

REVIEW

Perspective: machines for RNAi

Yukihide Tomari and Phillip D. Zamore¹

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA

RNA silencing pathways convert the sequence information in long RNA, typically double-stranded RNA, into ~21-nt RNA signaling molecules such as small interfering RNAs (siRNAs) and microRNAs (miRNAs). siRNAs and miRNAs provide specificity to protein effector complexes that repress mRNA transcription or translation, or catalyze mRNA destruction. Here, we review our current understanding of how small RNAs are produced, how they are loaded into protein complexes, and how they repress gene expression.

Three questions have guided the study of RNA silencing (Box 1) for nearly a decade: What is the molecular nature of the silencing trigger? What are the silencing targets? And what proteins comprise the RNAi machinery? These questions continue to organize the study of RNA silencing, even as new questions emerge about its mechanism and how the pathway is woven into the broader fabric of cell metabolism and plant and animal development.

Triggers and guides

Silencing triggers are nucleic acids that initiate the assembly of protein–RNA complexes that repress expression of specific target genes by reducing their rates of transcription, the stability of their mRNAs, or the translation of their mRNAs into protein. Silencing triggers identify their nucleic acid targets by sequence-specific base pairing. The best studied silencing trigger is double-stranded RNA (dsRNA), which can be produced by bidirectional transcription, transcription of an inverted repeat or “hairpin” sequence, or physically introduced into cells by injection, soaking, or even feeding dsRNA-expressing bacteria (Kennerdell and Carthew 1998; Montgomery and Fire 1998; Tabara et al. 1998; Timmons and Fire 1998; Waterhouse et al. 1998; Clemens et al. 2000; Billy et al. 2001). Transgenes intended to generate only

Box 1. RNA silencing primer.

RNA silencing was first observed in plants (**post-transcriptional gene silencing, PTGS**) and fungi (“**quelling**”), where attempts to overexpress endogenous genes by introducing transgenic copies of the endogene instead blocked expression of both (Cogoni et al. 1996; Jorgensen 2003). In animals, RNA silencing was first reported when Guo and Kemphues (1995) used antisense RNA to block *par-1* mRNA expression in *Caenorhabditis elegans* but found that the *par-1* mRNA itself also repressed *par-1*. Their paradoxical observation—subsequently dubbed **RNA interference (RNAi)**—inspired the experiments of Fire, Mello, and colleagues (Fire et al. 1998), who showed that **double-stranded RNA (dsRNA)**, was the **trigger** of gene silencing. RNAi is but one aspect of a larger web of sequence-specific, cellular responses to RNA known collectively as RNA silencing. All RNA silencing pathways are triggered by 21–27-nt-long “**small RNAs**”—a term that encompasses **small interfering RNAs (siRNAs)**, **repeat-associated small interfering RNAs (rasiRNAs)**, and **micro RNAs (miRNAs)**. These silencing pathways share a common set of proteins that produce or amplify small RNAs and couple small RNAs to specific regulatory outcomes. Small RNAs guide RNA silencing effector complexes, like the **RNA-induced silencing complex (RISC)** (Hammond et al. 2000) or the **RNA-induced initiation of transcriptional gene silencing (RITS)** complex (Verdel et al. 2004). A member of the **Argonaute** family of proteins lies at the heart of all RNA silencing effector complexes (Tabara et al. 1999, 2002; Hammond et al. 2001; Hutvagner et al. 2001; Catalanotto et al. 2002; Martinez et al. 2002; Mourelatos et al. 2002; Djikeng et al. 2003; Shi et al. 2004). RNA silencing proteins, often called the “**RNAi machinery**”, also include the multidomain ribonuclease III enzyme **Dicer**, which produces small RNAs and **RNA-dependent RNA polymerases (RdRPs)**, which amplify silencing triggers (see Box 2).

sense or antisense RNA also silence gene expression, especially in plants (Baulcombe 1996, 2004; Jorgensen 2003). The copying of these transgene-encoded single-stranded RNAs into dsRNA is thought to convert them into silencing triggers (Jorgensen 2003; Baulcombe 2004). We do not understand how cells can distinguish between abundant cellular mRNAs, which do not trigger silencing, and aberrant RNAs, which do. Current data support a threshold sensing model, in which “aberrant” single-

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¹**Corresponding author.**

E-MAIL phillip.zamore@umassmed.edu; FAX (508) 856-2003.

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stranded RNA accumulates in vivo beyond some critical level, triggering its copying into dsRNA (Zamore 2001; Jorgensen 2003). The absence of in vitro systems that recapitulate RNA silencing triggered by single-stranded RNA has limited progress on this problem, which is central to silencing in nematodes, fungi, and plants, but does not seem to play a role in flies or mammals (Box 2).

The path from dsRNA trigger to silenced gene is now broadly outlined. The discovery that silencing is heritable in worms (Grishok et al. 2000) and can spread the

Box 2. Amplification of silencing triggers.

RNA silencing pathways can be divided into those that require RdRPs and those that do not. In *Caenorhabditis elegans* and fungi like *Neurospora crassa* and *Schizosaccharomyces pombe*, genes encoding RdRP enzymes are required for silencing (Cogoni and Macino 1999; Dalmay et al. 2000; Mourrain et al. 2000; Smardon et al. 2000; Sijen et al. 2001; Hall et al. 2002; Volpe et al. 2002). In plants, RdRPs are required for silencing initiated by single-stranded RNA triggers (Dalmay et al. 2000), but perhaps not for silencing triggered by dsRNA (Waterhouse et al. 1998). In *C. elegans*, dsRNA is thought to first be diced into rare **primary siRNAs** and then amplified by RdRP enzymes to create more abundant **secondary siRNAs** (Sijen et al. 2001). Two models have been proposed to explain the role of RdRPs. First, RdRPs might use primary siRNAs to prime the synthesis of dsRNA using the target mRNA itself as a template. Primed synthesis of dsRNA by RdRPs can lead to **spreading**, the production of siRNAs encoded by the target gene but not by the trigger RNA. Such spreading along the target should only occur 5' to the dsRNA trigger, as is observed in worms (Sijen et al. 2001). In plants, however, siRNAs spread both 5' and 3' along the target, relative to the trigger RNA (Voinnet et al. 1998; Vaistij et al. 2002), hence the notion of an unprimed RdRP pathway, in which single-stranded RNA fragments are copied without the use of siRNA primers. Primed and unprimed RdRP activities have been detected for purified RdRP enzymes and in plant extracts (Schiebel et al. 1993a,b, 1998; Makeyev and Bamford 2002; Tang et al. 2003). Spreading of siRNAs is linked to **systemic silencing**, a phenomenon observed in worms and plants in which locally initiated silencing can be inherited or can spread systemically to distant parts of the organism (Boerjan et al. 1994; Fire et al. 1998). Recent evidence suggests that protein-bound siRNAs flowing through the vasculature cause systemic silencing in plants (Yoo et al. 2004). Thus, siRNAs function as both intracellular and extracellular signaling molecules.

