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Dicers at RISC: The Mechanism of RNAi

The pathway of RNA interference starts when Dicer cuts dsRNA into small interfering RNAs (siRNAs) that subsequently target homologous mRNAs for destruction. microRNA processing from stem loop precursors similarly requires Dicer activity. Two papers in this issue of *Cell* now demonstrate that Dicer is also essential for the execution phase of RNAi and explore the distinct requirements for Dicers in the siRNA and miRNA pathways.

The original picture is this: in response to dsRNA, cells trigger a two-step reaction. In the first step, long dsRNA is processed by a ribonuclease (RNase) III enzyme called Dicer into small interfering RNAs (siRNAs); these subsequently serve as the sequence determinants of the RNAi pathway by directing cleavage of homologous mRNA via an RNA-induced silencing complex (RISC) (Reviewed in Hannon [2002]).

Similar small RNA molecules that can silence gene activity are micro-RNAs (miRNAs). These single-stranded RNA molecules, which have widespread roles in growth and development, are also the result of Dicer activity, but in this case, the stem loop precursor molecules are encoded within the animal or plant genomes, and silencing can occur either via destruction of the mRNA (plants) or by blocking its translation (animals and plants) (see Carrington and Ambros [2003] for a recent review).

Much insight into the mechanism of RNAi has come from biochemical analysis within *Drosophila* cells and extracts. Forward genetic screens performed in other genetic systems, such as *C. elegans*, *Neurospora*, and *Arabidopsis* have led to the identification of crucial players in the RNAi pathway; unfortunately, however, the biochemistry for these systems does not, at present, match the level of finesse reached with *Drosophila* in vitro systems. Carthew and colleagues (Lee et al., 2004 [this issue of *Cell*]) now bring the two approaches together; these authors used genetically engineered *Drosophila* strains to identify mutants that had either a reduced or an enhanced response to eye-specific expression of a hairpin dsRNA corresponding to *white* sequences (*white* null mutants have white eyes whereas wild-type *Drosophila* eyes are red) and then analyzed them biochemically. There is a good correlation between the number of transgenes that express the hairpin and the level of *white* silencing (arguing against trigger amplification in this animal): one copy turns the eye pale orange, but two copies result in white eyes; this allows a combined enhancer and suppressor mutant search by screening animals that contain a single copy. Importantly, the screen is based on a mosaic-generating system that produces homozygous mutant compound eyes in an otherwise heterozygous animal. The ability to score RNAi phenotypes of mutations in essential genes (which when homozygous would result in lethality or sterility) seems crucial for further identification of RNAi components, considering the mechanistic overlap in the RNA silencing pathways triggered by siRNAs and miRNAs and the crucial regulatory roles of miRNAs in growth and development.

Screening three of *Drosophila*'s major autosomal arms yielded at least 15 suppressor loci including both Dicers in the animal's genome. Humans and *C. elegans* encode only one Dicer, which can process both dsRNA (in RNAi) and miRNA precursors, but *Drosophila* has two, and the *Arabidopsis* genome four. Lee et al. (2004) now find that there is division of labor in fly cells as the two Dicers have clearly different functions in RNA silencing: although Dcr-2 is the major siRNA producing enzyme in RNAi, Dcr-1 is vital in miRNA-triggered gene silencing. This differential function is reflected in the observed phenotypes: *dcr-2* null alleles have a complete RNAi defect in the eye and in the female germline, but have otherwise wild-type appearance (and a normal level of miRNA processing). In contrast, *dcr-1* animals display only mild RNAi defects but have halve-sized eyes with several morphological aberrations: pleiotropic defects that are indicative of impaired miRNA function. The authors strengthen this idea by showing that *dcr-1* animals fail to produce miR-2 miRNAs and that a set of specific reporter constructs that are likely the subjects of miRNA downregulation are not silenced in *dcr-1* fly eyes. A similar situation likely exists in *Arabidopsis*: here, mutation of the Dcr-1 homolog, DCL1, impairs miRNA but not siRNA production (Finnegan et al., 2003).

