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Small non-coding RNAs mount a silent revolution in gene expression

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During the past decade, it has become evident that small non-coding RNAs (ncRNAs) participate in widespread and essential regulatory mechanisms in most eukaryotic cells. Novel classes of small RNAs, their biogenesis pathways and cellular effects are continuously being described, and new properties of already established ncRNAs are still being discovered. As the list of small RNA molecules and their roles becomes more and more extensive, one can get lost in the midst of new information. In this review, we attempt to bring order to the small ncRNA transcriptome by covering some of the major milestones of recent years. We go through many of the new properties that have been attributed to already familiar RNA molecules, and introduce some of the more recent novel classes of tiny ncRNAs.

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Introduction

The discovery of microRNAs (miRNAs) and small interfering RNAs (siRNAs) established the field of small RNA (sRNA) research in animals at the turn of the 21st century. The first described small ncRNAs, miRNAs, siRNAs and Piwi-interacting RNAs (piRNAs), are classified by distinct biogenesis pathways and regulatory mechanisms, but they have in common the use of sequence complementarity to recognize target genes and silence their expression. Recent technical developments have enabled scientists to scan the small RNA transcriptome more thoroughly, which has revealed a multitude of novel types of RNA molecules that do not fit into the well-established classes. Here we review some of the new results that have emerged from large-scale sequencing studies as well as from more mechanistic approaches, and strive to categorize these recent advances in a meaningful context. The classes of sRNAs discussed in this review are listed in Table 1. As this review primarily focuses on animal small ncRNAs, the reader is directed to [1] for a discussion of plant ncRNAs.

miRNAs: diverse biogenesis and regulatory pathways

Since their discovery in *C. elegans* over a decade ago, miRNAs have emerged as one of the most abundant and important classes of small ncRNAs. Mis-regulation of specific miRNAs has been linked to cancer, heart ailments, diabetes and neurological defects in humans [2]. The growing number of miRNAs is curated at the miRBase database (http://www.mirbase.org/) [3]. The latest release (18) has 18 226 entries that give rise to 21 643 mature miRNAs from 168 species including animals, plants, unicellular algae and even viruses. Some miRNAs, such as let-7, are perfectly conserved across diverse species. Others share identical 5′ end sequences (typically nucleotides 2–7 called the seed region) and comprise families of miRNAs. While some individual miRNAs have proven to be essential, the redundancy of miRNAs within a family can often compensate for the loss of single members. However, it is unclear if the functional overlap stems from related miRNAs recognizing identical target sites or regulating genes in a common pathway.

The details of miRNA biogenesis have been extensively reviewed and the general pathway is summarized in Figure 1a [4]. Recent studies have revealed unexpected alternatives to the canonical pathway. The generation of mature miRNAs has been regarded to depend on the catalytic activity of Dicer with Ago proteins directing the repression of mRNAs recognized by the miRNAs. However, this view was challenged by several reports examining the biogenesis of vertebrate miR-451 [5•,6•,7•]. The precursor of miR-451 bypasses Dicer processing and, instead, undergoes final maturation by Ago2 – the only mammalian Argonaute that displays catalytic activity. miR-451 is co-transcribed with another miRNA, miR-144. Although both miRNAs share a common primary transcript, because of its structure, only the miR-451 precursor is subject to Ago2-dependent maturation. Reasons for this alternative miRNA processing pathway are currently unknown, but one of its consequences may be the preservation of a catalytically competent Argonaute during mammalian evolution [5•–7•].

