PERSPECTIVE

Expanding RNA physiology: microRNAs in a unicellular organism

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Until recently, the world viewed through the eyes of molecular biologists seemed simple with a well-known slogan hailing a central dogma: “DNA makes RNA makes protein.” This has been interpreted to mean that genetic output is entirely or almost entirely transacted by proteins (Mattick 2004). However, within a few years of the discovery of RNA interference (RNAi) (Fire et al. 1998), an explosion of data has lifted the veil off many previous puzzling findings. Thus, our view of the field was irrevocably changed with a new slogan—that went alongside the classical central dogma—“RNA makes small RNA makes no protein” (Fig. 1). Sequence information in long RNA transcripts is converted into small, ~20- to 30-nucleotide (nt)-long, non-protein-coding RNAs such as microRNAs (miRNAs) and small interfering RNAs (siRNAs). These small RNAs trigger various forms of sequence-specific gene silencing, including RNA cleavage and degradation, cleavage-independent mRNA decay, translational repression, methylation of protein-coding DNA, and heterochromatin formation in a variety of eukaryotic organisms ranging from fission yeast, plants, fungi, nematode, and fly to humans. This process is now collectively referred to as RNA silencing (Tomari and Zamore 2005; Zaratiegui et al. 2007). In addition to classical small non-protein-coding RNAs—including tRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs)—recent studies have shown that our cells express a surprisingly large number of new regulatory small RNAs that seem to be particularly abundant in roles that direct the binding of protein complexes to specific nucleic acid sequences (Hüttenhofer and Schattner 2006). These include miRNAs, several groups of natural or endogenous siRNAs, and Piwi-interacting RNAs (piRNAs) (Kim 2006). The ongoing efforts to uncover the full biological scope of RNA silencing have already modified our notions about how gene expression is controlled. It is becoming clear that RNA silencing comprises an important tier of gene expression in eukaryotes, enabling the integration and networking of complex suites of gene activity, thereby elaborating multicellular complexity (Bartel 2004; Kloosterman and Plasterk 2006).

The first small RNA trigger discovered in eukaryotes was the *lin-4* miRNA, which was found by mapping a

developmental timing (heterochronic) mutant *Caenorhabditis elegans* locus (Lee et al. 1993). The discovery of the phylogenetic conservation of *let-7* miRNA then really opened up the field (Pasquinelli et al. 2000). Thousands of miRNAs have now been identified in various multicellular organisms (miRBase; Griffiths-Jones et al. 2006), and an astonishing number of miRNA genes have been predicted to exist in our genome (Miranda et al. 2006). Transcripts of genes encoding self-complementary RNA that forms imperfect hairpins, termed primary microRNA (pri-miRNA), are cleaved by the Drosha-Pasha/DGCR8 complex in the nucleus to generate pre-miRNA. The pre-miRNA is exported to the cytoplasm and further processed by Dicer into an ~21- to 23-nt miRNA duplex. Then, one strand over the other is often preferentially loaded into a RNA-induced silencing complex (RISC), which contains at its core a member of the Argonaute family of proteins (Parker and Barford 2006). Once incorporated into the silencing complex, mature miRNAs interact with target mRNAs at specific sites to induce cleavage of the message, cleavage-independent mRNA decay, or inhibit translation (Tomari and Zamore 2005; Pillai et al. 2007). Cleavage of mRNA targets is catalyzed by Argonaute proteins (Liu et al. 2004; Parker and Barford 2006). In both animals and plants, miRNAs play important roles in diverse developmental processes, including developmental timing, cell death, cell proliferation, asymmetric cell fate decision, organogenesis, and patterning of the nervous system. However, the biological functions of most miRNAs remain unknown (Alvarez-Garcia and Miska 2005; Jones-Rhoades et al. 2006). Recent studies have shown that miRNAs target 30% or more of all animal protein-coding genes, most of which are regulated through base pairing between specific sites in the 3' untranslated region of the messenger RNAs (mRNAs) and a small region termed the “seed” (nucleotides 1–8, from the 5' end) near the 5' end of the miRNA (Brennecke et al. 2005; Lewis et al. 2005; Xie et al. 2005). These rather lax criteria can result in numerous targets being predicted for a given miRNA. Many genes have several target sites for either one miRNA or a few different miRNAs. Additionally, many miRNAs are expressed in a cell type- or tissue-specific manner, and miRNAs and their targets have, to a large extent, mutually exclusive expression patterns (“para-expression”) (Farh et al. 2005; Stark et al. 2005). Therefore, the general function of miRNAs may be that miRNAs diversify cell types or organ types by fine-tuning the proteome, and then retain

