The small RNA world

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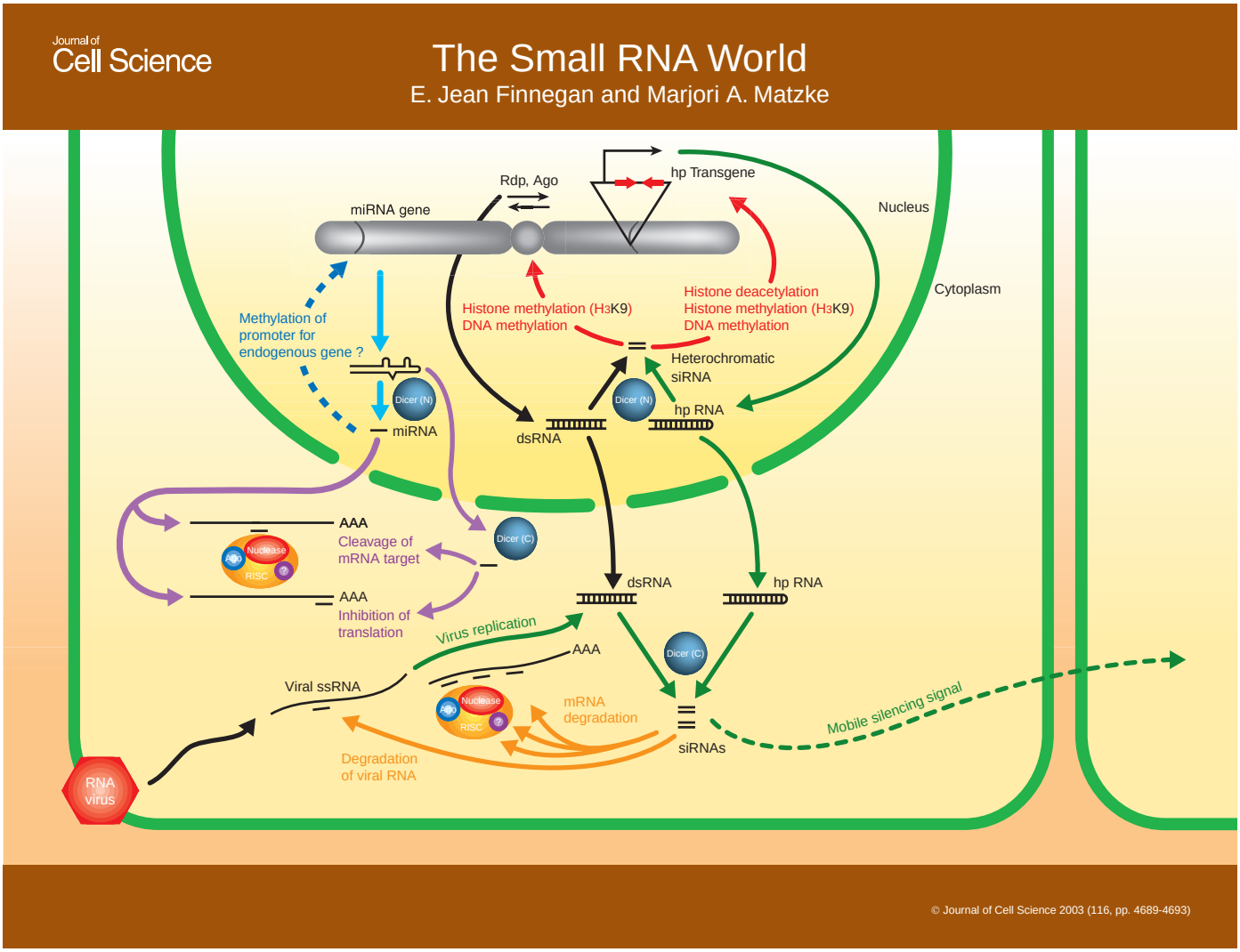
Small RNAs have burst on the scene as ubiquitous, versatile repressors of gene expression in plants, animals and many fungi. These tiny RNAs (~21-26 nt), which induce silencing through homologous sequence interactions, come in many guises: short interfering (si) RNAs (Elbashir et al., 2001), small temporal (st) RNAs (Pasquinelli et al., 2000), heterochromatic siRNAs (Reinhart and Bartel, 2002), tiny noncoding RNAs (Ambros et al., 2003a) and microRNAs (miRNAs) (Lee and Ambros, 2001; Lau et al., 2001; Lagos-Quintana et al., 2001). They can control mRNA stability or translation, or target epigenetic modifications to specific regions of the genome. Small RNAs and evolutionarily conserved RNA-mediated silencing pathways have established a new paradigm for understanding eukaryotic gene regulation and revealed novel host defenses to viruses and transposons.