Table 1

Classes of small non-coding RNAs

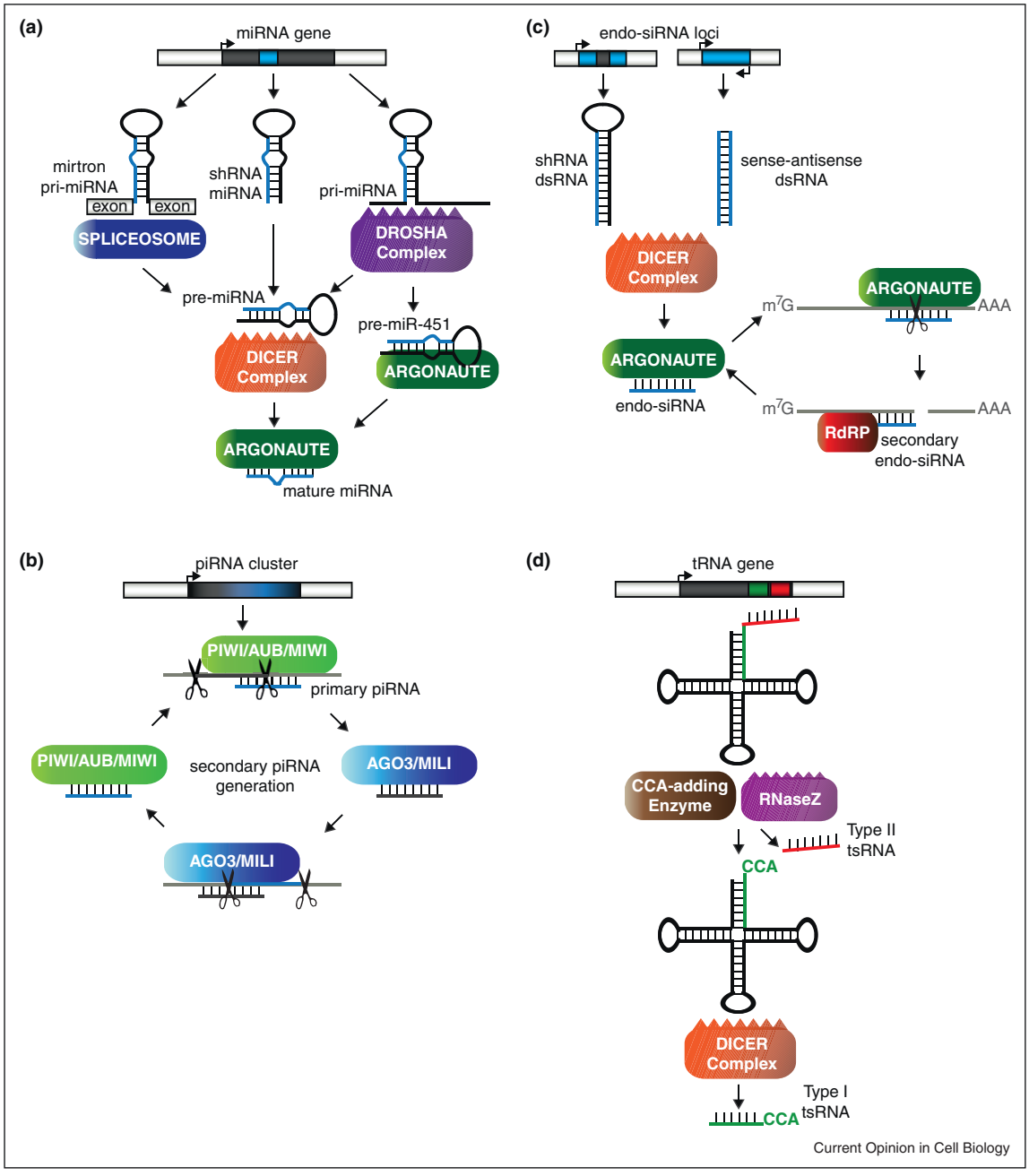
ncRNA	Abbreviation	Model organism(s)	References
microRNA	miRNA	Most eukaryotic organisms except fungi; viruses	[4,20]
miRNA-offset RNA	moRNA	*Ciona intestinalis*, mammals	[13,14]
Short hairpin-derived miRNA	shRNA-derived miRNA	*Mus musculus*	[10]
miRNA-like small RNA	milRNA	*Neurospora crassa*	[48•]
mirtron	–	*D. melanogaster*, *C. elegans*, mammals	[8•,9•,10]
Piwi-interacting RNA	piRNA	*D. melanogaster*, mammals, *Danio rerio*, *Xenopus laevis*, *Bombyx mori*	[23]
21U-RNA	–	*C. elegans*	[51]
Endogenous small interfering RNA	endo-siRNA	*D. melanogaster*, *C. elegans*, mammals	[29–33,34•,35•]
tRNA-derived RNA fragment, tRNA-derived small RNA	tRF, tsRNA	Mammals	[38•,39•,40•]
Promoter-associated small RNA, termini-associated small RNA	PASR, TASR	Mammals	[41]
Transcription start site-associated RNA, transcription initiation RNA	TSSa-RNA, tiRNA	Mammals, *Gallus gallus*, *D. melanogaster*	[42–44]
Splice-site RNA	spliRNA	Metazoans	[13,44]
snoRNA-derived RNA	sdRNA	Metazoans, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*	[13,45,46]
QDE-2-interacting small RNA	qiRNA	*N. crassa*	[47]
Small vault RNA	svRNA	*Homo sapiens*	[49]

