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Structural and Biochemical Advances in Mammalian RNAi

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Abstract RNAi is a collection of processes mediated by small RNAs that silence gene expression in a sequence-specific manner. Studies of processes as divergent as post-transcriptional gene silencing, transcriptional silencing through RNA-directed DNA methylation, or heterochromatin formation, and even RNA-guided DNA elimination have converged on a core pathway. This review will highlight recent structural and mechanistic studies illustrating siRNA and miRNA processing, RISC formation, the execution of RNAi by RISC, and the regulation of these pathways, with a specific focus on vertebrate systems. J. Cell. Biochem. 99: 1251–1266, 2006. © 2006 Wiley-Liss, Inc.

Key words: RNAi; Dicer; RISC; RNase IV; PACT; TRBP; ADAR; TGS

The initiation of RNAi requires the formation of a specific double-stranded (ds)RNA structure from diverse dsRNAs, without requirements of sequence specificity (Fig. 1). Two RNase III enzymes are employed in this process. In the nucleus, Drosha, assisted by its double-stranded RNA binding domain (dsRBD) partner, Pasha, cleaves RNA polymerase II-transcribed primary microRNA (pri-miRNA) transcripts into pre-miRNAs, ~80 nucleotide (nt) dsRNAs with a hairpin loop, and 3′ 2-nt overhang [Lee et al., 2003], which exit the nucleus in an Exportin-5-dependent manner [Yi et al., 2003]. In the cytosol, Dicer (DCR) processes pre-miRNA, and viral or cellular dsRNA to a ~21 dsRNA with 2-nt 3′ overhangs and 5′ phosphates [Bernstein et al., 2001]. One strand, the guide strand, is preferentially incorporated into an Argonaute (AGO) protein, while the other, the passenger strand, is released and destroyed. This strand bias is determined by thermodynamic factors, as the strand whose 5′ end is less stably paired to its complement is incorporated [Khvorova et al., 2003; Schwarz et al., 2003].

Argonaute proteins contain 5′ and 3′ binding sites for single-stranded (ss) RNA, and feature an RNaseH-like domain. The guide-strand Ago complex forms RISC [Rivas et al., 2005], the executor of RNAi, with the RNA endowing the generalized protein machinery of RNAi with sequence specificity. Should the guide strand bind with high affinity, and form a sufficient duplex with an mRNA target in a catalytically active Ago (e.g., hAGO2 [Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005]), that target is cleaved [Haley and Zamore, 2004]. This is typical of miRNAs in plants (see Vaucheret [2006] for an excellent review of RNAi mechanisms in plants) but generally, moderate-affinity binding of mRNA, and the failure to form a central miRNA/mRNA duplex in animals yields mRNA binding but not cleavage [Haley and Zamore, 2004]. Binding to the target mRNA (bioinformatics approaches predict targeting of the 3′ UTR [Lewis et al., 2005]) blocks cap-dependent translational initiation [Pillai et al., 2005]. The RISC-mRNA localizes to cytosolic P-bodies, where the mRNA is degraded [Liu et al.,

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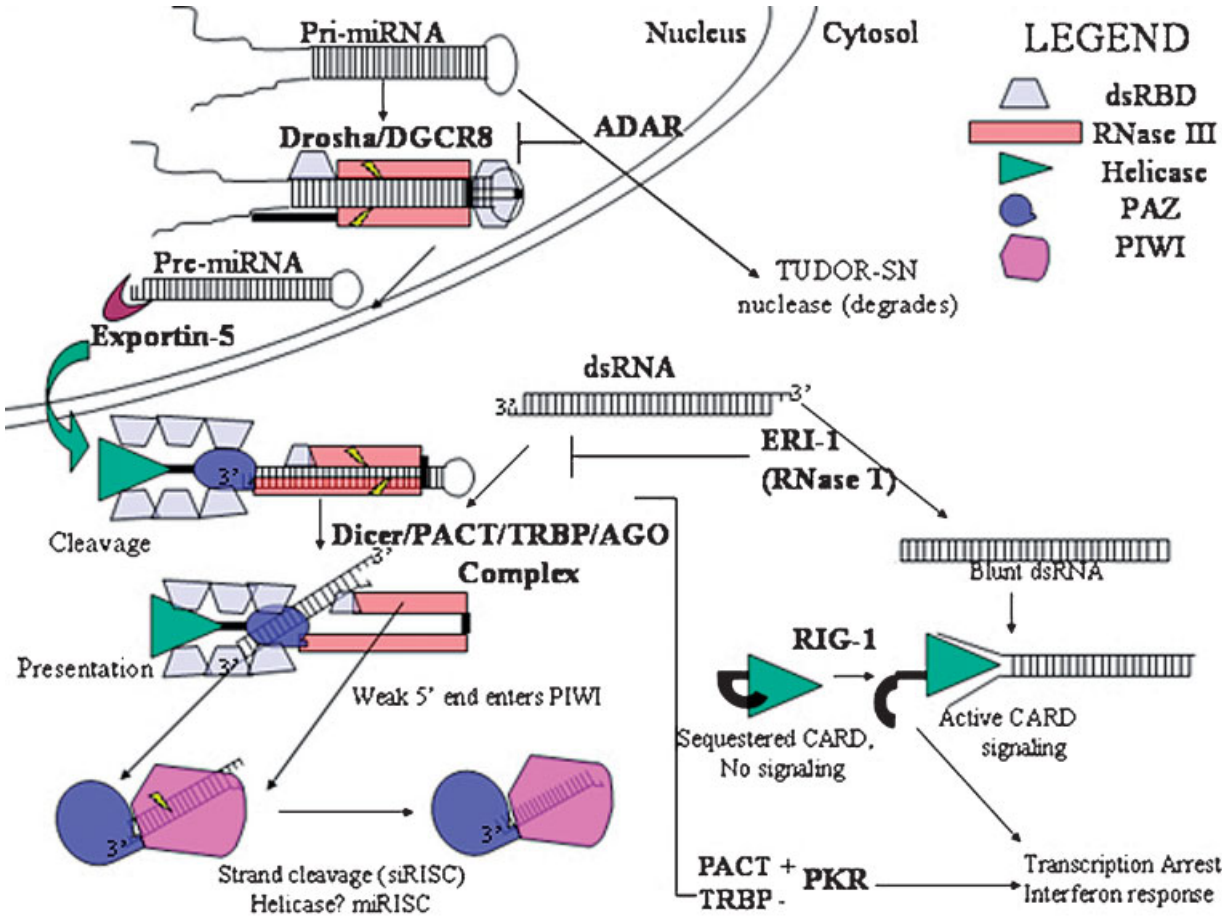

Fig. 1. Schematic of the RNAi and dsRNA surveillance pathways (see introduction). Primary transcripts are processed by Drosha in complex with a dsRBD containing protein, Drosha. Extensive adenine deamination (ADAR) blocks Drosha processing and fates pri-miRNA for degradation by Tudor-SN nuclease. Pre-miRNAs exit the nucleus in an Exportin-5-dependent manner. In the cytosol, pre-miRNA and viral and cellular dsRNA are processed by Dicer, which resides in complex with two 3-dsRBD containing proteins, PACT and TRBP. Dicer binds pre-existing 3′-2nt overhangs with its PAZ domain, and measures out a precise distance to its active site, where another 3′-2nt overhang end is created. The strand with the weaker 5′ end binding is incorporated into RISC. PACT and TRBP may sense this asymmetry, and with Dicer, load Argonaute proteins with dsRNA. Following loading, the passenger strand is cleaved (in catalytic RISCs) or removed by other means. ERI-1 is upregulated in response to excess dsRNA and blunts siRNAs, blocking their entry into the RNAi pathway. Blunt dsRNAs are a preferred substrate for RIG-1 helicase. RIG-1 and PKR are activated in response to dsRNAs, and activate a cellular response that includes type 1 interferon. Interestingly, PACT and TRBP have opposing effects on PKR, further reinforcing the interplay between the RNAi and RNA surveillance pathways.

2005]. Recently, studies of maternal mRNA degradation mediated by one miRNA expressed early in Zebrafish development suggests deadenylation is the key step in target mRNA elimination [Giraldez et al., 2006]. From early estimates of less than 50, bioinformatic approaches based on sequence conservation in evolution or pattern recognition of microRNA properties now predict thousands of miRNAs may exist [Bentwich, 2005]. Since imperfect base pairing is tolerated, one miRNA can have many targets, and algorithms that predict microRNA targets by analyzing the thermodynamics of base-pairing between miRNAs and putative mRNA targets predict only 148 conserved miRNAs could target as much as 30% of the genome [Lewis et al., 2005]. It comes as no surprise then, that the biological roles of miRNAs are expanding to diverse cellular processes, including development, differentiation, proliferation, and apoptosis (reviewed in Carthew, 2006). Relating to their roles as

cellular regulators, miRNAs are now confirmed oncogenes (reviewed in Hammond, 2006).