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Figure 1. RNA physiology. Recent studies show that the vast majority of our genome is transcribed (e.g., Carninci et al. 2005; Manak et al. 2006). This in turn produces a surprisingly large number of functional non-protein-coding RNAs, including small regulatory RNAs (Prasanth and Spector 2007). These observations have led to a new concept: that lying behind the origin of complexity of higher organisms is the dramatic expansion of the non-protein-coding RNA inventory, including the absolute number of siRNAs and miRNAs, rather than an increase in the protein-encoding inventory (Mattick 2004).

terminal differentiation programs in a stable end state by dumping expression of genes that should not be expressed in particular cell types (Kloosterman and Plasterk 2006). The rapidly expanding repertoire of miRNAs and their targets suggests that miRNAs played a role in the adaptive diversification of pre-existing transcriptional and cell-cell signaling pathways required for the evolution of new regulatory circuits and, ultimately, new patterns within body plans. Thereby, miRNAs appear to drive animal and plant diversity (Carthew 2006; Ambros and Chen 2007; Niwa and Slack 2007). Consistent with this notion that miRNAs are global regulators of cell differentiation and patterning, miRNAs have thus far only been identified in multicellular organisms. They are notably absent from unicellular organisms including *Schizosaccharomyces pombe* and *Tetrahymena thermophila*, in which RNA silencing mechanisms are known to operate. However, in this issue of *Genes & Development*, Qi and coworkers (Zhao et al. 2007) add an exciting new twist to this developing story, showing that the unicellular green alga *Chlamydomonas reinhardtii* encodes many miRNAs.

Long known for their roles in motility, flagella (or cilia) are increasingly being recognized as sites for transmitting signals to the cytoplasm and nuclei to control gene expression, cell function, animal development, and behavior (Snell et al. 2004; Scholey and Anderson 2006). The unicellular biflagellate alga *C. reinhardtii* has been a productive model system for molecular genetic studies

of flagellar assembly and function, as well as of basal body function, for more than five decades, mainly because its flagellated vegetative cells are haploid and its mutants can be genetically analyzed by sexual crosses between different strains (Schroda 2006). Mating is initiated when the cells are starved for nitrogen and involves the shedding of the cell wall prior to cytoplasmic fusion of opposite mating types. After nuclear and chloroplast fusion, flagella are lost and a zygotic cell wall begins to form. Meiosis is initiated in zygotes by reintroduction of nutrients and is completed to produce four meiotic zoospores. The development of reliable procedures for transformation has also facilitated the study of various aspects of cell biology of *C. reinhardtii*. Additionally, RNAi has been shown to be an effective reverse genetic approach in this organism. This indicates that *C. reinhardtii* possesses functional RNAi machinery.

Zhao et al. (2007) now report the identification and characterization of small RNAs in *C. reinhardtii* using a highly parallel pyrosequencing methodology (Margulies et al. 2005). Computational studies show that among 4182 unique small RNA sequences identified, 200 species were derived from genomic sequences with the potential to form hairpin structures that meet the requirements for an miRNA precursor. These RNAs share several common features with miRNAs, including having a 5' phosphate and a preference for uracil at the 5' end. In addition, several of the miRNAs identified have a sequenced pairing miRNA* located in the opposite arm of the precursor, which is capable of forming a duplex bearing the 2-nt 3' overhang—the hallmark of Dicer cleavage (Tomari and Zamore 2005; Kim 2006). These findings show that *C. reinhardtii* encodes miRNAs—the first evidence of miRNAs in a unicellular organism.

None of the *C. reinhardtii* miRNAs had an ortholog in other green algae, plants, or animals, suggesting that *C. reinhardtii* miRNA genes may have arisen independently after splitting from a common lineage. A substantial number of the *C. reinhardtii* miRNAs are located in relatively long hairpins, many of which have the potential to produce multiple small RNAs. These observations are similar to recent results from plants that suggest that some miRNA genes evolve de novo through inverted duplication of their future target genes (Allen et al. 2004). This hypothesis predicts that recently evolved, and therefore presumably nonconserved, miRNAs might still contain long segments with high similarity to the target gene sequence. Indeed, a number of nonconserved *Arabidopsis thaliana* miRNA loci have been identified to fulfill these criteria (Allen et al. 2004; Rajagopalan et al. 2006). Whether some of the *C. reinhardtii* miRNAs fulfill the criteria remains to be determined. Given their highly evolving nature, newly evolved miRNAs are ideally suited to convey rapid adaptation to an environment and, therefore, will probably be more abundant in their stress responses (Voinnet 2004). Interestingly, computational studies have also predicted many genes encoding flagellar-associated proteins as targets for the *C. reinhardtii* miRNAs. *Chlamydomonas* cells shed their flagella in response to many stressful conditions, presum-

ably serving a protective function for cells in the particular environment. Then, after the shedding, *Chlamydomonas* cells begin to regenerate flagella (Lefebvre 1995).