Other variations in miRNA processing pathways add to the complexity of miRNA biogenesis (Figure 1a). Some miRNAs bypass Drosha-mediated cleavage altogether, but undergo normal digestion by Dicer in the cytoplasm. The initial processing of this class of miRNAs is spliceosomal, and the RNAs have been assigned as 'mirtrons' for their miRNA-like and intron-like features [8•,9•,10]. In addition, some miRNAs are Dicer-dependent but Microprocessor-independent and splicing-independent. These miRNAs originate from RNAs that have the capacity to form extensive stem-loop structures and are known as short hairpin-derived miRNAs [10]. Moreover, many miRNAs display variation in their annotated 5′ or 3′ termini. These isomers (isomiRs) may arise from products of alternative 5′ processing or from the addition of non-templated nucleotides to the 3′ends of miRNAs [11,12]. In addition, the 5′ and 3′ arms of some *Ciona intestinalis* and human pre-miRNAs give rise to miRNA-offset RNAs (moRNAs) that apparently are products of Drosha processing [13,14]. Thus, miRNAs share the feature of deriving from intramolecular stem-loop structures capable of giving rise to ~22 nt RNA products but are not classified by a uniform processing pathway.

Mature miRNAs guide the RNA induced silencing complex (miRISC), which consists of Argonaute and other effector proteins, to target mRNAs through antisense base-pairing. In animals, most miRNAs only partially base-pair with their targets. The most common motif from computational predictions is perfect pairing of the miRNA 5′ end, called the seed region, usually to mRNA 3′UTR sequences [15]. Validating predicted target sites *in vivo*, however, has until recently only been feasible through reporter gene analyses. An advance in identifying endogenous miRNA target sites has been made possible through CLIP-seq (cross-linking immunoprecipitation coupled with high-throughput sequencing, also known as HITS-CLIP). This method has been used to isolate and map the sequences bound by Ago *in vivo* [16••,17••,18••,19••]. Results from CLIP-seq studies revealed that, in addition to 3′UTR sequences, coding exon sites make up a large fraction of Ago bound regions. Furthermore, seed pairing capacity was enriched in many but not all of the sequences associated with Ago, suggesting that other types of complementarity guide a considerable portion of miRISC interactions. While CLIP-seq narrows the miRNA target site to less than 100 nt and in some cases reveals the bases in contact with the Ago protein, the actual sequences paired to the miRNA cannot yet be resolved. Nonetheless, the extensive datasets of endogenous RISC binding activity provide a much needed experimental basis for further improvement of miRNA target prediction methods.