RdRPs do not seem to play any role in the *Drosophila* and mammalian RNAi pathways, where amplification and spreading have not been detected in vivo, perhaps because no RdRP homologs are present in the genomes of flies or mammals (Celotto and Graveley 2002; Chiu and Rana 2002; Schwarz et al. 2002; Roignant et al. 2003). In this regard, the *Drosophila* and mammalian RNAi pathways resemble the miRNA pathway in plants, which does not involve RdRPs or spreading (Parizotto et al. 2004; Xie et al. 2004). The absence of an RdRP-dependent amplification step may explain the relatively short duration of RNAi in dividing cells in these organisms (Holen et al. 2002). For an alternative perspective, the reader should consult Lipardi et al. (2001).

length of a branch through the vasculature of higher plants (Voinnet and Baulcombe 1997) suggested that the initial silencing trigger was converted into a stable specificity determinant, a novel intermediate with a structure or chemistry uniquely enabling it to target a gene for silencing. The work of plant virologists Hamilton and Baulcombe (1999) and of two teams of biochemists working with extracts of *Drosophila* cells (Hammond et al. 2000; Zamore et al. 2000) collectively demonstrated that the dsRNA is converted by endonucleolytic cleavage into a new class of intracellular signaling molecules, small interfering RNAs (siRNAs). These 21–25-nt double-stranded RNAs have a characteristic structure that reflects their production by the multidomain ribonuclease III (RNase III) enzyme, Dicer (Bernstein et al. 2001; Elbashir et al. 2001a,b; Ketting et al. 2001; Knight and Bass 2001; Xie et al. 2004). siRNAs and related small RNAs are the first class of RNA-based signaling molecules that couple an input—a single- or double-stranded RNA trigger—to a regulatory output, a reduction in gene expression (i.e., “silencing”). Moreover, the sequence and structure of a small RNA determines how it functions to effect silencing as well as the identity of its target genes.

The discovery that exogenous dsRNA is converted into siRNAs led directly to the recognition that microRNAs (miRNAs) and endogenous siRNAs regulate endogenous gene expression via the RNAi pathway (Fig. 1; Lee et al. 1993; Pasquinelli et al. 2000; Reinhart et al. 2000, 2002; Aravin et al. 2001, 2003; Lagos-Quintana et al. 2001, 2002, 2003; Lau et al. 2001; Lee and Ambros 2001; Llave et al. 2002a; Mourelatos et al. 2002; Reinhart and Bartel 2002; Brennecke and Cohen 2003; Brennecke et al. 2003; Chen 2004; Chen et al. 2004; Dostie et al. 2003; Johnston and Hobert 2003; Lim et al. 2003a,b; Palatnik et al. 2003; Chang et al. 2004). miRNAs are cousins of siRNAs: They are endogenous small RNA guides that repress the expression of target genes. miRNAs differ from siRNAs in their biogenesis, not in their functions (Hutvagner and Zamore 2002; Ambros et al. 2003; Aukerman and Sakai 2003; Chen 2004; Chen et al. 2004; Doench et al. 2003; Saxena et al. 2003; Zeng et al. 2003; Yekta et al. 2004). Like siRNAs, plant and animal miRNAs can direct cleavage of their mRNA targets when the two are extensively complementary (Hutvagner and Zamore 2002; Llave et al. 2002b; Tang et al. 2003; Xie et al. 2003; Zeng and Cullen 2003; Mallory et al. 2004; Okamura et al. 2004; Yekta et al. 2004), but repress mRNA translation when they are not (Olsen and Ambros 1999; Chen 2004; Doench et al. 2003; Saxena et al. 2003; Zeng et al. 2003; Doench and Sharp 2004). The sequence of a miRNA or an siRNA may determine how effectively it directs cleavage or translational repression.

Both siRNAs and miRNAs can also trigger methylation of the DNA of protein-coding genes that share their sequence in plants (Zilberman et al. 2003; Bao et al. 2004) and perhaps even in mammals (Kawasaki and Taira 2004; Morris et al. 2004). siRNAs can direct the construction of repressive heterochromatin, especially centromeric heterochromatin (Shiu et al. 2001; Hall et