Are the *C. reinhardtii* miRNAs functional? Zhao et al. (2007) fractionated total extracts from *C. reinhardtii* cultures by gel filtration and found that the *C. reinhardtii* miRNAs exist in complexes with a molecular size of ~150 kDa, a size that is similar to that of minimal RISC in human and *Drosophila* cells (Martinez et al. 2002). If miRNAs have sufficient complementarity to the target RNA, they can direct cleavage of the target between 10 and 11 nt from the 5' end of the miRNA, as is the case for siRNAs in RNAi (Yekta et al. 2004). *C. reinhardtii* miRNAs were cofractionated with the site-specific cleavage activity of their predicted mRNA targets, much like "Slicer" activity of some Argonaute proteins (Parker and Barford 2006). The *Chlamydomonas* genome encodes three Dicer-like and at least two Argonaute proteins (Schroda 2006). It remains to be determined whether an Argonaute protein resides in *C. reinhardtii* miRNP complexes and which cellular processes they are involved in. Do the *C. reinhardtii* miRNAs act to regulate genes involved in, for example, specification of flagellar assembly, functioning in a manner analogous to cell fate decisions in multicellular organisms that are triggered by signal transduction activity (Bartel 2004; Carthew 2006; Kloosterman and Plasterk 2006)? It is probably noteworthy that *Chlamydomonas* gametes use their flagella as sensory transducers during fertilization (Snell et al. 2004; Scholey and Anderson 2006). The interaction between the flagella of cells of opposite mating types initiates a series of morphological changes, including the release of their extracellular matrix (cell wall), modification and elongation of the flagellar tip, and erection of microvillus-like cell fusion organelles. Some of the *C. reinhardtii* miRNAs appear to be differentially expressed in a vegetative state and in gametes, suggesting that they might play roles in gametic differentiation. It will be interesting to see how these processes are affected when some or all of the *C. reinhardtii* miRNAs are depleted, and to analyze whether certain transcripts or proteins are differently expressed in such miRNA-depleted and control gametes.

In addition to miRNAs, Zhao et al. (2007) identified a large number of endogenous siRNAs. Their 5' ends also show the strong preference for uracil that is characteristic of small RNAs (Kim 2006). They are derived from various locations including protein-coding genes and intergenic regions. Many of them are enriched in several genomic loci and phased relative to each other, reminiscent of plant *trans*-acting siRNAs (tasiRNAs), whose biogenesis requires miRNA-directed cleavage (Allen et al. 2005; Yoshikawa et al. 2005; Axtell et al. 2006). Although many endogenous siRNAs are products of RNA-dependent RNA polymerases (RdRPs) in plants and *C. elegans* (Cerutti and Casas-Mollano 2006; Vazquez 2006), *Chlamydomonas* lacks an RdRP in its genome (Schroda 2006). Very few *C. reinhardtii* siRNAs arise from repetitive sequences and transposons. In fact, repetitive sequences are underrepresented. This situation

appears similar to mammalian piRNAs (Aravin et al. 2006; Girard et al. 2006; Lau et al. 2006). It remains to be determined whether these endogenous siRNAs in *C. reinhardtii* use a molecular mechanism similar to that of mammalian piRNAs to silence portions of the genome (Kim 2006) or whether they use a molecular mechanism similar to that of plant tasiRNAs to silence messages from loci that are unrelated to those from which the siRNAs derive (Vazquez 2006).

There are many more questions ensuing from these studies. Are miRNAs found in unicellular organisms other than *C. reinhardtii*? The giant unicellular alga *Acetabularia* may be one such candidate. Control of developmental morphogenesis in this enormous cell (2–4 cm) was studied by J. Hämmerling in the 1930s (Hämmerling 1934; Mandoli 1998), but the morphogenetic signals involved in his intriguing experiments are still to be explored using modern molecular techniques (Mandoli 1998). Are other classes of small RNAs found in *C. reinhardtii*? Zhao et al. (2007) isolated small RNAs from *C. reinhardtii* cultures under vegetative growth conditions. By analogy with piRNAs in the mammalian germline, *C. reinhardtii* gametes may express specific classes of small RNAs. Is there a class of siRNAs in *C. reinhardtii* that can direct chromatin modifications in the newly replicated "zygotic" nucleus and thus induce heritable silencing? Perhaps the most crucial question is how the surprisingly large number of siRNAs and miRNAs in *C. reinhardtii* connect between genotype and phenotype. Further investigation should reveal the physiology of small RNAs in *C. reinhardtii* and help expand our understanding of the physiology of small RNAs in multicellular organisms.

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