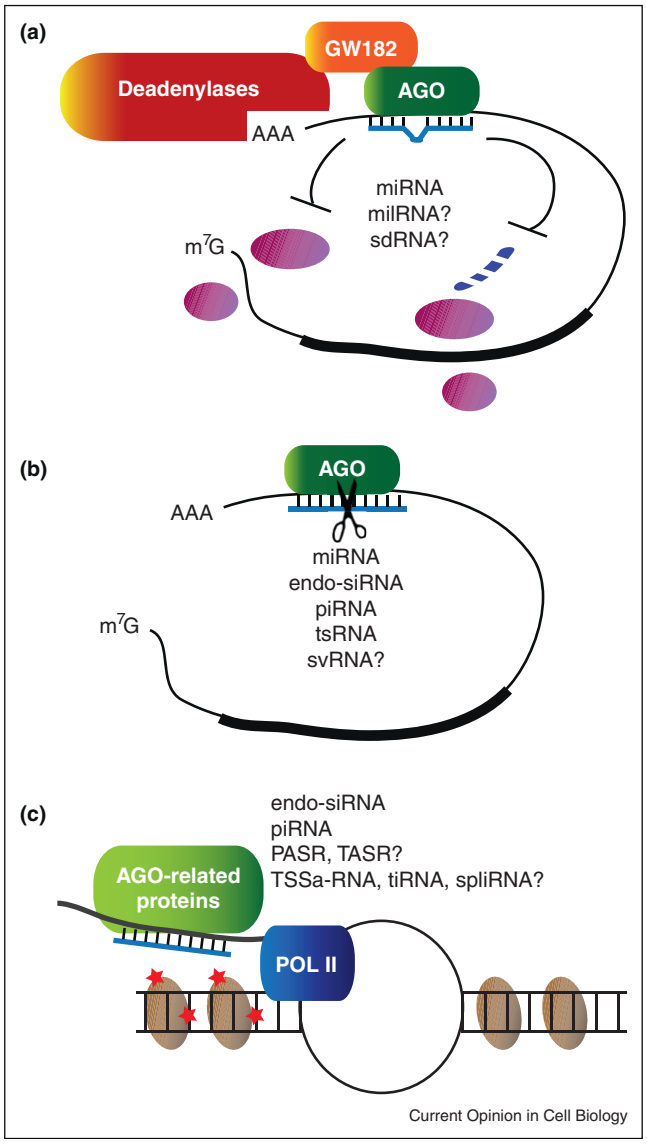
Originally, partial pairing of a miRNA with its target was thought to repress gene expression by inhibiting translation without inducing mRNA decay. It has been proposed that the miRNA complex can inhibit translation initiation or ribosome subunit joining, induce premature degradation of the nascent polypeptide chain and increase ribosome drop off (Figure 2a) [20]. Other studies have demonstrated that regulation by miRNAs can induce target mRNA deadenylation and destabilization (Figure 2a,b) [20]. A recent investigation suggests that miRNA-dependent mRNA destabilization may be a much more widely employed regulatory mechanism than

Figure 1



Many ways to make a silencer. Biogenesis pathways of small RNAs (sRNAs). **(a)** Following transcription of a primary miRNA (pri-miRNA) transcript, the Drosha complex or spliceosome releases the hairpin precursor miRNA (pre-miRNA) from typical or mirtron type miRNAs, respectively. The pre-miRNA or transcribed short hairpin (shRNA) miRNA transcripts undergo final processing by Dicer before incorporation into Argonaute complexes. The pre-miR-451 is an exception that utilizes Argonaute instead of Dicer in the final cleavage step. **(b)** Primary piRNAs from genomic clusters are loaded onto Piwi-related proteins and target homologous sequences for cleavage and generation of secondary piRNAs. These piRNAs are bound by distinct Piwi family proteins, which catalyze the generation of additional piRNAs through the cleavage and processing of RNAs with antisense sequences. **(c)** Endo-siRNAs derive from intramolecular hairpins or sense-antisense transcripts that base-pair and are cleaved by Dicer. The ~21 nt endo-siRNAs are bound by Argonaute proteins and in some organisms initiate the production of secondary endo-siRNAs by RNA-dependent RNA polymerases (RdRPs) that use cleaved target RNAs as templates. **(d)** Precursor tRNAs can be cleaved by RNaseZ to produce Type II tsRNAs from the 3′ termini. Following 3′-end CCA-addition, Dicer processes some tRNAs, potentially those that are misfolded, to produce Type I tsRNAs.