A growing number of protein cofactors for dsRNA processing and RISC loading are being discovered. For example, Drosha's dsRBD partner, Pasha/DGCR8, is required for pri-miRNA processing, likely by binding the stem-loop structure and presenting pri-miRNAs for cleavage [Landthaler et al., 2004]. Two human dsRBDs recently implicated in RISC loading, TRBP [Chendrimada et al., 2005; Gregory et al., 2005; Haase et al., 2005; Maniataki and Mourelatos, 2005] and PACT [Lee et al., 2006b], were previously described for their opposite influence on the mammalian dsRNA-signaling pathway, implying cross talk between the core pathway of RNAi and anti-viral dsRNA surveillance mechanisms.

Other RNAi cofactors and regulators are emerging. For example, a proteomics approach in *C. elegans* identified a number of DCR-interacting proteins that functionally influence RNAi [Duchaine et al., 2006]. In this study and others, regulated steps of the pathway, points of divergence, and competition for the AGO effector machinery are becoming known. In mammals, two mechanisms that regulate entry into the RNAi pathway have recently been discovered. Adenosine deaminase acting on RNA (ADAR) and ERI-1, a nuclease, are upregulated in response to excess exogenous siRNA exposure [Hong et al., 2005], suggesting a role in preserving limited RNAi resources for endogenous regulation.

RNAi is not limited to post-transcriptional gene silencing in some organisms. Argonaute RNAi effector complexes guide transcriptional gene silencing (TGS) in a number of organisms. This part of the pathway is perhaps the most divergent, and RNAi-mediated TGS in mammals remains controversial. We will examine recent contributions to this controversy, with an emphasis on alternatives mechanisms that explain certain key data. It is important to distinguish between RNAi-based pathways, and mechanisms that have an RNA component, but do not involve the RNAi machinery.

RNase III ENZYMES: DROSHA AND DICER

The RNase III enzymes Drosha and Dicer (DCR) are responsible for the initiation of the RNAi pathway. Drosha processes pri-miRNAs into pre-miRNAs with the help of a dsRBD protein, Pasha/DGCR8 [Lee et al., 2003; Gregory et al., 2004; Han et al., 2004]. Drosha itself consists of a long, variable N-terminal region, two RNase III domains, and a dsRBD [Cerutti and Casas-Mollano, 2006]. It should be noted plants lack Drosha, but process their pri-miRNAs with a DCR homolog and its dsRBD partner, HYL1 [Kurihara et al., 2006]. DCR further processes pre-miRNAs and dsRBDs, and functions in RISC loading. DCRs, in most species, consist of a N-terminal DEXD (DEAD-like) helicase domain, a conserved domain of unknown function, a PAZ domain, two RNase III domains, and a dsRBD [Cerutti and Casas-Mollano, 2006]. Recent data suggests the helicase domain is disposable for mammalian DCR RNase activity, but is important for cofactor interactions and RISC loading [Lee et al., 2006b].

RNase III proteins are notable for generating dsRNAs of fixed lengths (~11 bp for bacteria) with 2nt 3′ overhangs and 5′ phosphates. Bacterial RNAse III enzymes consist only of an endonuclease domain and a dsRBD. Early structural studies revealed bacterial RNases form intramolecular homodimers [Blaszczyk et al., 2001], that together form a large cleft for dsRNA binding. As a result, it was incorrectly suggested the homodimer would form two compound active sites, each with double-stranded RNase activity. In this model, the distance between active sites would measure out a product of fixed size. Using a structure-guided mutational approach, Zhang et al. [2004] show the bacterial homodimer, or the intramolecular dimer of the two DCR endonuclease domains, forms only one processing center capable of a double-stranded cleavage. Two new structures, one of a *Aquifex aeolicus* RNase III-product complex [Gan et al., 2006], and the other, of Giardia DCR [Macrae et al., 2006], reinforce this finding. Further, the Giardia DCR model lends structural evidence in support of biochemical data suggesting a two-metal catalytic mechanism in RNase III catalysis [Sun et al., 2005], and shows how the 3′ ssRNA binding activity of the PAZ domain may facilitate generation of products of appropriate length.

Aquifex aeolicus RNase III Bound to dsRNA

In order to form a substrate-RNase complex, catalytically impaired *Aquifex aeolicus* RNase

III, and RNA were co-crystallized. In the process, the residual activity of the mutant enzyme cleaved the RNA. The 3′ 2nt overhangs of the product overlap, forming a pseudo-continuous duplex (Fig. 2A,B). This mimics a product conformation, with one strand of RNA cut over each cleavage site. The positioning of the two sites is staggered to yield the 3′ 2-nt overhang. At each site, only one metal bound by four conserved acidic residues is observed, however, the absence of a second metal in a product conformation in a crystal structure is uninformative. The ligands for a second metal are apparent, based on similarity to other nucleases with two-metal catalysis. Upon dsRNA binding, a significant induced fit of the dsRBD domains is seen. The two dsRBDs cradle opposite sides of the RNA along the catalytic cleft. This position is at a right angle relative to another structure where the dsRBDs face outwards to contact dsRNA [Blaszczyk et al., 2004] (Fig. 2C,D). If this rotation about a flexible linker is conserved in the DCR dsRBD, it may have important implications for substrate binding, or product display as a function of RISC loading.

Structure of a Minimal Eukaryotic Dicer

Giardia DCR deviates from those of other Eukaryotes, as it lacks all but the PAZ and the two RNase III domains. However, it is fully functional, generating products of a specific size, and even rescuing RNAi in a strain of *S. pombe* with a DCR deletion [Macrae et al., 2006]. The RNase domains of Giardia DCR form a globular, all-helical intramolecular heterodimer that looks similar to the bacterial RNases (Fig. 3A). The two-endonuclease

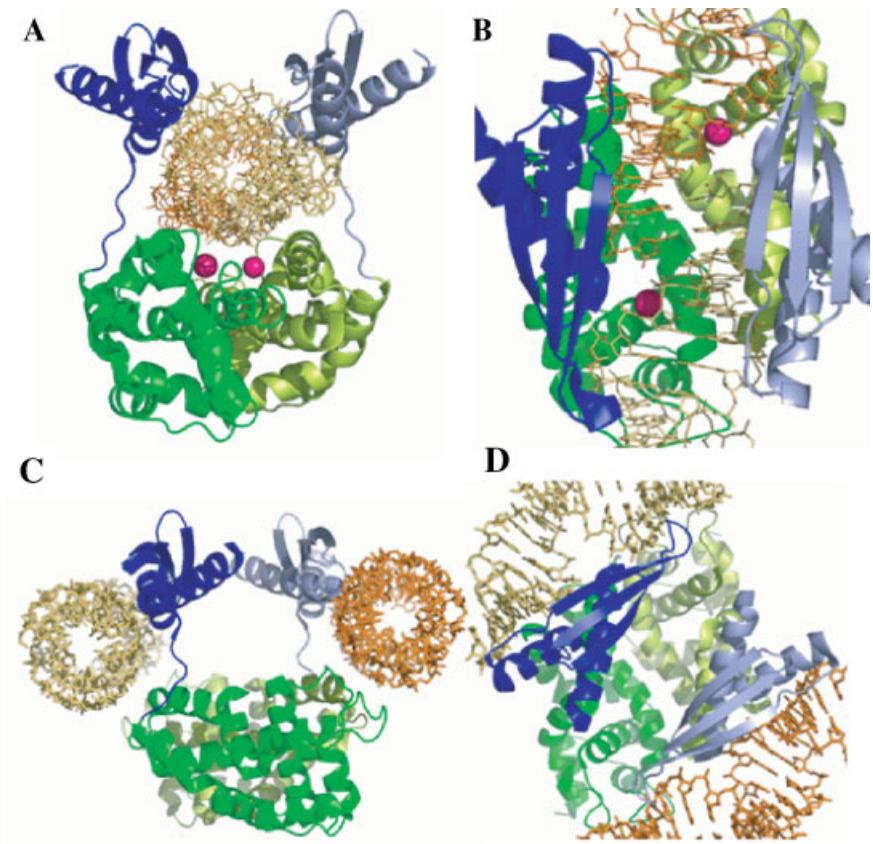

Fig. 2. Structures of *Aquifex aeolicus* RNase III bound to dsRNA. **A**: dsRNA bound in a product conformation to RNase III (PBD 2EZ6). **B**: A 90° rotation into towards the reader. Bacterial RNase III consists of a single RNase III domain (green/lime), and a dsRBD (blue/light blue) that homodimerize. Two double-stranded RNA products (orange/yellow) of RNAse III have rebound to the active site, marked by a pink sphere for the one metal observed per active site. Interestingly, the products have formed a psudo-duplex, with a staggered double-stranded cut. Also note the extensive cradling of the dsRNA by the dsRBDs. **C**: dsRNA bound to RNase III in a non-catalytic conformation (1RC7). The dsRBDs have rotated out on a flexible linker to contact dsRNA outside the catalytic groove. **D**: The two central helices of the RNase III domain have the same alignment as in panel B, and compare the respective positions of the dsRBDs.

