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RNAi: RISC Gets Loaded

When an siRNA or miRNA proceeds through the RNA-induced silencing complex assembly pathway, only one of the two ~21-nucleotide RNA strands survives in the final, active complex. In this issue of *Cell,* Matranga et al. (2005) and Rand et al. (2005) reveal the fate of the rejected passenger siRNA strand. Additionally, Gregory et al. (2005) define a heterotrimeric complex from humans that appears to execute dsRNA loading, strand selection, and target mRNA cleavage activities.

During RNA interference (RNAi), the RNA-induced silencing complex (RISC) assembly machinery faces a formidable challenge: to program an RNA endonuclease on demand to specifically target a genomic invader or endogenous transcript (for a recent review, see Filipowicz, 2005). These duties require the machinery to abstract from a long double-stranded RNA (dsRNA) a single-stranded RNA that is appropriately sized to confer specificity and then use it to guide target destruction. In this issue of *Cell,* Gregory et al. (2005), Matranga et al. (2005), and Rand et al. (2005) illuminate this process and reinforce the multitalented nature of the known RNAi components.

The dsRNAs that naturally initiate RNAi are processed by a Dicer (Dcr) family RNase III enzyme into ~20–25-nucleotide (nt) duplexes. These duplexes are called small interfering RNAs (siRNAs) if they are perfectly complementary and arise from long dsRNA or microRNAs (miRNAs) if they are imperfectly base-paired and arise from pre-miRNA hairpins. siRNA and miRNA duplexes are converted into a single-stranded form as they assemble into the RISC, where they provide the sequence specificity or "guide" for mRNA degradation. This transition from a double- to a single-stranded silencing trigger has been one of the more mysterious phases of the RNAi pathway, and the unwinding step itself has been frequently attributed to an unidentified ATP-dependent helicase. In this issue of *Cell,* Matranga et al. (2005) and Rand et al. (2005) now show that in at least some cases, dsRNA unwinding and RISC loading is facilitated by cleavage of the unincorporated "passenger" strand by Ago2 (Figure 1A).

An siRNA duplex is analogous to the duplex formed between the guide and the mRNA target within active RISC. This simple insight prompted Matranga et al. (2005) and Rand et al. (2005) to ask an intriguing question: does the passenger strand of an siRNA serve as the first target of RISC? Based on the site specificity of the endonuclease activity of RISC, siRNA cleavage would be predicted to yield 9- and 12-nt hydrolysis products with 5′-phosphate and 3′-hydroxyl termini. Both groups observed the predicted 9-nt 5′ cleavage product. Furthermore, cleavage was largely limited to the passenger strand that was thermodynamically disfavored for RISC assembly, in keeping with known siRNA asymmetry rules. Finally, siRNA cleavage preceded unwinding, as antisense RISC inhibitors that block binding and cleavage of targets after siRNA unwinding fail to protect the passenger strand.

Having left no doubt that passenger-strand cleavage occurs, Matranga et al. (2005) and Rand et al. (2005) go on to consider an important question: how critical is passenger-strand cleavage to the RISC assembly pathway? This question is all the more pressing because of evidence that three of the four Argonaute proteins in humans (Ago1, Ago3, and Ago4) lack endonuclease activity but are nonetheless loaded with single-stranded guide RNAs (Liu et al., 2004; Meister et al., 2004). Similarly, single-stranded miRNAs associate with Ago2, despite the expectation that mismatches within the unwound precursor should block passenger-strand cleavage activity of Ago2. To address this issue, Matranga et al. (2005) challenged the *Drosophila* RNAi machinery with siRNA carrying a phosphorothioate group at the passenger-strand cleavage site. This modification reduced the rate of passenger-strand hydrolysis by more than an order of magnitude. The rate of RISC assembly with these modified siRNAs (as measured by siRNA unwinding) was reduced approximately 3-fold relative to the unmodified control. This relatively modest effect led to significant impairment of RISC assembly at early time points. The phosphorothioate inhibition was more pronounced in human extracts, which are less efficient at assembling RISC. Similarly, when Rand et al. (2005) blocked passenger-strand cleavage with site-specific 2′-*O*-methyl modifications, they observed a reduction in RISC activity that correlated with the loss of the 9-mer product. They also showed that an active-site mutation in Ago2 that crippled the endonuclease activity impeded passenger-strand release. Together, the two studies indicate that passenger-strand cleavage plays a significant role in the formation of active Ago2-containing siRNA-programmed RISC (Figure 1A, left). However, the nonessential nature of passenger-strand hydrolysis led Matranga et al. (2005) to propose a slower, cleavage-independent "bypass" mechanism for RISC assembly (Figure 1A, right).