Figure 2



Many roads to silencing. Mechanisms used by sRNAs to silence gene expression. **(a)** Typically, miRNAs, and possibly milRNAs and sdRNAs, partially pair to mRNA target sequences and promote mRNA degradation through the recruitment of deadenylases by GW182 proteins bound to Argonaute. Alternatively, miRISC inhibits translation initiation, stalls translation elongation or stimulates proteolysis of nascent peptides encoded by the target mRNA. **(b)** Perfect pairing of many types of sRNAs results in cleavage of the target mRNA by certain Argonaute proteins. **(c)** Endo-siRNAs, piRNAs and possibly many of the sRNAs derived from protein-coding regions of the genome direct DNA and histone modifications (red stars) that regulate transcriptional activity.

previously appreciated [21••]. Here, the authors used ribosome profiling to determine the exact positions of ribosomes on mRNAs, and observed that at least in HeLa cells miRNA-mediated target repression is primarily due to decreased mRNA levels and translational repression alone is a rare event. However, others argue that the mRNA decay may be merely an outcome of translational inhibition [22]. At this point, it remains to be shown which of the several models of miRNA-mediated target regulation most accurately reflects *in vivo* conditions. It is likely that the regulatory outcome can differ depending on the cell type, developmental stage, cellular condition and context of the miRNA target site.

piRNAs: silencers of the transposable genome

piRNAs are characterized by their specific association with Argonaute proteins of the Piwi-clade, the absence of dsRNA precursors and independence of Dicer [23]. piRNAs have been shown to be crucial for early development, epigenetic regulation, gametogenesis and silencing of transposable elements (TEs) as well as some protein coding genes. Although the specifics of the pathways differ to some extent depending on the species, piRNAs have been mainly studied in *Drosophila*, where they were originally called repeat-associated siRNAs (rasiRNAs), *C. elegans*, where they are also called 21U-RNAs, and mouse. piRNAs were long considered to be confined in animal germline cells, but recently piRNAs have been detected in somatic tissues [24,25]. For a more thorough review of piRNA pathways, we recommend [23].

The piRNA pathway can be divided into initiation, effector and adaptation phases [26]. During the initiation phase, primary piRNAs that are mostly antisense to TEs are produced from piRNA clusters (Figure 1b). These RNAs are processed into 24–30 nt piRNAs that subsequently associate with specific Piwi proteins. In the piRNA pathway effector phase, the piRNA-loaded piRISCs base-pair to sense transposon transcripts and cleave them endonucleolytically. In *Drosophila* germline cells there is additionally an adaptation phase (a.k.a. the ping-pong amplification cycle) that depends partly on Ago3 and replenishes the piRNA pool while consuming the TE transcripts [23,24]. An intact piRNA pathway is continuously required for keeping expression of TEs at bay [26].

In addition to cleaving RNA transcripts directly, piRNA pathways induce TE silencing by establishing DNA methylation patterns within the genome (Figure 2b,c) [27]. piRNAs have also been shown to contribute to deadenylation and decay of maternal mRNAs in the *Drosophila* embryo [28]. Subcellular localization seems to be important for these processes, and components of the piRNA pathway are known to reside both in the nucleus and specific cytoplasmic foci. The mechanistic details of how piRNA complexes sort to particular subcellular locations and direct transcriptional and post-transcriptional silencing of TEs and other types of transcripts are yet to be deciphered.