Small regulatory RNAs are generated via processing of longer double-stranded RNA (dsRNA) precursors by an RNaseIII-like enzyme termed Dicer (Bernstein et al., 2001). Despite the overall conservation of RNA-mediated silencing mechanisms, this key enzyme has diversified structurally and numerically in different taxa (Schauer et al., 2002). While there is a single Dicer protein in mammals, *Caenorhabditis elegans* and fission yeast, *Drosophila melanogaster* has two, and in *Arabidopsis thaliana* there are four dicer-like (DCL) proteins (Schauer et al., 2002). Two *Arabidopsis* DCL proteins (DCL1 and DCL4) contain one or more predicted nuclear localization signals (NLS), suggesting both nuclear and cytoplasmic pathways for processing dsRNA in plants. Indeed, there is evidence that DCL1 is a nuclear protein (Papp et al., 2003). Nuclear processing of dsRNA might also occur in other organisms: one of the two Dicers in *Drosophila* contains a predicted NLS as does the single mammalian Dicer



(See poster insert)

(Schauer et al., 2002). Although the mammalian Dicer has been reported to be located in the cytoplasm (Billy et al., 2001), a recent study has suggested that it can process nonpolyadenylated dsRNA in the nucleus (Shinagawa and Ishii, 2003). Therefore, both nuclear (N) and cytoplasmic (C) Dicer activities are depicted here, the caveat being that both forms may not be present in all organisms. Although not illustrated here, Dicer acts in complexes with other proteins, including members of the Argonaute family (Carmell et al., 2002) and possibly HEN1 (Park et al., 2002; Boutet et al., 2003), to produce small RNAs.

siRNAs are associated with silencing triggered by transgenes, microinjected RNA, viruses, and transposons, and hence can be considered intermediaries in host defense pathways against foreign nucleic acids. siRNAs were first detected in plants (Hamilton and Baulcombe, 1999). In general, siRNAs can be derived from all regions of perfect duplex RNAs and, at least in plants, they accumulate in both sense and antisense polarities. Perfect duplex RNAs can be produced by transcription of a 'hairpin' (hp) transgene, which produces a corresponding hpRNA (see nuclear green pathway). In *Arabidopsis*, the DCL activity that produces siRNAs has not yet been identified; however, DCL1 does not appear to be involved (Finnegan et al., 2003; Papp et al., 2003). Although not illustrated here, plants make two functionally distinct size classes of small RNA. A shorter class, 21-22 nt, has been implicated in mRNA degradation, and a longer size class, 24-26 nt, in directing DNA methylation and in systemic silencing (Hamilton et al., 2002). Distinct DCL activities may be required for the production of these two size classes of small RNA (Tang et al., 2003; Papp et al., 2003).

miRNAs are small RNAs that downregulate endogenous genes important for implementing developmental programs in animals and plants (Carrington and Ambros, 2003; Bartel and Bartel, 2003; Hunter and Poethig, 2003). The classic miRNAs, *lin-4* and *let-7* [originally termed stRNAs (Pasquinelli et al., 2000)], were discovered by way of their heterochronic mutant phenotypes in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000). Efforts to clone size-selected RNAs have revealed numerous miRNAs in *C. elegans*, *Arabidopsis* and mice (Lagos-Quintana et al., 2001; Lee and Ambros, 2001; Lau et al., 2001; Llave et al., 2002a; Reinhart et al., 2002; Lagos-Quintana et al., 2002; Park et al., 2002; Ambros et al., 2003a). miRNAs are derived via Dicer cleavage of imperfect duplex RNAs, ~70-200 nt in length (Hutvágner et al., 2001; Grishok et al., 2001; Ambros et al., 2003b), that are encoded in intergenic regions of plant and animal genomes. miRNAs accumulate in one orientation and often from one region only of the dsRNA precursor. In *Arabidopsis*, DCL1 has been shown to process miRNA precursors (Rhoades et al., 2002; Park et al., 2002). There is indirect evidence that this can occur in the nucleus, which is consistent with the apparent nuclear location of DCL1 (Papp et al., 2003) (light blue pathway). Other miRNA precursors might be processed by Dicer activities in the cytoplasm (Lee et al., 2002) (see purple pathway). The expression of many miRNA genes is developmentally regulated in *Arabidopsis*, *C. elegans*, mice and *Drosophila* (Reinhart et al., 2002; Park et al., 2002; Llave et al., 2002a; Pasquinelli and Ruvkun, 2002; Lagos-Quintana et al., 2002; Ambros et al., 2003a; Brennecke et al., 2003). This might be due to the presence of temporal regulatory elements in the promoters of miRNA genes, as has been shown for *let-7* miRNA in *C. elegans* (Johnson et al., 2003).