domains are linked by a helical domain. The RNA sits on a flat, basic surface, rather than in a valley flanked by dsRBDs. This flat surface extends over a linker domain, a long α helix engulfed by anti-parallel β-sheets, to the PAZ domain. The Giardia 'platform structure' bears some sequence homology to a domain-of-unknown-function conserved in Dicers, suggesting a function for that domain. By use of a heavy, trivalent cation, two pairs of metal binding sites were identified [Macrae et al., 2006]. One metal of each pair corresponds to the manganese-binding site identified in other structures, which is defined by four acidic residues. The second metal at each site lies a reasonable distance from its partner for a two-metal mechanism of catalysis. Additionally, a dsRNA model suggests the two two-metal active sites would lie on opposite sides of a dsRNA, offset by about 2-nt between scissile phosphates.

A number of structures of the PAZ domain have been solved (reviewed in Collins and Cheng, 2005). The Giardia DCR PAZ domain is like other PAZ domains in fold and 3′ overhang binding pocket. DCR PAZ contains a

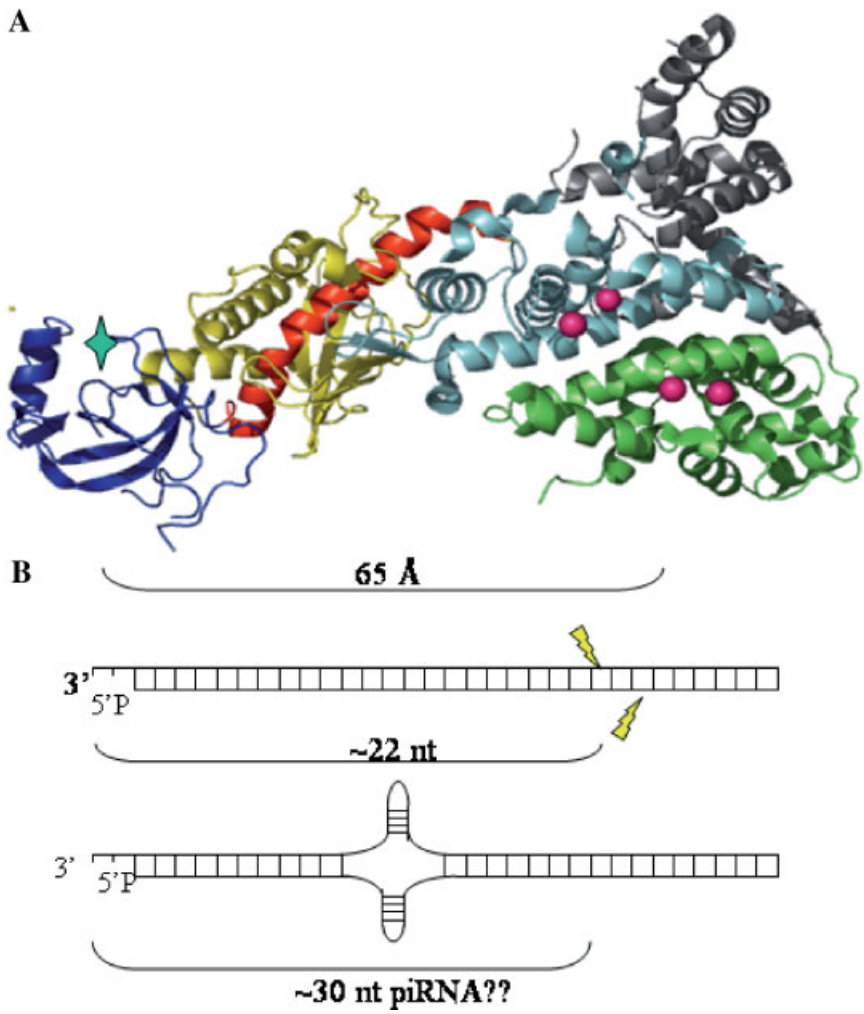

Fig. 3. Giardia Dicer as a 'molecular ruler'. **A**: Crystal structure of Giardia Dicer (PDB 2FFL). The PAZ domain (blue), contains the 3′ overhang binding site (star), and attaches to the RNase III domains by a long helix (red) flanked by residues from the N-terminus of the protein (yellow). The RNase domains are rendered in green and cyan, and are connected by a linker domain, rendered in gray. Four metals (pink) are observed in two active sites, supporting a two-metal catalytic mechanism. **B**: The distance between the 3′ binding site and active sites is 65 Å, which corresponds to 25 nt (human Dicer products are typically shorter). Further, the active sites are staggered, accounting for the 3′-2nt product produced by RNase III enzymes, and are positioned to flank either side of a RNA helix. In some cases, Dicer seems to produce diverse products. This may include a long (~30nt) class of miRNA-like silencing RNAs called piRNAS. We speculate Dicer, as a molecular ruler, measures out end-to-end distances, but internal structuring of the dsRNA could yield products with more nucleotides in the same linear distance.

basic loop that is absent in Argonaute proteins [Song et al., 2003]. This loop lies at the dsRNA groove seen in AGO PAZ structures, and it is unclear how it DCR and AGO PAZ RNA interactions may differ as a result. The presence of the PAZ domain in DCR has suggested a mechanism where the distance between PAZ and the active site would function as the molecular ruler that determines product length [Zhang et al., 2004] (Fig. 3B). Indeed, a dsRNA modeled into the Giardia DCR perfectly aligns such that the distance from its 3′ binding site in the PAZ domain to the cleavage site is 25 nucleotides. This model fits biochemical data suggesting DCR requires dsRNA termini for cleavage, prefers 3′ 2-nt overhangs to blunt termini, and reduces dsRNAs by small RNA sizes, never cutting internally [Zhang et al., 2002; Vermeulen et al., 2005]. Returning to the pathway, the RNAse III processed product from Drosha is a perfect substrate for DCR. A more difficult problem may be viral dsRNAs without processed ends. Data suggests DCR can process these slowly, with the help of its dsRBD. Once a first cut is made, the subsequent ends are then recognizable by PAZ, and rapidly processed [Zhang et al., 2004]. This, and the preformed binding mode for RNA seen in the DCR structure, suggests the primary role of PAZ may be to facilitate binding of substrate (as opposed to a conformational change licensing activity). Indeed, dsRBD truncated DCR became more dependent on 3′ overhang dsRNA recognition by PAZ, and could not process blunt dsRNA [Zhang et al., 2004].

The model of Dicer as a static molecular ruler is challenged by the finding of a ~30nt class of small RNA in the male mouse germline (piRNAs). They derive from long primary transcripts, show hallmarks of precise RNase III processing, and are loaded into members of the PIWI-subfamily of AGO proteins [Aravin et al., 2006; Girard et al., 2006]. Additionally, the PIWI proteins interact with Dicer when overexpressed [Sasaki et al., 2003]. Unless an unknown bypass pathway for DCR processing and RISC loading exists, this implies some flexibility in DCR processing. We suggest a purely speculative model, that a piRNA with hairpin structures in one or both stem strands would have the same length in end-to-end distance as a 21-nt dsRNA in a fully helical conformation (Fig. 3B). In this case, the DCR molecular ruler could generate products of different length, depending what conformation the substrate takes. These studies verified models of the two-metal RNase III center and product specificities of DCR. We look forward to structural and biochemical studies of RNase III in a substrate conformation, of DCR with RNA bound, and of Drosha and non-minimal DCRs with accessory domains. Of particular interest is the role of DCR in RISC loading.

dsRBDs AND RISC LOADING: CHOOSING THE RIGHT STRAND

Insights from *Drosophila*: R2D2 and siRNA Strand Asymmetry

Functional studies and isolation of DCR-Argonaute complexes have demonstrated dsRBD cofactors bridge Dicing and formation of a functional RISC. Both miRNAs and siRNA show strand bias, with the proper strand incorporated into RISC, and the passenger strand disposed of and rapidly destroyed. This is accomplished without sequence specificity, and without apparent bias imparted by the polarity of Drosha/DCR processing. Instead, the RNA strand with whose 5′ end is less stably paired with the opposite strand is chosen for incorporation [Khvorova et al., 2003; Schwarz et al., 2003]. *Drosophila* R2D2/DCR2 has become a model for understanding the molecular recognition of the thermodynamics of siRNAs and RISC loading.