Endogenous siRNAs: a natural RNAi pathway

Animal endogenous siRNAs (endo-siRNAs) are reminiscent of piRNAs in the sense that they often target TEs or

repetitive elements. Indeed, mammalian and fly endo-siRNAs are derived from dsRNA intermediates that result from 'natural' sources such as bidirectional transcription, inverted repeats or pseudogenes hybridized to mRNAs (Figure 1c) [29–33]. The dsRNA is cleaved by Dicer and the siRNAs associate mostly with the catalytically competent Ago2. Presumably, the siRNA loaded Ago2 complex can direct cleavage of targets that pair perfectly with the endo-siRNA, although direct evidence for this mode of action in animal cells is limited (Figure 2b). Endo-siRNAs are also abundant in *C. elegans*, but their mechanism of biosynthesis is unique. Most nematode endo-siRNAs are secondary and result from unprimed RNA-dependent RNA polymerase (RdRP) activity, and Dicer-dependent primary endo-siRNAs are scarce (Figure 1c) [34•,35•]. In *C. elegans* and *Drosophila*, endo-siRNAs also have a role in chromatin regulation and thus can act as co-transcriptional silencers of gene expression (Figure 2c) [36,37].

New tricks for tRNAs: tRFs and tsRNAs

High-throughput sequencing efforts of small RNAs always yield hits to abundant RNA species, such as rRNAs, tRNAs and snoRNAs, that are usually regarded as random degradation products and omitted from the final analyses. However, recent studies have shown that these housekeeping RNAs may give rise to biologically relevant small RNAs. Sequencing the small RNAs of 17–26 nt from two prostate cancer cell lines revealed reads that matched mature tRNAs or pre-tRNAs [38•]. These tRNA-derived RNA fragments (tRFs) were the second-most copious class of small RNAs in these cell types, and they displayed distinct expression patterns. Interestingly, blocking the expression of an abundantly expressed tRF (tRF-1001) by siRNAs reduced cell viability and proliferation. These effects were rescued by synthetic tRF-1001, which suggests that tRFs may be essential for certain biological processes [38•]. Another study identified two types of tRNA-derived small RNAs (tsRNAs): type I tsRNAs are Dicer-dependent, whereas type II tsRNAs are Dicer-independent but dependent on RNaseZ and RNA polymerase III transcription termination (Figure 1d) [39•]. The two types of tsRNAs seemed to associate with separate sets of Ago proteins, which affected their ability to induce silencing of a reporter gene (Figure 2b). The physiological functions of these tRFs/tsRNAs remain to be determined, but the authors suggest the possibilities of Dicer-mediated cleavage of tRNAs as a regulatory or quality control mechanism, canonical RNA silencing, or regulation of cellular homeostasis by fine-tuning the small RNA pool and Dicer/Ago occupancy [38•,39•,40•].

Transcription meets silencing: PASRs, TASRs, TSSa-RNAs, tiRNAs and spliRNAs

Recent studies have revealed new types of small RNA species that originate from different regions of annotated protein-coding genes. Promoter-associated sRNAs (PASRs) and termini-associated sRNAs (TASRs) are 22–200 nt in length, and are apparently derived from longer precursor RNAs [41]. Transcription start site-associated RNAs (TSSa-RNAs) have the mean length of 20 nt and are of both sense and antisense orientation [42], while transcription initiation RNAs (tiRNAs) are about 18 nt long and originate largely from the same genomic strand as the TSS [43]. TSSa-RNAs are thought to be products of RNA polymerase II (RNAPII) divergent transcription and pausing. Productive RNAPII transcription elongation occurs unidirectionally, and divergent transcription and TSSa-RNAs might be a transcription regulatory mechanism [42]. Another group of RNAs that may be related to RNAPII backtracking is splice-site RNAs (spliRNAs) [13]. SpliRNAs are small RNAs whose 3′ termini map precisely to the splice donor site, are enriched in the nucleus and are present at internal exons. The authors suggest that the accumulation of both tiRNAs and spliRNAs might reflect the relation between RNAPII activity and nucleosome positioning [13]. A recent study suggests that TSSa-RNAs/tiRNAs are unlikely to be derived from RNAPII backtracking, but instead emerge as a result of RNAPII protecting nascent RNAs from degradation [44]. Similarly, in this study spliRNAs are considered as remnants of splicing intermediates [44].