miRNAs and siRNAs silence at the post-transcriptional level by virtue of their sequence complementarity to target mRNAs. siRNAs associate with an endonuclease-containing complex, RISC (RNA induced silencing complex) (Hammond et al., 2000), and cause degradation of cognate mRNAs and, in plants, viral RNAs (see orange pathway). This process is termed RNAi in animals, post-transcriptional gene silencing (PTGS) in plants, and quelling in the filamentous fungus *Neurospora crassa* (Zamore, 2002; Waterhouse and Helliwell, 2003; Denli and Hannon, 2003; Pickford and Cogoni, 2003). miRNAs, which also appear to associate with a RISC-like complex (Denli and Hannon, 2003), can either base pair with the 3′ UTR of mRNAs and block translation (Olsen and Ambros, 1999) or act in the manner of siRNAs and guide mRNA degradation (Llave et al., 2002b; Kasschau et al., 2003; Boutet et al., 2003) (see purple pathway). The choice between these two pathways is probably determined by the degree of complementarity between a given miRNA and its target mRNA (Carrington and Ambros, 2003). Since most animal miRNAs base pair imperfectly with their targets, the predominant mode of silencing in these organisms is translational repression. By contrast, plant miRNAs frequently show perfect complementarity to their target and hence trigger mRNA degradation (Llave et al., 2002b; Tang et al., 2003; Kasschau et al., 2003). However, plant miRNAs complementary to 3′ UTRs can probably guide translational repression (Chen, 2003). Although the core reactions of RNA-mediated silencing are known to be common to plants and animals (Tang et al., 2003; Voinnet, 2003), the exact components of RISC in different organisms and how this complex functions to execute two seemingly disparate modes of silencing are still under investigation (Denli and Hannon, 2003). Proteins that might determine whether siRNAs or miRNAs are used as substrates by RISC are various members of the Argonaute (Ago) family (Denli and Hannon, 2003). Argonaute proteins have been isolated in RISC complexes from *Drosophila* and are required for RNAi and related phenomena in diverse organisms (Carmell et al., 2002).

Computational methods estimate the total number of miRNA genes in humans to be 200-255 and in *C. elegans* up to 123 (Lim et al., 2003a; Lim et al., 2003b). Identifying the targets of these miRNAs is a high priority. In *C. elegans*, *lin-4* represses LIN-14 and LIN-28, which regulate early developmental transitions (Carrington and Ambros, 2003), and *let-7* represses LIN-41, which is involved in controlling later transitions (Banerjee and Slack, 2002). A recently validated target of an miRNA in *C. elegans* is the *hunchback-like* gene *hbl1-1*, which controls developmental timing (Abrahante et al., 2003; Lin et al.,

2003). The *bantam* miRNA in *Drosophila* targets the *Hid1* gene involved in apoptosis (Brennecke et al., 2003). Hes1, a basic helix-loop-helix transcriptional repressor, is regulated by miRNA-23 in human neuronal cells (Kawasaki and Taira, 2003). Although computational approaches have not yet been used to estimate the number of miRNAs in plants, cloning of small RNAs has recovered 19 unique miRNAs encoded by 41 miRNA genes (Bartel and Bartel, 2003), which is likely to be only a fraction of the total number. Many of the *Arabidopsis* miRNAs are complementary to mRNAs of transcription factors that control fate decisions in the shoot meristem (Rhoades et al., 2002; Llave et al., 2002b). Others regulate RNA metabolism (Bartel and Bartel, 2003), including an miRNA that regulates the level of DCL1 mRNA (Xie et al., 2003).

Recent work has identified links between RNAi and epigenetic alterations of the genome, such as DNA methylation and histone modifications. RNA-directed DNA methylation (RdDM), first discovered in plants (Wassenegger et al., 1994), requires a dsRNA that is processed to 21-24 nt small RNAs. In *Arabidopsis*, links between locus-specific small RNAs, DNA methyltransferases, and histone modifications, including deacetylation (Aufsatz et al., 2002) and histone H3 lysine 9 (H3K9) methylation (Jackson et al., 2002; Zilberman et al., 2003) have been identified (see red pathway). Some small RNAs identified so far in *Arabidopsis* might target native promoters of endogenous genes (Park et al., 2002) (see dashed blue pathway).