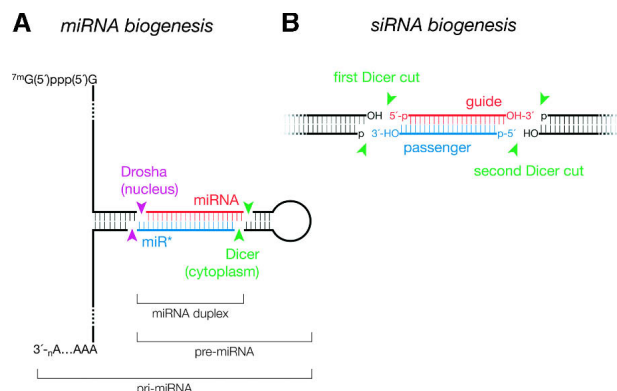


Figure 1. Small RNA biogenesis in animals. (A) miRNA are produced by the successive actions of two RNase III ribonucleases. After their transcription by RNA polymerase II, primary miRNA (pri-miRNA) are cleaved in the nucleus by Drosha, likely as a heterodimeric complex with the dsRNA-binding protein, Pasha, which forms the whimsically named microProcessor complex. Drosha cleavage generates the pre-miRNA, which binds Exportin 5 and is exported to the cytoplasm. In the cytoplasm, Dicer is thought to bind the base of the pre-miRNA stem defined in the nucleus by Drosha. Dicer cleavage liberates a duplex comprising the miRNA and miR* strands of the pre-miRNA. The miRNA must then be unwound and selectively incorporated into RISC by the miRNA-specific RISC assembly machinery. (B) Long dsRNA is a substrate for Dicer, but not for Drosha. Dicer must make two successive pairs of cuts to yield an siRNA duplex. Dicer is thought to preferentially initiate dsRNA cleavage at the ends of dsRNA, a phenomenon that sometimes produces a phased string of siRNAs along the dsRNA. The siRNA-specific RISC assembly machinery selectively loads the guide strand into RISC; the passenger strand is degraded.

al. 2002; Mochizuki et al. 2002; Volpe et al. 2002; Schramke and Allshire 2003; Liu et al. 2004b; Noma et al. 2004; Verdel et al. 2004). As a class, miRNA and siRNA guide strands are functionally indistinguishable, but it is still unknown if all miRNAs and siRNA sequences are capable of effectively guiding all RNA silencing functions.

Cellular miRNAs are derived from long primary RNAs (pri-miRNAs) transcribed by RNA Polymerase II (Bracht et al. 2004; Cai et al. 2004; Lee et al. 2004a; Parizotto et al. 2004). Animal viruses also encode miRNAs (Ben-nasser et al. 2004; Pfeffer et al. 2004); these may be transcribed by RNA Polymerase II or III. pri-miRNAs contain stem-loop structures that harbor the miRNA in the 5' or 3' half of the stem (Fig. 1; Lee et al. 2002). In animals, the stem-loops are ~70 nt long, and Drosha, a nuclear RNase III endonuclease, liberates the stem-loop from the primary transcript to yield a pre-miRNA (Lee et al. 2003). The pair of cuts made by Drosha establishes one end of the miRNA (Basyuk et al. 2003; Lee et al. 2003). In these efforts, Drosha collaborates with a double-stranded RNA-binding protein, called Pasha in flies and DGCR8 in humans (Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). Pasha is required for Drosha function, but why is not yet known. The pre-

miRNA liberated by Drosha is exported from the nucleus by the protein Exportin 5 (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004; Zeng and Cullen 2004), then cut by Dicer in the cytoplasm to produce the mature miRNA (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Park et al. 2002). In some organisms, such as worms and people, the same Dicer that makes siRNAs processes pre-miRNA; in plants and flies, different Dicer paralogs make siRNAs and miRNA (Lee et al. 2004b; Xie et al. 2004). In plants, a nuclear Dicer may double as both Drosha and Dicer (Finnegan et al. 2003; Papp et al. 2003; Kurihara and Watanabe 2004; Xie et al. 2004).

All current evidence suggests that both miRNAs and siRNAs are born double-stranded, but accumulate in functional complexes as single-strands. The most compelling evidence that miRNAs initially comprise two strands is that miRNA biogenesis can be blocked in both plants and animals by the viral protein p19, a suppressor of the RNA silencing defenses that plants mount against viral infection. p19 binds double-stranded siRNA-like structures but not single-stranded RNAs (Vargason et al. 2003; Ye et al. 2003; Lakatos et al. 2004). Thus, the simplest explanation for p19 protein blocking miRNA function is that miRNAs are transiently double-stranded. Each miRNA or siRNA duplex can potentially yield two single-stranded ~21-nt RNAs capable of directing RNA silencing, so new vocabulary is needed to describe the strands. Only one of the two possible small RNAs residing in the stem of a pre-miRNA usually accumulates in vivo; this is the mature miRNA. The underrepresented strand is the miR* ("meer star"). (The corresponding strands of an siRNA are the guide strand, which accumulates in RISC, and the passenger strand, which is largely destroyed.) Consistent with the view that miRNA are initially born as duplexes, from which the mature miRNA is selected and the miR* strand degraded, p19 protein and another suppressor of RNA silencing, p21 protein, stabilize miR* strands and lead to the accumulation of miR/miR* duplexes in plants (Chapman et al. 2004; Dunoyer et al. 2004).

The hallmarks of highly effective siRNA

In 2003, two types of experiments to understand siRNA function converged on a single insight: siRNA structure and sequence determine which siRNA strand enters RISC as the guide strand and which is excluded from RISC, becoming the passenger strand. Several groups, but most comprehensively, the team of Khvorova and colleagues, used informatic methods to understand why some siRNAs successfully knock-down their intended mRNA targets while others do not (Aza-Blanc et al. 2003; Khvorova et al. 2003). They compared the thermodynamic profiles for hundreds of siRNAs—active and inactive—in order to decipher the signature of siRNAs that successfully function in the RNAi pathway in cultured mammalian cells. Thermodynamic profiles of siRNAs provide a graphical representation of how tightly base

paired one siRNA strand is to its complement at each position of the 19-bp helix that links the two strands. The local thermodynamic stability of nucleic acid helices is a function of the base pairs flanking an individual G:C or A:U base pair in an RNA helix. Thus, a “nearest-neighbor” analysis can predict the local stability at each position in an siRNA (Xia et al. 2001).