When considering the *Drosophila* RNAi pathways as a model for mammalian RNAi, one must remember the *Drosophila* RNAi pathway is bifurcated. Long dsRNAs and siRNAs are processed by DCR-2, and loaded into AGO2 with R2D2 as a cofactor, while pre-miRNAs are processed by DCR-1 and loaded into Argonaute-1, with Loquacious as cofactor (reviewed in Hammond, 2005), with only some functional overlap detected in genome-wide microarray experiments [Rehwinkel et al., 2006]. Additionally, *Drosophila* DCR-2 appears to have a PAZ domain with mutations that could perturb RNA binding, and DCR-1 lacks a functional helicase domain [Hammond, 2005]. Humans only have one DCR that processes miRNA and siRNAs. Nevertheless, R2D2, Loquacious, and the mammalian dsRBD cofactors, TRBP and PACT, are homologs and consist of three dsRBDs each.

The role of R2D2 in siRNA strand selection for entry into RISC is established [Liu et al., 2003; Tomari et al., 2004]. Following Dicing, siRNAs

remain bound to a DCR/R2D2 heterodimer. R2D2 specifically binds the end of the siRNA with the greater stability (stronger double-stranded character), and DCR-2 binds the other duplex end [Tomari et al., 2004]. This complex, but not DCR-2 alone, appears capable of enhancing RISC loading [Liu et al., 2003]. It was suggested a helicase could unwind the siRNA duplex only from the end not bound to R2D2, that is, the end with a poorly paired duplex. In most models, unwinding of this strand would be coupled to its Ago2 loading. Two studies have shown that Ago2 is loaded not with a single strand, but with both strands of the siRNA [Matranga et al., 2005; Rand et al., 2005]. The guide strand then directs cleavage of the passenger strand, as it would an mRNA target, and the cleaved passenger strand dissociates. RISC loading was shown to be ATP (and therefore helicase) independent [Rand et al., 2005]. It is unclear how this mechanism relates to non-catalytic Argonautes (hAgo1, hAgo3, hAgo4 [Meister et al., 2004]), or miRNAs, which fail to pair well with their targets and direct cleavage. In the cases where cleavage does not occur, Matranga et al. [2005] propose a 'slow bypass' mechanism. Whether this reflects slow release of miRNAs with imperfect pairing mimicking a strand cleavage or activity of some helicase in the extract is unclear. Performing the assays of bypass activity in ATP-depleted extracts should shed light on this. If the bypass mechanism is a helicase, then it must act on Ago-bound duplexes, rather than unwinding prior to RISC loading. This is interesting, as it is unclear how non-catalytic RISC–mRNA complexes are released, and for that matter, it is unclear in a slicing RISC how the cleavage products are de-annealed from the guide strand. There is disagreement on the requirements of ATP for both RISC loading and catalytic turn over. We address this issue in a separate section below.

Regardless of the finding that both guide and passenger strands are loaded into RISC, the function of R2D2 and the asymmetry of loading is unchanged. Preall et al. [2006] find that DCR or polarity of dsRNA processing does not impart which siRNA strand is selected as the RISC guide. DsRNA ends capped with long ssRNA sequences produce the RISC loaded with the appropriate guide strand, regardless of which end of the dsRNA was capped (i.e., which end DCR processed from). This implies DCR must cut the dsRNA in one mode of binding, and present both ends of the dsRNA to R2D2 in a second mode. Indeed, Preall et al. [2006] found at least a transient release step, where DCR preloaded with dsRNA would not load RISC with a strand from that dsRNA in the presence of a non-cognate siRNA 'quench,' implying dsRNA processing and Ago2 loading can be decoupled. The quenchable release-and-rebind step is likely a transient intermediate, as double-stranded siRNAs are not released by the DCR-2/R2D2 heterodimer in *Drosophila* embryo lysates lacking Ago2 [Tomari et al., 2004]. Following this release step, R2D2 is bound to the thermodynamically favored end and DCR to the other. Both strands are handed off to Ago2, with the non-R2D2 bound 5′ end loading into the Ago 5′ binding site, and the R2D2 bound 3′ end entering the PAZ binding site. Strand cleavage and/or the 'bypass' mechanism then removes the passenger strand.

Is Asymmetric Binding a General Feature dsRBDs?

No other dsRBD homolog of R2D2 has been shown to sense asymmetry in dsRNA ends, although this ability could be a general feature of dsRBDs. Structures detailing dsRBD/dsRNA interactions show a requirement for helix formation [Ryter and Schultz, 1998]. The dsRBD reads a major and two flanking minor grooves, with significant insertion into the dsRNA helix to read 2′ hydroxyls and make non-specific base contacts. It is difficult to reconcile the specificity for dsRNA with high-affinity binding of ssRNA, or de-annealed ends of a thermodynamically weak siRNA end.

In DCRs predicted to have functional PAZ domains, processing asymmetry could be more significant then in *Drosophila* DCR-2. It has been demonstrated the processing asymmetry of human DCR can influence strand selection for RISC, using siRNAs capped by blunt-ends containing DNA bases [Rose et al., 2005]. However, in vertebrate cell lines and *Drosophila* lysates, both miRNAs and siRNAs follow the 5′ end rule for strand incorporation into RISC [Khvorova et al., 2003; Schwarz et al., 2003]. Significant asymmetry in microRNA processing would be problematic, as the primary transcript dictates 5′ and 3′ polarity of the Drosha product. If the 3′ end that DCR PAZ binds is the strand that becomes the Ago-bound miRNA, all miRNAs would be derived from the 3′ side of the hairpin. Alternatively, the opposite

could be true, and all miRNAs would be 5′, but mature miRNAs originating from either 3′ or 5′ of the hairpin exist, implying there must be a thermodynamic evaluation independent of processing polarity. MiRNA precursors obey the 5′ end rule so well that duplex miRNAs with equivalent thermodynamic ends will result in RISCs loaded with miRNA from either strand [Krol et al., 2004]. Notably, all DCRs appear to have dsRBD binding partners. The *Drosophila* miRNA pathway that utilizes DCR-1 and Ago1 requires a dsRBD cofactor, Loquacious. Loquacious is required for endogenous miRNA silencing, and pre-miRNAs accumulate without it. DCR-1 alone lacks specificity towards miRNAs versus dsRNAs, but addition of Loquacious rescues specificity and full activity [Forstemann et al., 2005; Saito et al., 2005]. From these studies, it appears Loquacious is required for Dicing, not just RISC loading, though these functions may overlap. If DCR is unable to pass its product to Ago1, and remains bound, further rounds of catalysis could be thwarted.

Mammalian dsRBD Cofactors: PACT and TRBP

In mammals, the single DCR processes both miRNA and siRNAs, and has at least two dsRBD partners, PACT (Protein Kinase R protein activator) and TRBP (HIV TAR RNA Binding Protein). These proteins contain three dsRBDs each, and were previously described for their stimulation (PACT) [Patel and Sen, 1998] or repression (TRBP) [Park et al., 1994] of the dsRNA-dependent protein kinase (PKR). PKR phosphorylates eIF-2, thereby inhibiting protein synthesis, and activates interferon in response to exogenous dsRNA, including siRNAs [Sledz et al., 2003]. It would be advantageous for exogenous viral RNA to activate dsRNA signaling and endogenous microRNA to escape detection, suggesting a model where TRBP handles miRNA, and PACT is responsible for exogenous dsRNA. This model is appealing, but not totally consistent with available data. TRBP knockout mice have mild phenotypes (partial lethality at weaning and male sterility) [Zhong et al., 1999], in contrast to DCR knockout mice, which exhibit embryonic lethality, with embryos failing to gastrulate [Bernstein et al., 2003]. This is inconstant with TRBP being required for all miRNA processing.