In contrast to the siRNA pathway, miRNAs are loaded into RISC entirely via the cleavage-independent "bypass" mechanism (Figure 1B). Matranga et al. (2005) again used phosphorothioate substitution to block passenger-strand cleavage in duplexes with varying degrees of miRNA-like character. This analysis showed that the degree of base-pairing of the so-called "seed" region of the guide (nts 2–8) is a key determinant in channeling the duplex into either the cleavage-dependent or cleavage-independent pathway.

In the course of their investigations into RISC activation, Rand et al. (2005) cast unexpected doubt on the roles of ATP during this process. When RNAi is analyzed in unfractionated *Drosophila* embryo lysates, ATP appears to function at multiple stages of the RNAi pathway (Filipowicz, 2005), including the progression of the RISC-loading complex (RLC) intermediate into holo-RISC (Pham and Sontheimer, 2005 and references

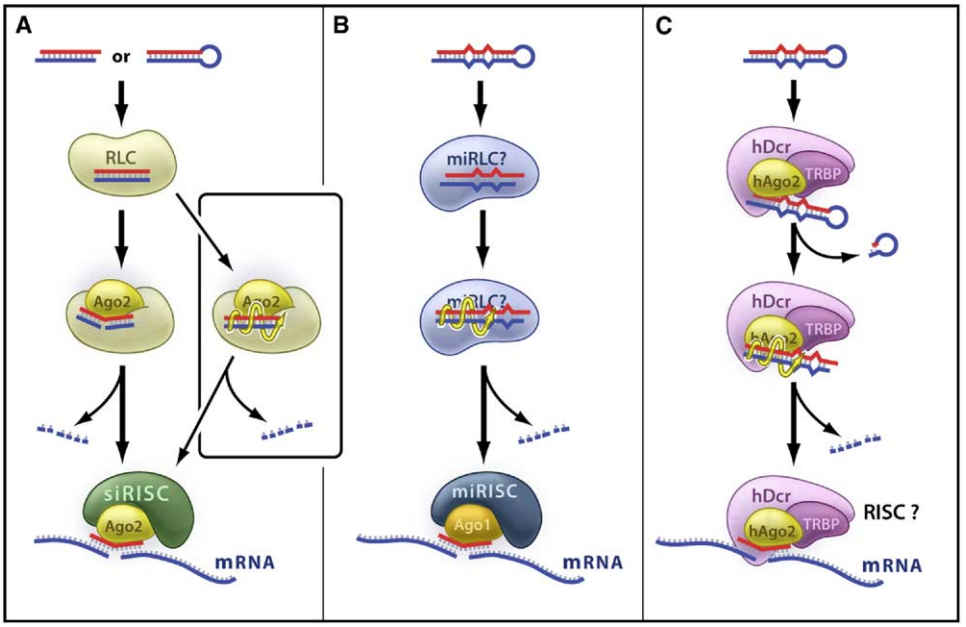

Figure 1. Mechanisms of Guide RNA Loading and Activation within RISC

(A) In *Drosophila*, perfectly base-paired siRNAs proceed through the RISC-loading complex (RLC) and engage Ago2. The precise details of the assembly of this complex are unknown. Passenger-strand cleavage by Ago2 precedes and presumably facilitates siRNA strand dissociation (left), leading to the formation of siRNA-programmed RISC (siRISC) that goes on to target the cleavage of specific mRNA. A slower, cleavage-independent "bypass" pathway (right) also generates active siRISC, perhaps by unwinding of the siRNA (yellow spiral arrow). The passenger strands are degraded by cellular nucleases.

(B) In *Drosophila*, imperfectly base-paired miRNA precursors generally assemble into Ago1-containing miRNA-programmed RISC (miRISC), perhaps through an miRISC-loading complex (miRLC). In this case, the bulges and mismatches in the duplex prevent passenger-strand cleavage, and the active miRISC forms via the cleavage-independent route.