Simultaneously, our own laboratory used in vitro biochemistry in *Drosophila* embryo lysates to understand how RISC, which contains single-stranded siRNA, is produced from siRNA duplexes (Schwarz et al. 2003). The biochemical and informatics efforts reached a single conclusion: The sequence and structure of an siRNA determines which of its two strands participates in the RNA silencing pathway. Consequently, some siRNAs appear inactive in vivo because the wrong strand has entered the RNAi pathway. Strategies for designing siRNAs that load the useful strand (the antisense strand) into RISC have been reviewed elsewhere (Silva et al. 2003; Gong and Ferrell 2004), and we implore readers to explore these “design rules” before synthesizing siRNAs to reduce expression of a favorite gene (Khvorova et al. 2003; Amarzguoui and Prydz 2004; Hohjoh 2004; Naito et al. 2004; Reynolds et al. 2004; Ui-Tei et al. 2004; Yiu et al. 2005). siRNAs designed to load one strand into RISC to the near exclusion of the other are called “functionally asymmetric.”

The informatics and biochemical studies also revealed a structural feature of functional siRNAs: Their ends look like those of miRNA/miR* duplexes (Khvorova et al. 2003). The strand with the less-tightly base-paired 5' end usually corresponds to the mature miRNA, whereas the miR* strand has the more-tightly paired 5' end and is degraded (Schwarz et al. 2003; Krol et al. 2004).

Building the RNAi effector complex

The central step of RISC assembly is the loading of one of the two siRNA strands into RISC. RISC assembly has only been studied in vitro for *Drosophila*, so we do not yet know if data from flies can be generalized to other organisms. In flies, siRNA and miRNA are produced by distinct Dicer enzymes; *Drosophila* Dcr-1 produces miRNAs, whereas Dcr-2 makes siRNAs (Lee et al. 2004b). Dcr-2 also acts to load one of the two siRNA strands into RISC. The function of Dcr-2 in RISC assembly is genetically distinct from its more famous role in producing siRNAs (Lee et al. 2004b). Human Dicer may also function in loading siRNA into RISC, because siRNA do not elicit silencing in human cells lacking Dicer (Doi et al. 2003). In contrast, siRNAs function to reduce target gene expression in Dicer knockout ES cells (Kanellopoulou et al. 2005). Perhaps Dicer is only required in human cells to load Ago2-containing RISC complexes, much as Dcr-2 is required only to load Ago2 in *Drosophila*.

Two similar ordered pathways have been proposed for *Drosophila* RISC assembly (Pham et al. 2004; Tomari et al. 2004a). In one scheme, RISC assembly begins with the binding of the double-stranded siRNA to as yet un-

identified protein(s) to form “complex B” (Tomari et al. 2004a). In vitro, complex B is a kinetic precursor to a more well understood complex, the RISC-loading complex (RLC; formerly “complex A”). While the case for complex B as an early intermediate in the RISC assembly pathway is incomplete, the idea that the RLC plays a central role in loading siRNA into RISC is well supported (Tomari et al. 2004a,b). The RLC contains the proteins Dcr-2 and its partner R2D2, which contains tandem dsRNA-binding domains (Tomari et al. 2004a,b). Both Dcr-2 and R2D2 are needed for RNAi in vivo (Lee et al. 2004b; Liu et al. 2003; Pham et al. 2004), for the formation of the RLC (Tomari et al. 2004a), and for siRNA unwinding (Tomari et al. 2004b). The RLC contains both double-stranded siRNA and a smaller amount of single-stranded siRNA, suggesting that siRNA unwinding initiates in this complex (Tomari et al. 2004b). In *Caenorhabditis elegans*, Dicer and the R2D2-like protein RDE-4 have been detected in a complex with RDE-1, an Argonaute protein, so Dicer may also function in RISC assembly in worms, although RDE-4 does not appear to bind siRNA, unlike R2D2 (Tabara et al. 2002).

Sontheimer and colleagues propose a RISC assembly pathway that begins with complex R1 and proceeds via complex R2 to RISC (Pham et al. 2004). Like the RLC, complexes R1 and R2 contain Dcr-2 and R2D2. Lysates prepared from embryos lacking Dcr-2 make neither R1 nor R2, whereas embryos lacking Dcr-1, which is required for the production of miRNA but not siRNA, assemble R1 but not R2 (Lee et al. 2004b; Pham et al. 2004). Which if either of these two complexes corresponds to the RLC? The molecular weight of complex R1 and its requirement for 5' phosphates but not ATP suggest that it is the same complex detected earlier as a RISC precursor (Nykänen et al. 2001), and recent data suggest that both complexes correspond to the Dcr-2/R2D2 heterodimer bound to siRNA (J.W. Pham and E.J. Sontheimer, pers. comm.). Complex R2 might then correspond to the RLC, whose assembly requires both the Dcr-2/R2D2 heterodimer and ATP. However, Complex R2 forms even when ATP is depleted (Pham et al. 2004).

The requirement for Dcr-1 for the formation of complex R2 is intriguing. Extracts from *Drosophila* S2 cells in which Dcr-1 has been depleted by RNAi still dice long dsRNA into siRNAs (Liu et al. 2003). In these lysates, Dcr-2 and R2D2 levels are normal. Yet, genetic depletion of Dcr-1 in *Drosophila* embryos reduces the efficacy of RNAi triggered by either long dsRNA or siRNA. Perhaps other components of the RNAi pathway are destabilized by the absence of Dcr-1, rather than Dcr-1 itself being required for siRNA-guided RNAi. When lysates are programmed with exogenous siRNA, Dcr-1, Dcr-2, and R2D2 cosediment as an 80S complex, suggesting they are present in a holo-RISC “assemblosome” that may load both siRNA and miRNA into RISC (Pham et al. 2004). Consistent with this view, the core RISC protein Ago2 and the auxiliary factors dFMR1 (also known as dFXR) TSN and VIG cofractionate with the 80S complex. The large size of holo-RISC may reflect the association of RISC with ribosomes. In fact, the *Drosophila* RISC com-

ponent dFMR1 interacts directly with the ribosomal proteins L5 and L11 (Ishizuka et al. 2002).

