and plasma from pancreatic and colorectal carcinoma. qRT-PCR based assays correctly identified both tumor types with a very high sensitivity and specificity [140], demonstrating that also small RNA fragments may be used as fluid-based biomarkers in future prospective screening studies.

6.2. Therapeutic potential

At present, attention for RNA-based therapeutics that mimic or regulate miRNA activity is growing. Strategies, that utilize modified antisense oligonucleotides like locked nucleic acid (LNA) oligonucleotides to inhibit miRNA function, or miRNA mimics to restore it, are routinely used in miRNA research. Therapeutic agents based on tiny LNAs for the treatment of hepatitis C [141], lymphoma [142] and metastatic solid tumors [143] have entered clinical trials. Similar strategies can be easily adopted in the future for cancer-associated miRNA-like sdRNAs or tRFs to inhibit the function of oncogenic sdRNAs/tRFs or to restore the cellular levels of tumor-suppressive ones.

Elucidation of the molecular mechanisms by which sdRNAs and tRFs function in regulation of gene expression can generate novel molecular tools for the modulation of functional restoration of other oncogenes or tumor-suppressor genes in the malignant cell. For example, snoRNA-based vector systems for the (simultaneous) targeted knockdown of one or more RNA transcripts already exist. They have been proven effective in the specific knockdown or replacement of the targeted endogenous proteins and could be utilized in basic gene expression research and target validation, but also for gene therapy [144]. Furthermore, if at least some "orphan" sdRNAs are proven to regulate cancer associated alternative *cis*- or *trans*-splicing events, synthetic sdRNA-like constructs can be developed and used for the repair of transcripts that are subject to misprocessed splicing in cancer. Such strategies based on splicing modulation mediated by antisense oligonucleotides are currently used to restore cryptic splicing, to change levels of alternatively spliced genes, or, in case of Duchenne muscular dystrophy for exon-skipping in order to restore a disrupted reading frame [145].

A better understanding of the function of tRNAs in apoptosis, and the involvement of tRFs in stress signaling, may help to develop methods for the repair of malfunctioning signaling pathways associated with cancer and can lead in the future to novel treatment strategies in early stages of cancer.

In conclusion, the further development of NGS technologies, computational algorithms for the prediction and analysis of ncRNA, and the expanding data on the (aberrant) expression of known and newly discovered small RNAs, will without doubt deepen our knowledge of their function in the normal and malignant cell. For now however, the mechanistic pathways in which sdRNAs and tRFs are generated and function, remain to be elucidated, while existing hypotheses are still to be scrutinized and functionally examined *in vitro* and *in vivo*, before we gain a comprehensive view of the biology of small non-coding RNA.

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