(C) Affinity purification of Dicer-containing complexes from human cells indicates the existence of an assembly consisting of Dicer (hDcr), TRBP, and hAgo2. This complex is capable of dicing dsRNA precursors, loading the guide strand into hAgo2, and cleaving mRNA targets, suggesting that it may function as a form of RISC.

therein). It has been presumed that the reported ATP requirement for progression beyond the RLC reflects an ATP-dependent RNA helicase activity that unwinds the siRNA. In contrast to the embryo lysate results, Rand et al. (2005) observed no absolute requirements for ATP in siRNA-programmed *Drosophila* S2 cell extracts. These results are surprising not only because they differ from those seen in embryo lysates but also because RNA duplexes as short as several base pairs (such as those found in the spliceosome) require ATPases for unwinding. The authors conclude that, in S2 cell lysates, RISC assembly per se does not require ATP. In this case, siRNA unwinding during RISC activation could be driven in part by the binding energy that is released as Ago2 engages the siRNA guide strand. To explain the conflicting ATP requirements of RISC assembly in lysates from S2 cells and embryos, Rand et al. speculate that ATP is required to maintain the 5′-phosphorylated state of siRNAs in the presence of an embryo extract-specific phosphatase. This explanation seems incomplete, as recent native gel analyses indicate that holo-RISC assembly requires ATP even when the integrity of the siRNA 5′-phosphate is directly monitored with a radiolabel (Pham and Sontheimer, 2005). Given that the S2 cell extracts are prepared as S100 postribosomal supernatants, they probably assemble more minimal forms of RISC than the holo-RISC observed in embryo lysates; perhaps holo-RISC assembly requires ATP, whereas formation of the smaller active complexes does not.

From these two papers, it appears that the world of the RNAi machinery may be getting smaller: in at least some cases, Ago2 may partially substitute for the hypothetical helicase thought to be involved in RISC activation. Still, many questions remain. Is the cleavage-independent "bypass" pathway related to the ATP-dependent mechanism that stimulates multiple turnover (Haley and Zamore, 2004), and if so, what is the relevant ATPase? To what extent are the cleavage and bypass mechanisms in kinetic competition with each other? As weak 5′-end base pairing by the guide strand seems to obviate the need for passenger cleavage, and as many Ago proteins are inactive as endonucleases, can we really tell which is the "primary" mechanism for RISC loading and which is the "bypass"?

The first two papers suggest that the most familiar *Drosophila* RNAi proteins—Dcr-2, the dsRNA binding domain (dsRBD) protein R2D2, and Ago2—may collectively fulfill more functions than previously thought. In a third paper in this issue of *Cell*, Gregory et al. (2005) put the human orthologs of these three proteins (hDcr, TRBP, and hAgo2, respectively) to the test and provide

evidence that they comprise the minimal machinery required for a complete RNAi response. In this study, the authors identified only two proteins that specifically copurified with epitope-tagged hDcr: TRBP and hAgo2. The purified ternary complex could be programmed with either exogenous siRNA or unprocessed dsRNA to recapitulate RNAi activity in vitro (Figure 1C). Thus, it appears that the minimal RNAi machine in humans has shrunk to the familiar trinity—hDcr, TRBP, and hAgo2—though the participation of low-abundance copurifying factors has not yet been categorically excluded. We await the final nail in the coffin: the production of an entirely recombinant human RNAi machine, which should now be achievable.

The current study also bolsters the possibility that hDcr/TRBP can play a role as a RISC-loading platform, despite the fact that mammalian Dcr is not strictly required for an siRNA response (Kanellopoulou et al., 2005; Murchison et al., 2005). In particular, Gregory et al. (2005) found that the preassembled ternary complex is significantly more potent when programmed with a dsRNA processing substrate than with a "pre-diced" duplex. Although this idea is not new (Rose et al., 2005 and references therein), this study adds biochemical credence to the notion of direct coupling between dsRNA processing and RISC loading as an autonomous function of hDcr/TRBP.

For its final trick, the minimal RNAi machine characterized by Gregory et al. (2005) could take up an siRNA and cleave a cognate target without the aid of ATP, similar to the observations of Rand et al. (2005) in *Drosophila* S2 lysates. The authors estimate that, even in the absence of ATP, the purified complex could carry out multiple rounds of catalysis, though ATP boosted RISC activity. Preliminary analyses with GTP, nonhydrolyzable ATP analogs, and even pyrophosphate and inorganic phosphate indicate that we still have much to learn about possible roles of NTP binding and hydrolysis in the human RNAi pathway.

Taken together, these three studies suggest a simplified view of siRNA-programmed RISC activation and posit a minimal, three-component RNAi machine that nominally fulfills the basic biochemical requirements of a silencing response (dicing dsRNA, separating the two strands, and executing multiple rounds of mRNA cleavage). Still, questions remain about accessory factors that could serve to grease the machine's wheels or adapt it for multiple silencing outputs (Filipowicz, 2005). Genetic and biochemical data have implicated a host of proteins in both *Drosophila* and humans as components of mature, active forms RISC. It remains to be seen what new functions these other factors confer to RNAi machines, and how they modulate the biochemical steps that are executed by the core Dicer/dsRBD protein/Argonaute heterotrimer.

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