In lysates to which no siRNA has been added, Dcr-1, Ago2, dFMR1, TSN, and VIG all cosediment with the 80S complex, but not Dcr-2 and R2D2. Why is the Dcr-2/R2D2 heterodimer present in holo-RISC only when exogenous siRNA is present? Perhaps endogenous small RNAs, such as miRNAs, are produced and assembled into RISC in vivo in the holo-RISC, but siRNAs are generated separately from exogenous dsRNA triggers by the free Dcr-2/R2D2 heterodimer or by the larger, Dcr-2/R2D2-containing RLC (Fig. 2).

As unwinding of siRNA proceeds, the Dcr-2/R2D2 heterodimer, probably in the RLC, is exchanged for the core RISC protein, Ago2 (Tomari et al. 2004b). Ago2 is not thought to be a component of the RLC, because the RLC forms in its absence. However, the RLC formed in lysates lacking Ago2 does not contain any single-stranded siRNA (Tomari et al. 2004b) because Ago2 is required for siRNA unwinding (Okamura et al. 2004). Moreover, Dcr-2 and R2D2 remain stably bound to the siRNA in the absence of Ago2, as if Ago2 is required for their release (Tomari et al. 2004b). Given that the Piwi domain of human Ago2 and the RNase III domain of human Dicer interact directly (Tabbaz et al. 2004), the Dcr-2/R2D2 heterodimer containing siRNA may be recruited to the holo-RISC by its binding Ago2. Docking of Dcr-2 with Ago2 may deliver the siRNA products of an

exogenous dsRNA to the holo-RISC, perhaps changing the conformation of Ago2, Dcr-2, R2D2, or even the siRNA, thereby initiating unwinding.

Beyond its role in making siRNA, the Dcr-2/R2D2 heterodimer appears to sense siRNA thermodynamic asymmetry, because the orientation of these proteins on the siRNA duplex reflects which strand is incorporated into RISC (Tomari et al. 2004b). For functionally asymmetric siRNAs, the orientation of the heterodimer on the siRNA is determined largely by R2D2, which prefers to bind the siRNA end with greater double-stranded RNA character (Fig. 2). R2D2 binds more strongly to 5' phosphorylated siRNA ends (Tomari et al. 2004b), while the presence of a 5' phosphate does not influence Dcr-2 binding (Fig. 2; Pham et al. 2004; Tomari et al. 2004b). Thus, R2D2 also acts to authenticate siRNAs as products of Dicer, which leaves 5' phosphates on both strands (Elbashir et al. 2001a). The preference of R2D2 for 5' phosphorylated siRNA may indicate that R2D2 directly contacts the phosphate group—as has been observed for the siRNA-binding protein p19 (Vargason et al. 2003)—or may simply reflect the stabilizing effect of a 5' phosphate on RNA:RNA helices (Freier et al. 1983).

Flies lacking Dcr-2 or R2D2 show no major developmental defects (Liu et al. 2003; Lee et al. 2004b), suggesting that these proteins are not required for the biogenesis or function of miRNAs, many of which have essential developmental roles. In worms, the R2D2 homolog RDE-4 and the Argonaute protein RDE-1 are required for silencing responses to exogenous dsRNA, but not for miRNA-directed pathways (Tabara et al. 1999). By keeping the production and assembly of siRNA into RISC separate from the miRNA pathway for RISC components. The miRNA and siRNA pathways are similarly distinct in plants (Vazquez et al. 2004; Xie et al. 2004). Yet, the biogenesis of miRNA is well explained by postulating an R2D2-like sensor for miRNA/miR* duplex thermodynamic asymmetry. What then senses the asymmetry of miRNA duplexes? Mammalian cells contain a single Dicer, which generates both miRNA and siRNA. An R2D2-like partner for human Dicer remains to be identified, but could conceivably act as a sensor for both siRNA and miRNA duplex asymmetry. However, flies have two Dicers. Perhaps Dcr-1 alone senses miRNA duplex asymmetry. Unlike Dcr-2, whose PAZ domain has drifted from the canonical sequence, Dcr-1 has a canonical PAZ domain. The PAZ motif is thought to bind single stranded RNA (Lingel et al. 2003; Song et al. 2003; Yan et al. 2003; Lingel et al. 2004; Ma et al. 2004). Thus, Dcr-1 might capture the end of the miRNA duplex that has more single-stranded character. Alternatively, Dcr-1 may have a yet-to-be-identified dsRNA-binding protein partner. An R2D2-like protein, Pasha, copurifies with Drosha, the ribonuclease III enzyme that cleaves pre-miRNA from their primary transcripts and thus defines one of the two ends of each miRNA (Fig. 1; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004;

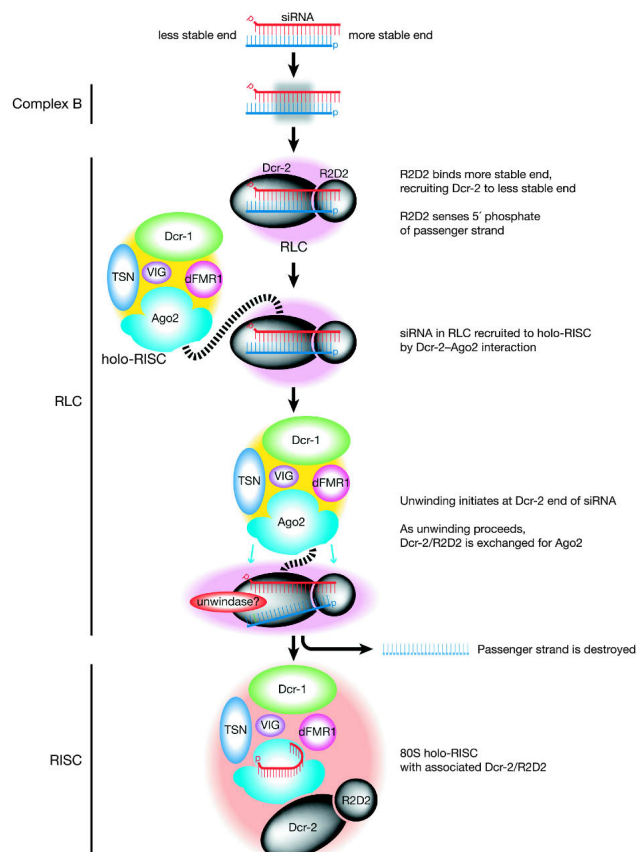


Figure 2. The RISC assembly pathway for siRNA in flies.