Other newly established small RNA classes

In addition to the small RNA species presented above, several distinct classes of sRNAs have been described lately. Small nucleolar RNAs (snoRNAs) are normally needed for alternative splicing and RNA modifications, but they also give rise to snoRNA-derived RNAs (sdRNAs) [45,46]. The snoRNA ACA45 is a precursor for a Dicer-dependent sdRNA that acts as a miRNA in a reporter assay, indicating that sdRNAs may function in gene expression regulation (Figure 2a) [45]. In the filamentous fungus *Neurospora crassa*, DNA damage induces the synthesis of a class of RNAs known as QDE-2-interacting small RNAs (qiRNAs) [47]. qiRNAs match almost exclusively to rDNA loci, and are probably essential components of the DNA damage response pathway of the fungus. Small RNA sequencing in *Neurospora* has also recently detected fungal miRNA-like small RNAs (milRNAs) [48•]. milRNAs are synthesized by at least four distinct pathways and are able to regulate the levels of specific target genes (Figure 2a). Vault particles are conserved organelles found in most animals that are composed of several proteins and three vault RNAs (vRNAs). Small RNAs matching to vRNAs (svRNAs) were discovered in human breast cancer samples [49]. svRNAs are synthesized by a Dicer-dependent pathway, and they are capable of guiding sequence-specific target RNA cleavage *in vitro*. These various examples indicate that high-throughput sequencing projects continuously add complexity to the already extensive list of small RNAs.

Perspectives

During the recent years, next-generation sequencing efforts have resulted in a plethora of small RNA reads. The analysis of these data has shown that regulation of gene expression is an exceedingly complex phenomenon and we are still in the midst of discovering the executers of this process. Therefore, caution should be exercised in categorizing these sequence reads. It may well be that many reads of abundant housekeeping RNAs are not unspecific degradation products, but represent *bona fide*, biologically relevant RNA species. On the contrary, as the libraries originate from various organisms, cell types and developmental or physiological stages, there may be functional or mechanistic overlap that is not immediately evident at the sequence level. It is possible that seemingly novel classes of small RNAs might actually represent previously described molecules. Thus, scientists should be conservative in assigning novel names for newly described small RNAs, as this will eventually lead to an overload of terminology.

Many of the small noncoding RNAs presented above show similar characteristics. As an example, miRNAs, tRFs, sdRNAs, svRNAs, qiRNAs and some endo-siRNAs seem to be Dicer-dependent, but their Microprocessor-dependence, subcellular localization and affinity to Argonautes may differ. Moreover, miRNA-like characteristics are continuously described in small RNAs that are not canonical miRNAs (e.g. mirtrons, sdRNAs, svRNAs, milRNAs). It is conceivable that the abundance of small RNAs is a way to control the silencing machinery by regulating Dicer/Drosha activity and Ago occupancy. In *S. pombe*, there is a surveillance mechanism that prevents unwanted small RNA molecules from entering the silencing pathway [50]. Other organisms may accomplish the same goal by 'titration' of the silencing components.

Remarkably, the predominant function of sRNAs is to induce silencing of gene expression. While there are some examples to the contrary, base-pairing of a sRNA to a target sequence typically results in repressed gene expression through a variety of mechanisms (Figure 2) [20]. It remains to be discovered if in the context of specific proteins or cellular conditions sRNAs might also have widespread roles in gene activation.

All in all, regulation of gene expression in a eukaryotic cell appears to be largely dictated by the composition of its small RNA repertoire. As the roster of small RNAs keeps on growing, there is a dire need for a set of guidelines for their systematic classification. We are only beginning to appreciate the amazing complexity of these tiny molecules and the discernment of common features will surely accelerate our understanding of the biological functions of specific sRNAs.

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