TRBP, AGO2, and DCR have been demonstrated to exist in complex by co-immunoprecipitation, gradient sedimentation, and gel filtration [Chendrimada et al., 2005; Gregory et al., 2005; Haase et al., 2005; Maniataki and Mourelatos, 2005]. Knockdown of TRBP causes loss of hairpin-construct or siRNA-mediated RNAi silencing, as detected by reporter constructs [Chendrimada et al., 2005; Haase et al., 2005]. Recombinant Dicer processes pre-miRNA and long dsRNA without TRBP in vitro, but cell extracts with knocked down TRBP only minimally process pre-miRNA [Haase et al., 2005]. TRBP knockdown in one study, caused failure to accumulate mature miRNA [Chendrimada et al., 2005]. Chendrimada et al. [2005] report Dicer is destabilized with TRBP knockdown, but use AGO2 immunoprecipitates as the source of material. In whole cell lysates, Dicer is unaffected by TRBP knockdown [Haase et al., 2005]. This suggests TRBP is not required for DCR stability, but is required as a physical link between DCR and AGOs. If TRBP is required for RISC loading, perhaps Diced products are not off loaded to RISC, accounting for the lack of activity in cell extracts (as DCR would have endogenous RNA bound). This would predict the DCR activity seen in vitro might only be one round of reaction, or rate-limited by slow product dissociation. The complex containing AGO2-DCR-TRBP is able to process pre-miRNA, recognize guide versus passenger strand, and forms a mature RISC, all without input of ATP [Gregory et al., 2005; Maniataki and Mourelatos, 2005]. Further, human RISC apparently dissociates from DCR [Maniataki and Mourelatos, 2005], in contrast to the holoRISC of flies [Tomari et al., 2004]. There was a substantially higher rate of RISC formation using miRNA versus siRNA, however, these studies employed pre-miRNA and 21nt duplex siRNA [Gregory et al., 2005; Maniataki and Mourelatos, 2005]. The pre-miRNA is a Dicer substrate and the siRNA a Dicer product. If the Dicer conformer used in dsRNA display (as in R2D2 loading) and RISC loading is effected by Dicer catalysis, a substantial preference in RISC loading for substrate over product could exist. We are curious if this complex would function on long dsRNAs.

These studies clearly demonstrate a mammalian RISC-loading complex that recognizes (at least) miRNA, guides the proper strand into Argonaute, and dissociates from RISC, without apparent energy input. A specific role of in miRNA processing is consistent with the role TRBP as a suppressor of PKR, and the suspicion

TAR RNA is a viral miRNA that TRBP aids in processing (reviewed in Weinberg and Morris, 2006). However, the mild phenotype of the TRBP mouse is hard to reconcile with total abrogation of miRNA silencing, and the lethal Dicer phenotype. A second dsRBD cofactor of DCR, PACT has been identified [Lee et al., 2006b]. PACT exists in complex with TRBP, DCR, AGO2, and DCR. PACT is not required for DCR activity, but is required for miRNA accumulation and siRNA-induced RNAi. However, this effect may exceed PACT's function, as PACT knockdown reduces DCR levels in whole-cell extract. This is hard to reconcile with the mild phenotype of the PACT knockout compared to the Dicer knockout. PACT knockout mice are small and deaf (microtia), but fertile [Rowe et al., 2006]. Finally, both PACT and TRBP interact with the Helicase domain of DCR, a region not required for DCR activity, but now implicated in RISC loading [Lee et al., 2006b].

These studies suggest two dsRBD cofactors, PACT and TRBP contribute to RISC loading, as part of a complex containing PACT, TRBP, DCR, and RISC. The human RISC-loading complex shows strand specificity and disengages from mature RISC. Despite their mild knockout phenotypes and opposite effects on PKR, both seem required for miRNA and siRNA mediated RNAi. We await reconstitution of recombinant RISC loading complexes from purified components, and precise definition of their activities, as well as further exploration of the interplay between the RNAi and dsRNA surveillance pathways.

ARGONAUTE PROTEINS: CORE EFFECTORS OF RNAi

Mammalian Argonaute Families: AGO and PIWI

Humans have eight Argonaute proteins: four AGO subfamily members: hAGO1, hAGO2, hAGO3, hAGO4, and four PIWI subfamily members: HIWI, HILI, PIWIL3, and HIWI2 [Sasaki et al., 2003]. All eight human AGOs conserve the PAZ-PIWI domain structure. The human PIWI subfamily of Argonaute should not be confused with the prokaryotic PIWIs utilized by structural biologists, which lack the PAZ domain. The structural biology of Ago proteins has been reviewed in detail elsewhere [Collins and Cheng, 2005; Hall, 2005]. The PAZ domain is responsible for 3′ guide strand binding, while the PIWI domain harbors a RNase H-like active site and 5′ guide strand binding site. Full-length AGO proteins from two prokaryotes without RNA bound and the structure of RNase H have been solved, suggesting a number of paths guide and target strands could take in AGO/RNA complexes. As the structure of AGO loaded with a guide strand is imminent, we will not speculate further here.

All eight AGOs co-immunoprecipitate with Dicer if overexpressed [Sasaki et al., 2003], but hAGO2 is the only hAGO demonstrated to possess Slicer activity [Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005]. Human hAGO1 and hAGO2 are both implicated in miRNA-mediated silencing and localize to p-bodies, cytosolic sites of RNA degradation [Sen and Blau, 2005].

All AGO subfamily members are broadly expressed, while PIWI family expression is testis specific [Sasaki et al., 2003]. Limited detection from tissue-specific expression analysis may diminish the significance of some hPIWIs in maintaining small numbers of stem cells in a proliferative niche in many tissues. However, mouse HILI and HIWI (MILI and MIWI) are specifically required for spermatogenesis, and bind a large class of longer (~30nt), germline-specific small RNAs now called piRNAs [Aravin et al., 2006; Girard et al., 2006]. HILI and HIWI overexpression is functionally linked to cancers, and when knocked down in their associated cancer cell lines cell-cycle arrest and apoptosis was induced [Liu et al., 2006; Lee et al., 2006a].

Energetics of RISC Loading and Catalytic Turnover

Several studies (described above) suggest RISC loading is ATP independent and forms functional RISC. This implies guide strand release and does not support the requirement of a helicase. Interestingly, recombinant human AGO2 does not bind duplex siRNA, but will bind ssRNA, forming a RISC capable of a single target strand cleavage [Rivas et al., 2005]. Passenger strand cleavage is associated with RISC loading, suggesting the RISC loading complex forces AGO to bind both strands of the siRNA. If so, this implies a conformational change, and the energy imparted may contribute to cleavage and the release of product. A RISC composed of guide-strand loaded AGO2 alone is capable of only a single turnover, even

in the presence of ATP [Maniataki and Mourelatos, 2005; Rivas et al., 2005], and in *Drosophila* extracts, an ATP-dependent activity allows RISC to have multi-turnover kinetics [Haley and Zamore, 2004]. This is highly suggestive of helicase activity. However, there have been reports of multi-turnover kinetics in the absence of ATP [Gregory et al., 2005]. The suggestion by Matranga et al. [2005] that two mechanisms, a fast one dependent on guide strand cleavage and a slower one is interesting. Does this imply AGO2 might sometimes couple the energy of helix formation between guide and target strand, or target strand cleavage with product release, and in the absence of cleavage, a helicase removes the target? This is intriguing, but the 'fast' and 'bypass' mechanisms may be the same, with the helicase acting faster on a cleaved product with more accessible ends. Also, if AGO can couple passenger-strand cleavage with product release, why then, is target-strand release rate limiting? Perhaps the RISC-loading machinery itself assists in passenger strand release. We are curious what activity will restore multi-turnover kinetics to recombinant RISC. Although helicase activity is suggested, there are alternatives. RNA chaperones that melt RNA without energy input exist. It has been proposed RNA chaperones are intrinsically unstructured, and couple the energy of RNA melting to the formation of structured RNA-protein domains. However, to release the RNA, it must form a higher-energy RNA structure (hence chaperone) [Mir and Panganiban, 2006]. Alternatively, RNA chaperones could return to their low energy state by energy-assisted product release, or destruction of the bound strand. Nuclease digestion is a valid option for target strand removal, coupled either to a RNA-chaperone mechanism, or as an independent mechanism. Indeed, holo–RISC complexes in many species contain a functional nuclease, Tudor-SN [Caudy et al., 2003]. The reconstitution of a multi-turnover RISC should settle this question.