Landthaler et al. 2004), but Droscha and Pasha are believed to function in the nucleus, whereas miRNA loading into RISC is thought to be cytoplasmic.

Does Dicer release siRNAs, only to rebind them?

If the Dcr-2/R2D2 heterodimer acts both in siRNA production from long dsRNA and in choosing which siRNA strand is assembled into RISC, do siRNAs fall off Dicer after they are made and then rebind, or does Dicer hold onto an siRNA after dicing, loading it directly into RISC? Since Dicer seems to pay no heed to siRNA thermodynamic asymmetry when producing siRNAs, siRNAs can be generated in two orientations relative to their thermodynamics: with their more weakly paired 5' end facing forward with respect to the end of the dsRNA where Dicer is thought to initiate (Elbashir et al. 2001a; Zhang et al. 2002, 2004), or with this 5' end facing backwards. Yet, the orientation of the Dcr-2/R2D2 heterodimer in the RLC is fixed by thermodynamic differences in the ends of an siRNA duplex. What is the fate of an siRNA that is made "backwards" with respect to its thermodynamic asymmetry and therefore backwards relative to its orientation within the Dcr-2/R2D2 heterodimer in the RLC? If such an siRNA has an opportunity to leave Dicer, then rebind in the "forward" direction, then even "backwards" siRNAs could enter the RISC loading pathway. Recently, Cr  t   and colleagues (Vazquez et al. 2004) discovered an example of such a backwards siRNA in plants. They identified small RNAs arising from the intron of the gene At2g27400, which regulate expression of at least five other protein coding genes. Remarkably, siRNA production from At2g27400 is phased. The phasing of the siRNAs, with one siRNA every 21 nt, suggests that the entire cluster is made by Dicer moving in one direction. Only one of the two predicted strands of each of these siRNAs can be detected in vivo, generally in the forward direction. But for one siRNA, only the reverse strand accumulates in vivo. The relative thermodynamic stabilities of the 5' ends of each siRNA accurately predict why most of the siRNAs accumulate as forward strands, but the one accumulates in the reverse orientation. The simplest explanation for the data is that each siRNA dissociates from the Dicer active site soon after it is produced, its thermodynamics are evaluated by the RNA silencing machinery, and then one strand is selectively loaded into RISC and the other strand destroyed. Khvorova et al. (2003), interpreting the data of Carrington and coworkers (Llave et al. 2002a), reached a similar conclusion for siRNAs generated in vivo in plants into which exogenous long dsRNA was introduced.

Dcr-2 alone can dice long dsRNA but does not stably bind siRNA, whereas the Dcr-2/R2D2 heterodimer binds siRNA tightly (Liu et al. 2003; Tomari et al. 2004b). These data suggest that siRNA are free to leave Dcr-2 after they are produced. They also suggest that siRNAs may subsequently be recaptured by the Dcr-2/R2D2 heterodimer via R2D2 binding siRNA. siRNAs inhibit Dcr-2 activity in cell lysates (Tang et al. 2003), suggest-

ing that after the Dcr-2/R2D2 heterodimer has re-engaged an siRNA, it cannot interact with long dsRNA until it finishes loading the siRNA into RISC. The notion that siRNAs are made by Dicer, released, then bound again by the Dcr-2/R2D2 heterodimer is also consistent with the accumulation of free, double-stranded siRNAs observed when siRNAs are generated from long dsRNA in vitro in *Drosophila* embryo lysates (Nyk  nen et al. 2001), and with the ability of the double-stranded siRNA-binding protein, p19, to block RNAi initiated by long dsRNA in vivo in both plants and in cultured human cells (Chapman et al. 2004; Dunoyer et al. 2004; Lakatos et al. 2004). p19 protein also blocks RNAi initiated by long dsRNA or by siRNAs in vitro in *Drosophila* embryo lysates, but does not disrupt the function of Dicer in producing siRNAs or interfere with the activity of mature RISC in cleaving target RNA (Lakatos et al. 2004).

RISC function

Once loaded into RISC, small RNAs guide at least three distinct modes of silencing. In the RNAi pathway, small RNAs direct RISC to cut target RNAs. Cleavage by RISC requires Mg²⁺, leaves 3' hydroxy and 5' phosphate termini, and is inhibited by phosphorothioates at the scissile phosphate, all hallmarks of a role for Mg²⁺ in catalysis. (Martinez and Tuschl 2004; Schwarz et al. 2004). Cleavage is thought to be catalyzed by the Piwi domain of a subclass of Argonaute proteins. This domain is a structural homolog of RNase H, a Mg²⁺-dependent endoribonuclease that cleaves the RNA strand of RNA-DNA hybrids (Liu et al. 2004a; Song et al. 2004). Unlike RNase H, each cleavage-competent RISC can break only one phosphodiester bond (the "scissile phosphate") in its RNA target (Elbashir et al. 2001a,b). RISC is a multiple-turnover enzyme; the siRNA guide delivers RISC to the RNA target, the target is cleaved, then the siRNA departs intact with RISC (Hutv  gner and Zamore 2002; Haley and Zamore 2004; Martinez and Tuschl 2004).