The connection between RNAi and chromatin modifications has been solidified by results from fission yeast showing that small RNAs complementary to centromeric repeats and proteins of the RNAi pathway [Dicer, RNA-dependent RNA polymerase (Rdp) and Ago] are required for histone H3K9 methylation and centromere function (Volpe et al., 2002; Volpe et al., 2003; Hall et al., 2003). Similarly, a copy of the centromeric repeat at the mating type locus in *S. pombe* is also a target of RNAi-mediated heterochromatin formation (Hall et al., 2002). The centromeric siRNAs, termed 'heterochromatic siRNAs' to indicate their involvement in epigenetic modifications, are derived from overlapping transcripts of centromere outer repeats (Reinhart and Bartel, 2002) (see black pathway). RNAi-dependent chromatin modifications can also target long terminal repeats of retrotransposons in *S. pombe*, thereby repressing adjacent meiotically induced genes in vegetative cells (Schramke and Allshire, 2003). RNAi-induced heterochromatin formation thus appears to be a general means for regulating gene expression in *S. pombe*. The extent to which this holds for higher organisms remains to be determined. In *Drosophila*, an Ago protein, Piwi, is required for transcriptional gene silencing mediated by polycomb group proteins (Pal Bhadra et al., 2002). Small RNAs in the category 'tiny noncoding RNAs' detected in *C. elegans* might also target chromatin modifications (Ambros et al., 2003b). The involvement of small RNAs in guiding DNA sequence elimination during nuclear development in *Tetrahymena* (Mochizuki et al., 2002; Yao et al., 2003) further widens the scope of RNA-mediated genome alterations.

RNA-mediated silencing pathways combat 'foreign' nucleic acids, as shown by silencing-deficient mutants, some of which unleash transposons (Plasterk, 2002) or, in plants, exhibit enhanced susceptibility to virus infection (Vance and Vaucheret, 2001; Voinnet, 2002). Most plant viruses have an RNA genome and synthesize dsRNA during their replication cycle, which can potentially trigger siRNA production and eventually viral genome degradation (see green and orange pathways). Substantiating a role for RNA-mediated silencing in defense against viruses is the fact that many plant viruses encode proteins that can suppress silencing at different points in the pathway (Hamilton et al., 2002; Mallory et al., 2002) and potentially interfere with plant development by blocking miRNA-guided mRNA cleavage (Kasschau et al., 2003). Although an antiviral role for RNAi has been suggested in *Drosophila* (Li et al., 2002), we do not yet know whether RNAi has a similar function in mammalian cells (Gitlin and Andino, 2003).

A final aspect of small RNAs considered here is systemic silencing, which has been documented in plants (Palauqui et al., 1997; Voinnet et al., 1998) and *C. elegans* (Timmons and Fire, 1998; Winston et al., 2002) but does not occur in *Drosophila* (Roignant et al., 2003). A silencing signal, probably siRNA (Himber et al., 2003), is able to move from cell to cell and, in plants, enter the vascular system to induce sequence-specific silencing at distant sites (Mlotshwa et al., 2002) (see dotted green pathway).

The astonishing diversity of regulatory pathways directed by small RNA molecules has been unraveled through a combination of genetic and biochemical approaches. This diversity is achieved through the association of these RNAs with various protein partners in complexes that degrade cognate viral or mRNAs, block translation or modify chromatin structure. Many components of these complexes – for example, the endonuclease component of the RISC complex – have yet to be identified. These ribonucleoprotein complexes are directed to their targets through sequence complementarity between the target and small RNA. Challenges that remain are to identify the targets of many of the putative miRNAs and to determine how small RNAs are channeled into the various pathways by association with the appropriate protein partners.

Note added in proof

Cloning of small RNAs from *Drosophila* has recovered 62 non-redundant microRNAs and 178 repeat-associated small interfering RNAs (rasiRNAs), which are derived from every known type of transposon in the *Drosophila* genome. These rasiRNAs potentially regulate transposon mobility and assembly of heterochromatin at transposon-rich regions such as telomeres and centromeres (Aravin et al., 2003).

The *Drosophila* protein R2D2, which is homologous to *C. elegans* RNAi protein RDE-4, associates with Dicer to facilitate siRNA binding and sequence-specific mRNA degradation by the

RNA-silencing induced complex (RISC) (Liu et al., 2003).

Although initial reports indicated that only miRNAs with perfect sequence complementarity to their targets cause cleavage of target mRNA, a recent study showed that this is not always the case. *Arabidopsis* miR-JAW regulates five members of the TCP family of transcription factors by mRNA cleavage, even though it has incomplete sequence complementarity with all five *TCP* transcripts (Palatinik et al., 2003).

The C. elegans protein SID-1 is sufficient to mediate the uptake of dsRNA into cells and is required for systemic RNAi. Expression of SID-1 in *Drosophila*, which lack systemic RNAi, confers the ability to take up dsRNA on *Drosophila* cells (Feinberg and Hunter, 2003).

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