TRANSCRIPTIONAL GENE SILENCING (TGS)

RNAi is not limited to post-transcriptional mechanisms in at least some organisms and regulates gene expression at the transcriptional level. In Arabidopsis, AGO4 controls RNAi-mediated DNA and histone methylation [Zilberman et al., 2003]. Similarly, AGO1, as part of the RITS complex, drives heterochromatin formation and epigenetic silencing in *S. pombe* [Verdel et al., 2004]. In *Drosophila*, Piwi and polycomb group proteins cooperate in repeat-induced silencing, and heterochromatin formation is dependent on the RNAi pathway [Pal-Bhadra et al., 2002, 2004]. In mammals mechanisms of TGS as a result of RNAi remains elusive. Given the retractions of a number of seminal papers in mammalian TGS (http://news.nature.com//news/2006/060403/440720a.html), we consider several studies proposing RNAi does not contribute to TGS in mammals.

Evidence for the contribution of RNAi to heterochromatin formation in humans first came from a Human-chicken hybrid cell line lacking Dicer. This cell line manifested heterochromatic defects in heterochromatin protein 1 (HP1) dissociation from chromatin, and transcription through what should be otherwise silent pericentromeric heterochromatin [Fukagawa et al., 2004]. Dicer-null mouse embryonic stem cells were reported to have similar phenotypes by one group [Kanellopoulou et al., 2005], and relatively normal methylation and heterochromatic silencing by another [Murchison et al., 2005]. However, this evidence is indirect: human miRNA targets include histone methyltransferases, and other key chromatin regulators [Lewis et al., 2005], and it is possible there are substantial secondary effects to the Dicer knockout.

Most recently, RNAi-independent heterochromatin formation and gene silencing has been demonstrated in Mammalian cells [Wang et al., 2006]. In this experiment, a transgenic repeat locus forms heterochromatin, complete with DNA methylation, Histone 3 lysine 9 methylation, and HP1 binding. No transgene small RNAs originating from the array were detected. Knockdown of Dicer caused no transcriptional leakage or heterochromatin decondensation. Finally, with the inducible transgene activated, expression of transgene-targeted siRNA induces post-translational silencing but not TGS heterochromatin formation. In this same context, HP1 tethering to the locus is sufficient to silence expression through induction of heterochromatin.

The authors of this study note that even in organisms with functional RNAi, mechanisms for heterochromatin formation independent of RNAi exist. This is the case in Neurospora [Freitag et al., 2004], *S. pombe* [Jia et al., 2004;

Kanoh et al., 2005], and Dictyostelium [Kaller et al., 2006]. Further, it is notable that centromeric small RNAs have not been isolated in the small-RNA cloning studies in mammals [Bernstein and Allis, 2005], despite the presence of RNA transcripts of both strands of centromeric satellite repeats.

These findings contrast with reports that siRNA can drive gene silencing through epigenetic mechanisms in Mammalian cells (e.g., Weinberg et al., 2006; Morris et al., 2004). A common theme from these studies is that single-stranded RNA antisense to a promoter directs DNA methylation and/or histone modification if delivered directly to the nucleus. This mechanism requires RNA-polymerase II, and one model suggests the Argonaute-guide strand complex recruits DNA methyltransferases and histone modification enzymes to the promoter. This is intriguing, but seems distant from the consensus RNAi pathway. The pathway for nuclear delivery of siRNA is unknown, and seems at odd with the cytosolic localization of Dicer and the RISC loading complex. This also implies an unloaded AGO waits in the nucleus to bind guide-strand RNA. Clearly, these are all possibilities, but it may also consider the possibility that the silencing effect of promoter targeted antisense oligonucleotides is not related to RNAi. In mammals, small RNA originating from natural antisense transcripts has not been identified, despite the abundance of natural sense/antisense transcription and its importance in processes such as X-inactivation. The natural antisense transcripts of IGF2R [Sleutels et al., 2003] hypoxia inducing factor and thymidylate synthase [Faghihi and Wahlestedt, 2006] have been examined, and do not form dsRNAs that enter the RNAi pathway, but do regulate gene expression. It is possible sense/antisense transcription plays a role in heterochromatin formation distinct from RNAi. A substantial portion of heterochromatin has an RNA component: HP1 dissociates from chromatin with RNase treatment and binds RNA through a conserved hinge region [Maison et al., 2002; Muchardt et al., 2002]. Other heterochromatin effectors, including DNA methyltransferases and DNA-methyl binding domain proteins may bind dsRNA [Jeffery and Nakielny, 2004], suggesting a pathway of heterochromatin formation mediated by nuclear dsRNAs, where the components and modifiers of heterochromatin bind RNA. This contrasts with AGO/guide strand targeting of complexes. Delivery of ssRNA antisense to promoters could act through the former mechanism. To demonstrate RNAi-mediated TGS in mammals, the possibility that it is tapping into an RNAi-independent (but RNA dependent) pathway of heterochromatin formation will have to be excluded.

REGULATION RNAi PATHWAY IN VERTEBRATES

The RNAi pathway, as a major regulator of cell proliferation and fate, must be tightly regulated. MicroRNAs are regulated at the transcriptional level, either as transcription-factor responsive independent units of transcription [Zhao et al., 2005] or for miRNAs deriving from introns, as a function of the regulation of their parent gene (reviewed in Ying and Lin, 2006). Genetic screens and proteomic approaches in lower Eukaryotes have defined large numbers of proteins involved in RNAi, and suggest the RNAi pathway is regulated at multiple levels. Distinct regulation of siRNA and miRNA pathways is apparent, as is competition between pathways [Duchaine et al., 2006].

Two mammalian pathways that are suppress RNAi have recently been described. One, adenosine deamination of RNAs (ADAR) can inhibit miRNA and entry into the RNAi pathway, forcing its degradation by Tudor-SN nuclease [Scadden, 2005; Yang et al., 2006]. In a second mechanism, a nuclease, ERI-1, removes the 2-nt 3′ overhangs of siRNAs. This may block entry of excess exogenous RNA into the RNAi pathway, sparing resources for endogenous sources [Kennedy et al., 2004]. Interestingly, the ERI-1 product, a blunt dsRNA, is a potent activator of dsRNA signaling via RIG-1 helicase [Marques et al., 2006], suggesting this 'safety valve' would not terminate cellular anti-viral responses.

ADAR and RNAi

ADARs deaminate adenosine in RNA, yielding inosine, and are not specific to miRNAs. Rather, their dsRBDs convey specificity for dsRNA in general. ADAR can be discreet, acting at limited sites which can diversify protein products of a single mRNA thorough the introduction of missense mutations. Alternatively, it can broadly edit dsRNAs, which has been proposed as a mechanism of nuclear

retention and gene silencing, perhaps even contributing to heterochromatin formation (reviewed in DeCerbo and Carmichael, 2005). Yang et al. [2006] demonstrate ADAR editing of pri-miRNAs blocks their processing by Drosha, presumably by introducing I-U pairs that distort the dsRNA helix beyond what Drosha will recognize and process. ADAR editing also fates pri-miRNA to be degraded by Tudor-SN, which had already been implicated in degradation of hyper-edited RNAs [Scadden, 2005]. Tudor-SN, incidentally, is a bona-fide member of RISC complexes in a number of organisms [Caudy et al., 2003]. ADAR-1 and ADAR-2 knockout mice show deregulation of certain miRNAs. Further, pri-miRNA editing is not uniform, with ADARs demonstrating substrate specificity that differs between isoforms [Yang et al., 2006]. This establishes ADAR editing as a means of blocking certain miRNAs from entering RNAi, but it may have other roles as well. ADAR-1 has a cytosolic isoform, and is upregulated in response to siRNAs in mice [Hong et al., 2005]. ADAR-1 antagonizes siRNA efficacy by binding siRNAs with high affinity (they may be too short to trigger enzymatic activity) [Yang et al., 2005], and treatment of longer dsRNAs with ADAR reduces their ability to be Diced [Scadden and Smith, 2001]. Alternatively, discreet editing of pri-miRNAs could yield diversification of miRNAs, and thus the number of target they recognize. Early searches for edited human pri-miRNAs suggest about 6% of adenosines were deaminated, with 50% of these occurring in the mature miRNA [Blow et al., 2006]. ADAR could even reverse strand selection for RISC loading by inducing a thermodynamically disfavorable I-U base pair at a duplex end. Prediction of, and design against ADAR modifications in RNAi technologies may be necessary for effective silencing, and minimization of off-target effects.