The first position of a small RNA in RISC does not contribute to binding, as was first noted in studies designed to predict miRNA targets (Enright et al. 2003; Lewis et al. 2003; Stark et al. 2003). Instead, bases 2–8 form a "seed" that can be used computationally to pair miRNA with target candidates. The preeminent role of these bases was first proposed by Lai (2002), who noted that bases 2–8 of many miRNAs were complementary to conserved sequence elements in the 3' untranslated regions (UTRs) of post-transcriptionally repressed mRNAs in flies. It is now clear that most of the binding energy that tethers RISC to a target RNA comes from bases in the 5' half of the small RNA (Doench and Sharp 2004; Haley and Zamore 2004). This is a key difference between siRNAs or miRNAs and antisense oligonucleotides, where all bases contribute equally to specificity. In fact, complete pairing of the 3' half of an miRNA or siRNA to its target RNA is not required for translational repression, provided that multiple small RNAs are bound to the target (Doench and Sharp 2004), nor for

target mRNA destruction, if the bases surrounding the scissile phosphate can form an A-form helix locally (Haley and Zamore 2004). Technologically, this means that most active siRNAs will not only down-regulate their intended mRNA targets but also reduce expression of other mRNAs possessing partial complementarity to the siRNA guide strand (Jackson et al. 2003). Solving this vexing siRNA “off-target” problem will most likely require modifying siRNA structure or sequence to maximize affinity for the target while reducing off-target binding, as well as the use of the lowest possible siRNA dose, so as to increase the discrimination between the desired target knock-down and undesirable “off-target” effects.

A two-state model for Argonaute function

Why do the 5' nucleotides of an siRNA contribute disproportionately to target binding? Why don't the 3' nucleotides contribute more binding energy? Figure 3 suggests a mechanism by which different regions of the siRNA guide strand or mature miRNA can make distinct contributions to small RNA function. This “two-state” model envisions that the 3' end of an siRNA or miRNA is tightly bound to an Argonaute protein, the core constituent of the RISC. The small RNA is drawn with its 3' end embedded in the PAZ domain. Cross-linking experiments suggest that in *Drosophila* siRNAs bind Ago2 through their 3' ends (Tomari et al. 2004b), and structural studies make the Argonaute PAZ domain the best candidate for this binding (Lingel et al. 2003, 2004; Song et al. 2003; Yan et al. 2003; Ma et al. 2004). The siRNA would then extend along the positively charged binding cleft predicted by the structure of the *Pyrococcus furiosus* Argonaute protein (Song et al. 2004), with its 5' phosphate resting in a pocket within or near the Piwi domain (Parker et al. 2004). In fact, Barford and colleagues (Parker et al. 2004) propose that a divalent cation in a conserved region of the Piwi domain may mark the site of phosphate recognition. Such a phosphate-binding pocket is predicted because the identity of the scissile phosphate on the target RNA is measured from the 5'

end of the siRNA before RISC encounters its RNA target (Elbashir et al. 2001a,b; Haley and Zamore 2004; Martinez and Tuschl 2004). A 5' phosphate on the siRNA guide strand is required for an siRNA to function (Boutla et al. 2001; Nykänen et al. 2001; Chiu and Rana 2002; Schwarz et al. 2002; Liu et al. 2004a).

We envision the 5' half of the small RNA guide to be bound to Argonaute protein in a quasi-helical shape. In other words, about half of the small RNA guide is proposed to be pre-organized for binding an RNA target. In contrast, the 3' half of the siRNA is predicted to be bound to Argonaute in a way that antagonizes its base pairing with the RNA target. Therefore, pairing of the 3' half of the siRNA with the target RNA requires dislodging it from a binding pocket in Argonaute, perhaps the PAZ domain. Such pairing is required for efficient target cleavage but not the binding of small RNAs that repress translation. Energetically, the favorable energy produced by pairing the 3' region of a small RNA with its target would be offset by the energy spent expelling these bases from their nook in Argonaute. The model helps explain why base pairs between the 3' end of the siRNA and its RNA target influence k_{cat} but not K_M (Haley and Zamore 2004).

Silencing modes

In the translational repression pathway, small RNAs direct RISC to bind an mRNA and repress its translation into protein, rather than cleave it. Animal miRNAs typically, but not always, mediate translational repression rather than cleavage. In contrast, most plant miRNAs direct target RNA cleavage. In all of the well-studied examples of translational repression by animal miRNAs or by siRNAs, translational repression is a response to the binding of multiple small RNA-programmed RISCs to sites in the 3' UTR of the mRNA target. In fact, the presence of multiple candidate sites in 3' UTR sequences is a useful predictor of an mRNA being regulated by miRNA (Lewis et al. 2003). Complementarity between a small RNA and its RNA target helps determine if the small RNA directs target cleavage or represses mRNA translation, because an A-form helix must be formed with the target RNA at the center of an siRNA guide strand for cleavage to occur (Chiu and Rana 2003; Haley and Zamore 2004). In the absence of such a helix, as occurs when the central region of the small RNA cannot pair with bases in the target, translational repression may ensue. Translational repression occurs at some step after translational initiation, because the distribution of ribosomes along the length of the repressed mRNA is thus far indistinguishable from the same mRNA undergoing active translation (Olsen and Ambros 1999). A tantalizing proposal from Sharp and colleagues (cited in Ketting and Plasterk 2004) is that RISC somehow directs degradation of the nascent polypeptide, rather than simply stalling translational elongation. Both models are currently without experimental support.

Small RNAs can also direct the formation of repressive heterochromatin. The only complex thus far described

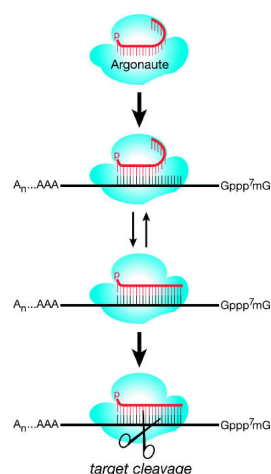


Figure 3. A two-state model for Argonaute function in RISC.

for this pathway is the *Schizosaccharomyces pombe* RITS, a RISC-like complex that contains siRNA, an Argonaute protein (Ago1), the chromodomain protein Chp1, and Tas3, a protein of unknown function (Noma et al. 2004; Verdel et al. 2004). How the RITS directs modification of histones to promote the establishment of repressive heterochromatin is unknown. In fact, it is not known if the RITS binds the DNA of the targeted gene or simply binds nuclear RNA transcribed from the targeted DNA sequences. DsRNA targeting promoter sequences also trigger transcriptional silencing in plants (Mette et al. 2000). siRNA-directed transcriptional silencing via DNA methylation was recently reported in animals, too (Kawasaki and Taira 2004; Morris et al. 2004). The complexes that mediate these effects are not known, and such effects were not observed for long dsRNA in mouse oocytes (Svoboda et al. 2004).