ERI-1 Nuclease Blunts RNAi Response

Like ADAR, ERI-1 (named, as its mutation Enhances RNAi in *C. elegans*) is upregulated in mammalian cells in response to exogenous siRNAs [Hong et al., 2005]. ERI-1 is a RNase T that removes the 3′ 2nt overhangs that characterize siRNAs. These blunt products are not licensed for entry into RNAi [Kennedy et al., 2004]. The suppressive effect of excess siRNA has on RNAi is, at least partially, ERI-1 dependent [Hong et al., 2005]. This suggests ERI-1 and cytosolic ADAR may be a safety valve to protect the RNAi machinery from total take-over by viral dsRNA in case of infection. The outcome of animal trials of a Hepatitis B RNAi therapy provide a dramatic demonstration of a RNAi safety valve is critical. Overload of the miRNA pathway by a number of different viral-delivered short hairpin vectors caused fatality in mice in a dose-dependent manner [Grimm et al., 2006]. As endogenous miRNA is subject to transcriptional regulation, cells may have no defense against over saturation by nuclear-transcribed hairpin RNAs. In contrast, cells appear to have mechanisms to spare some RNAi machinery for endogenous sources in the case of viral infection. In mammals viral (and scientist supplied) dsRNA not only generates anti-viral RISCs, but activates dsRNA signaling, a program to inhibit viral replication (reviewed in Karpala et al., 2005). This includes induction of Type 1 interferon and PKR (RNA-activated Protein kinase) phosphorylation of eif2α, which blocks protein translation. Ultimately, dsRNA signaling can induce apoptosis [Marques et al., 2005]. Taken together, these pathways allow an infected cell to clear the virus by use of RNAi and dsRNA signaling, and in the mean time, not sacrifice its miRNA-controlled transcriptional program (and therefore maintain genomic stability, cellular differentiation, and a non-proliferative state). Interestingly, although the blunt product of ERI-1 is not useful for RISC loading, it could be a potent activator of dsRNA signaling through RIG-1 helicase. RIG-1 contains a helicase and caspase recruitment domain (CARD). When the helicase domain encounters a dsRNA, as it engages and unwinds that target, the CARD domain is free to initiate dsRNA-signaling cascades [Yoneyama et al., 2004]. DsRNAs with 3′ 2nt overhangs cannot be unwound by RIG-1, and therefore fail to cause a response, while blunt dsRNA is an ideal activator of RIG-1 signaling [Marques et al., 2006]. This suggests a physical mechanism allowing RIG-1 to distinguish between Drosha/Dicer products, blunt viral dsRNA, and perhaps ERI-1 products, though this pathway has not been formally demonstrated. These processes present an obstacle for RNAi therapy. Nuclear-transcribed hairpin RNAs can overload the miRNA pathway with lethal effects, while cytosolic siRNA delivery is recognized as a viral invasion, and activates dsRNA signaling. At high doses, siRNA results in a downregulation

of RNAi. Precise biochemical and structural characterization of the pathways that affect these outcomes may allow design of RNAi therapies that evade these problems.

PROSPECTS

Structural and biochemical studies have, and will continue to shed light on the RNAi pathway. The studies described herein have come to the brink of reconstituting the dsRNA processing, RISC loading, and mature RISC complexes from recombinant components. This should resolve the controversies resulting from characterizing activities in undefined cell extracts. In vitro screening of small RNAs for efficacy could be a immediate result of this accomplishment. RNAi mechanisms intertwine with the RNA surveillance pathway, and other pathways involving RNA. It will be necessary to elucidate which are bona-fide RNAi pathways and which are not. The regulation of the mammalian RNAi pathway is just becoming apparent, but already has therapeutic implications. No doubt, many more regulatory mechanisms exist that have not yet been exposed in mammals.

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REFERENCES

Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, Morris P, Brownstein MJ, Kuramochi-Miyagawa S, Nakano T, Chien M, Russo JJ, Ju J, Sheridan R, Sander C, Zavolan M, Tuschl T. 2006. A novel class of small RNAs bind to MILI protein in mouse testes. Nature.

Bentwich I. 2005. Prediction and validation of microRNAs and their targets. FEBS Lett 579:5904–5910.

Bernstein E, Allis CD. 2005. RNA meets chromatin. Genes Dev 19:1635–1655.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363–366.

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. 2003. Dicer is essential for mouse development. Nat Genet 35:215–217.

Blaszczyk J, Tropea JE, Bubunenko M, Routzahn KM, Waugh DS, Court DL, Ji X. 2001. Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure (Camb) 9:1225–1236.

Blaszczyk J, Gan J, Tropea JE, Court DL, Waugh DS, Ji X. 2004. Noncatalytic assembly of ribonuclease III with double-stranded RNA. Structure (Camb) 12:457–466.

Blow MJ, Grocock RJ, van Dongen S, Enright AJ, Dicks E, Futreal PA, Wooster R, Stratton MR. 2006. RNA editing of human microRNAs. Genome Biol 7:R27.

Carthew RW. 2006. Gene regulation by microRNAs. Curr Opin Genet Dev 16:203–208.

Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, Tops BB, Silva JM, Myers MM, Hannon GJ, Plasterk RH. 2003. A micrococcal nuclease homologue in RNAi effector complexes. Nature 425:411–414.

Cerutti H, Casas-Mollano JA. 2006. On the origin and functions of RNA-mediated silencing: From protists to man. Curr Genet.

Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436:740–744.

Collins RE, Cheng X. 2005. Structural domains in RNAi. FEBS Lett 579:5841–5849.

DeCerbo J, Carmichael GG. 2005. Retention and repression: Fates of hyperedited RNAs in the nucleus. Curr Opin Cell Biol 17:302–308.

Duchaine TF, Wohlschlegel JA, Kennedy S, Bei Y, Conte D, Jr., Pang K, Brownell DR, Harding S, Mitani S, Ruvkun G, Yates JR III, Mello CC. 2006. Functional proteomics reveals the biochemical niche of *C. elegans* DCR-1 in multiple small-RNA-mediated pathways. Cell 124:343–354.

Faghihi MA, Wahlestedt C. 2006. RNA interference is not involved in natural antisense mediated regulation of gene expression in mammals. Genome Biol 7:R38.

Forstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, Klattenhoff C, Theurkauf WE, Zamore PD. 2005. Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 3:e236.

Freitag M, Lee DW, Kothe GO, Pratt RJ, Aramayo R, Selker EU. 2004. DNA methylation is independent of RNA interference in Neurospora. Science 304:1939.

Fukagawa T, Nogami M, Yoshikawa M, Ikeno M, Okazaki T, Takami Y, Nakayama T, Oshimura M. 2004. Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat Cell Biol 6:784–791.

Gan J, Tropea JE, Austin BP, Court DL, Waugh DS, Ji X. 2006. Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. Cell 124: 355–366.

Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, Schier AF. 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312:75–79.

Girard A, Sachidanandam R, Hannon GJ, Carmell MA. 2006. A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature 7099:199–202.

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. 2004. The Microprocessor complex mediates the genesis of microRNAs. Nature 432:235–240.

Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. 2005. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123:631–640.

Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441:537–541.

Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, Gatignol A, Filipowicz W. 2005. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep 6:961–967.

Haley B, Zamore PD. 2004. Kinetic analysis of the RNAi enzyme complex. Nat Struct Mol Biol 11:599–606.

Hall TM. 2005. Structure and function of argonaute proteins. Structure 13:1403–1408.

Hammond SM. 2005. Dicing and slicing: The core machinery of the RNA interference pathway. FEBS Lett 579:5822–5829.

Hammond SM. 2006. MicroRNAs as oncogenes. Curr Opin Genet Dev 16:4–9.

Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. 2004. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18:3016–3027.

Hong J, Qian Z, Shen S, Min T, Tan C, Xu J, Zhao Y, Huang W. 2005. High doses of siRNAs induce eri-1 and adar-1 gene expression and reduce the efficiency of RNA interference in the mouse. Biochem J 390:675–679.

Jeffery L, Nakielny S. 2004. Components of the DNA methylation system of chromatin control are RNA-binding proteins. J Biol Chem 279:49479–49487.

Jia S, Noma K, Grewal SI. 2004. RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. Science 304:1971–1976.

Kaller M, Euteneuer U, Nellen W. 2006. Differential effects of heterochromatin protein 1 isoforms on mitotic chromosome distribution and growth in dictyostelium discoideum. Eukaryot Cell 5:530–543.

Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM, Rajewsky K. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev 19:489–501.

Kanoh J, Sadaie M, Urano T, Ishikawa F. 2005. Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. Curr Biol 15:1808–1819.

Karpala AJ, Doran TJ, Bean AG. 2005. Immune responses to dsRNA: Implications for gene silencing technologies. Immunol Cell Biol 83:211–216.

Kennedy S, Wang D, Ruvkun G. 2004. A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. Nature 427:645–649.

Khvorova A, Reynolds A, Jayasena SD. 2003. Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209–216.

Krol J, Sobczak K, Wilczynska U, Drath M, Jasinska A, Kaczynska D, Krzyzosiak WJ. 2004. Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. J Biol Chem 279:42230–42239.

Kurihara Y, Takashi Y, Watanabe Y. 2006. The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. RNA 12:206–212.

Landthaler M, Yalcin A, Tuschl T. 2004. The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis. Curr Biol 14:2162–2167.

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. 2003. The nuclear RNase III Drosha initiates microRNA processing. Nature 425:415–419.

Lee JH, Schutte D, Wulf G, Fuzesi L, Radzun HJ, Schweyer S, Engel W, Nayernia K. 2006a. Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. Hum Mol Genet 15:201–211.

Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN. 2006b. The role of PACT in the RNA silencing pathway. EMBO J 25:522–532.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20.

Liu Q, Rand TA, Kalidas S, Du F, Kim HE, Smith DP, Wang X. 2003. R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science 301:1921–1925.

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science 305:1437–1441.

Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. 2005. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol 7:719–723.

Liu X, Sun Y, Guo J, Ma H, Li J, Dong B, Jin G, Zhang J, Wu J, Meng L, Shou C. 2006. Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. Int J Cancer 118:1922–1929.

Macrae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, Adams PD, Doudna JA. 2006. Structural basis for double-stranded RNA processing by Dicer. Science 311:195–198.

Maison C, Bailly D, Peters AH, Quivy JP, Roche D, Taddei A, Lachner M, Jenuwein T, Almouzni G. 2002. Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. Nat Genet 30:329–334.

Maniataki E, Mourelatos Z. 2005. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev 19:2979–2990.

Marques JT, Rebouillat D, Ramana CV, Murakami J, Hill JE, Gudkov A, Silverman RH, Stark GR, Williams BR. 2005. Down-regulation of p53 by double-stranded RNA modulates the antiviral response. J Virol 79:11105–11114.

Marques JT, Devosse T, Wang D, Zamanian-Daryoush M, Serbinowski P, Hartmann R, Fujita T, Behlke MA, Williams BR. 2006. A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. Nat Biotechnol 24:559–565.

Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. 2005. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123:607–620.

Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. 2004. Human Argonaute2 mediates RNA

cleavage targeted by miRNAs and siRNAs. Mol Cell 15:185–197.
Mir MA, Panganiban AT. 2006. The bunyavirus nucleocapsid protein is an RNA chaperone: Possible roles in viral RNA panhandle formation and genome replication. RNA 12:272–282.
Morris KV, Chan SW, Jacobsen SE, Looney DJ. 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. Science 305:1289–1292.
Muchardt C, Guilleme M, Seeler JS, Trouche D, Dejean A, Yaniv M. 2002. Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. EMBO Rep 3:975–981.
Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. 2005. Characterization of dicer-deficient murine embryonic stem cells. Proc Natl Acad Sci USA 102: 12135–12140.
Pal-Bhadra M, Bhadra U, Birchler JA. 2002. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. Mol Cell 9:315–327.
Pal-Bhadra M, Leibovitch BA, Gandhi SG, Rao M, Bhadra U, Birchler JA, Elgin SC. 2004. Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. Science 303:669–672.
Park H, Davies MV, Langland JO, Chang HW, Nam YS, Tartaglia J, Paoletti E, Jacobs BL, Kaufman RJ, Venkatesan S. 1994. TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR. Proc Natl Acad Sci USA 91:4713–4717.
Patel RC, Sen GC. 1998. PACT, a protein activator of the interferon-induced protein kinase, PKR. EMBO J 17: 4379–4390.
Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309:1573–1576.
Preall JB, He Z, Gorra JM, Sontheimer EJ. 2006. Short interfering RNA strand selection is independent of dsRNA processing polarity during RNAi in *Drosophila*. Curr Biol 16:530–535.
Rand TA, Petersen S, Du F, Wang X. 2005. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell 123:621–629.
Rehwinkel J, Natalin P, Stark A, Brennecke J, Cohen SM, Izaurralde E. 2006. Genome-wide analysis of mRNAs regulated by Drosha and Argonaute proteins in *Drosophila* melanogaster. Mol Cell Biol 26:2965–2975.
Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L. 2005. Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Biol 12:340–349.
Rose SD, Kim DH, Amarzguioui M, Heidel JD, Collingwood MA, Davis ME, Rossi JJ, Behlke MA. 2005. Functional polarity is introduced by Dicer processing of short substrate RNAs. Nucleic Acids Res 33:4140–41456.
Rowe TM, Rizzi M, Hirose K, Peters GA, Sen GC. 2006. A role of the double-stranded RNA-binding protein PACT in mouse ear development and hearing. Proc Natl Acad Sci USA 103:5823–5828.
Ryter JM, Schultz SC. 1998. Molecular basis of double-stranded RNA–protein interactions: Structure of a dsRNA-binding domain complexed with dsRNA. EMBO J 17:7505–7513.
Saito K, Ishizuka A, Siomi H, Siomi MC. 2005. Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. PLoS Biol 3:e235.
Sasaki T, Shiohama A, Minoshima S, Shimizu N. 2003. Identification of eight members of the Argonaute family in the human genome small star, filled. Genomics 82:323–330.
Scadden AD. 2005. The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. Nat Struct Mol Biol 12:489–496.
Scadden AD, Smith CW. 2001. RNAi is antagonized by A → I hyper-editing. EMBO Rep 2:1107–1111.
Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115:199–208.
Sen GL, Blau HM. 2005. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nat Cell Biol 7:633–636.
Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. 2003. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol 5:834–839.
Sleutels F, Tjon G, Ludwig T, Barlow DP. 2003. Imprinted silencing of Slc22a2 and Slc22a3 does not need transcriptional overlap between Igf2r and Air. EMBO J 22:3696–3704.
Song JJ, Liu J, Tolia NH, Schneiderman J, Smith SK, Martienssen RA, Hannon GJ, Joshua-Tor L. 2003. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat Struct Biol 10:1026–1032.
Sun W, Pertzev A, Nicholson AW. 2005. Catalytic mechanism of *Escherichia coli* ribonuclease III: Kinetic and inhibitor evidence for the involvement of two magnesium ions in RNA phosphodiester hydrolysis. Nucleic Acids Res 33:807–815.
Tomari Y, Matranga C, Haley B, Martinez N, Zamore PD. 2004. A protein sensor for siRNA asymmetry. Science 306:1377–1380.
Vaucheret H. 2006. Post-transcriptional small RNA pathways in plants: Mechanisms and regulations. Genes Dev 20:759–771.
Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, Moazed D. 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303:672–676.
Vermeulen A, Behlen L, Reynolds A, Wolfson A, Marshall WS, Karpilow J, Khvorova A. 2005. The contributions of dsRNA structure to Dicer specificity and efficiency. RNA 11:674–682.
Wang F, Koyama N, Nishida H, Haraguchi T, Reith W, Tsukamoto T. 2006. The assembly and maintenance of heterochromatin initiated by transgene repeats are independent of the RNA interference pathway in mammalian cells. Mol Cell Biol 26:4028–4040.
Weinberg MS, Morris KV. 2006. Are viral-encoded microRNAs mediating latent HIV-1 infection? DNA Cell Biol 25:223–231.
Weinberg MS, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen ZX, Riggs AD, Rossi JJ, Morris KV. 2006. The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. RNA 12:256–262.
Yang W, Wang Q, Howell KL, Lee JT, Cho DS, Murray JM, Nishikura K. 2005. ADAR1 RNA deaminase limits short

interfering RNA efficacy in mammalian cells. J Biol Chem 280:3946–3953.

Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, Nishikura K. 2006. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol 13:13–21.

Yi R, Qin Y, Macara IG, Cullen BR. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17:3011–3016.

Ying SY, Lin SL. 2006. Current perspectives in intronic micro RNAs (miRNAs). J Biomed Sci 13:5–15.

Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737.

Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W. 2002. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J 21: 5875–5885.

Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. 2004. Single processing center models for human Dicer and bacterial RNase III. Cell 118:57–68.

Zhao Y, Samal E, Srivastava D. 2005. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature 436:214–220.

Zhong J, Peters AH, Lee K, Braun RE. 1999. A double-stranded RNA binding protein required for activation of repressed messages in mammalian germ cells. Nat Genet 22:171–174.

Zilberman D, Cao X, Jacobsen SE. 2003. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299:716–719.