If small RNAs mediate three distinct functions, are there three corresponding RNA silencing effector complexes? Current data suggest both yes and no. In human cells, only Ago2, of four closely related Argonaute proteins, contains the key amino acids thought to catalyze target RNA cleavage. But Ago2 is bifunctional, since it can both direct mRNA cleavage and repress translation. This discovery was made in an elegant experiment by Filipowicz and coworkers (Pillai et al. 2004). They fused human Ago2 to the small, basic RNA-binding N-peptide from bacteriophage λ . The fusion protein retained the capacity for small RNA-guided target RNA cleavage. Next, they placed multiple N-peptide-binding sites in the 3' UTR of a reporter mRNA and introduced both the Ago2 fusion protein and the reporter into human cells. The Ago2 fusion protein bound the 3' UTR sites through the N-peptide rather than via a small RNA guide and silenced reporter protein production without reducing reporter mRNA levels. Translational repression by the Ago2-N fusion protein resembled translational repression directed by small RNAs in that more than three binding sites were required to achieve robust repression. Thus, Ago2 can direct both cleavage and translational repression in this engineered system. Purified Ago2 alone suffices for small RNA-guided target cleavage (Liu et al. 2004a), but perhaps accessory proteins assist Ago2 in other functions like translational or transcriptional repression. In contrast, human Ago1-, Ago3-, or Ago4-containing complexes cannot cleave (Liu et al. 2004a; Meister et al. 2004). Each of these three Argonaute proteins differs from Ago2 at one or more putative catalytic amino acids (Liu et al. 2004a). The function of human Ago1 remains to be determined, but Ago4 and perhaps Ago3 likely silence by repressing translation (Pillai et al. 2004).

Two of the five *Drosophila* Argonaute proteins, Ago1 and Ago2, are associated with RISCs capable of cleavage (Okamura et al. 2004), although we do not know if *Drosophila* Ago1- or Ago2-containing RISC can also cause translational repression. Current data suggest that Ago1 is the core component of miRNA-programmed RISCs and Ago2 is the core component of siRNA-programmed RISCs, implying that the assembly machinery restricts

miRNAs to bind Ago1 and siRNA to bind Ago2 in flies (Okamura et al. 2004). At odds with this simple view is the requirement for the *Drosophila* Argonaute protein, Aubergine, in both RNAi in vivo and RISC assembly in vitro (Kennerdell et al. 2002; Tomari et al. 2004a). Moreover, experiments using tagged, over-expressed human Argonaute proteins in cultured mammalian cells suggest that at least four human Argonaute proteins bind indiscriminately to miRNAs and siRNAs (Liu et al. 2004a; Meister et al. 2004). The worm Argonaute proteins RDE1 and ALG1/ALG2 provide a counter example. RDE1 is required for RNAi triggered by exogenous dsRNA, but plays little or no role in miRNA function (Tabara et al. 1999, 2002). The ALG1 and 2 proteins play no role in RNAi, but are essential for miRNA biogenesis and/or function (Grishok et al. 2001), likely because the ALG1 and 2 proteins bind miRNAs (Hutvagner et al. 2004).

Data from *S. pombe* suggest that Argonaute proteins might even be tri-functional. Translational repression has not yet been detected in fungi, but small RNAs direct both transcriptional and post-transcriptional silencing in *S. pombe*. Transcriptional silencing of large swaths of DNA occurs at the *S. pombe* centromere and the related mating-type locus. Transcription of these regions is blocked by the formation of repressive heterochromatin, which is enriched for Histone H3 methylated at Lys 9 (H3-K9) (Elgin and Grewal 2003). Small RNAs initiate the formation of heterochromatin in *S. pombe*, and the RNAi pathway is critical for building the *S. pombe* centromere (Verdel et al. 2004). Three "RNAi" genes are required for this process: RNA-dependent RNA polymerase (Rdp1), Dicer (Dcr1), and Argonaute (Ago1). Rdp1 is thought to amplify or produce the initial dsRNA trigger for centromeric silencing in *S. pombe*, Dcr1 is presumed to generate siRNAs from the trigger dsRNA, and Ago1 is a component of the RITS complex, the presumptive effector complex of siRNA-directed transcriptional silencing. The RNA silencing pathway may play a general role in the construction of centromeres, because Dicer is required for the formation of centromeric heterochromatin in cultured vertebrate cells (Fukagawa et al. 2004; Kanellopoulou et al. 2005).

Ago1 is the sole *S. pombe* Argonaute protein and is also required for dsRNA-triggered post-transcriptional silencing in this yeast (Sigova et al. 2004). PTGS in *S. pombe* also requires Rdp1, Dcr1, and siRNAs, like classical RNAi in worms. Thus, a single Argonaute protein can act in both transcriptional and post-transcriptional RNA silencing pathways. However, Tas3 and Chp1 are not required for PTGS, suggesting either that Ago1 is the sole protein required for *S. pombe* RNAi or that distinct TGS and PTGS complexes, both containing Ago1, coexist in *S. pombe*.

Questions for the future

Despite all we have learned about the mechanism of RNA silencing, many remarkably simple questions remain unanswered. Is there really an siRNA unwindase, a protein that uses ATP-energy to part the two siRNA

strands? What proteins compose RISC in vivo, and what is the molecular function of each RISC component? How does RISC repress mRNA translation? How does it trigger changes in chromatin structure or DNA methylation? The more we explore RNA silencing, the more these pathways reveal their remarkable complexity. Which raises the most important question of all: What is the biological meaning of all this complexity?

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