This separation of function for *Drosophila* Dicers is, however, not absolute; although it remains to be seen whether Dcr-2 has any contribution in miRNA function (what do the double mutant look like?), Dcr-1 is certainly also required for efficient RNAi. Surprisingly, the role of Dcr-2 in RNAi is not limited to processing long dsRNA into siRNAs. By direct injection of synthetic siRNAs into *Drosophila* eggs, one can bypass the dsRNA-processing activity of Dicers. Whereas wild-type eggs exhibited a profound RNAi response, *dcr-2* null mutant eggs displayed an impaired response, implying a role for Dcr-2 downstream of siRNA formation, backing up recent observations made in mammalian cells where siRNAs failed to induce RNAi upon cotransfection of siRNAs directed against Dicer (Doi et al., 2003). Interestingly, the helicase domain present in Dcr-2 (but not in Dcr-1) that is required for dicing activity is dispensible for siRNA-induced mRNA degradation.

What is Dicer's function at these later steps? Apparently, it's not cutting the mRNA via its RNase activity since mutations in the RNase III domains do not impair siRNA-induced cleavage in vitro (the responsible protein for mRNA cutting ["slicer"] has not yet been identified). Liu et al. (2003) recently provided striking data that fit a scenario in which Dcr-2 complexed to the Dcr-2-associated protein R2D2 binds to siRNAs and facilitates its loading onto RISC. Sontheimer and colleagues (Pham et al., 2004 [this issue of *Cell*]) now leap forward by

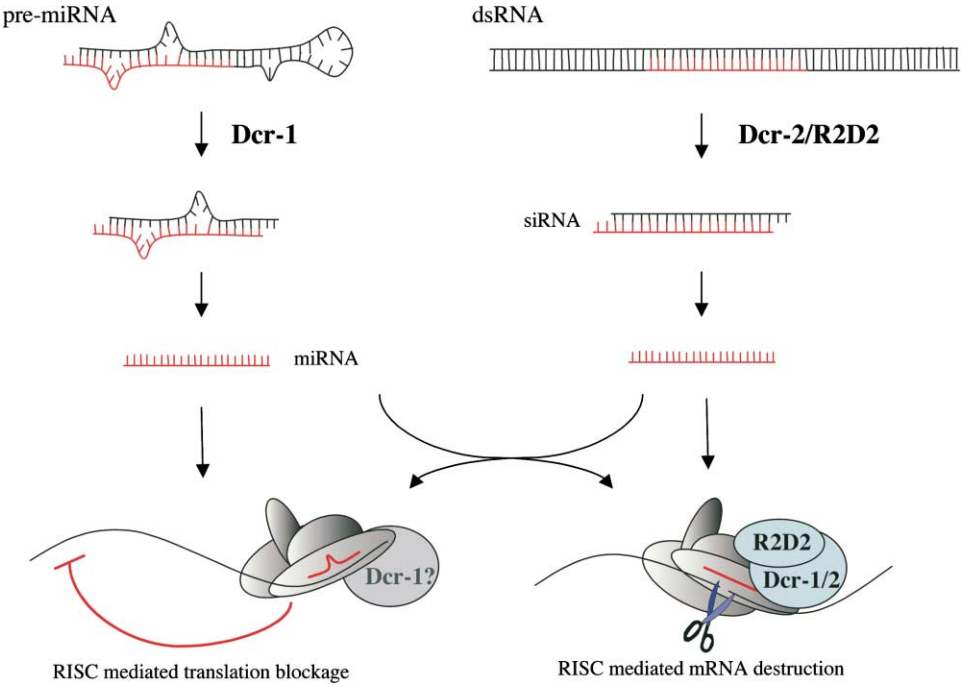

Figure 1. Model for RNA Silencing in *Drosophila*
In an ordered biochemical pathway, miRNAs (left panel) and siRNAs (right panel) are processed from double-stranded precursor molecules by Dcr-1 and Dcr-2, respectively, and stay attached to Dicer-containing complexes, which assemble into RISC. The degree of complementarity between the RNA silencing molecule (in red) and its cognate target determines the fate of the mRNA: blocked translation or immediate destruction.

visualizing an RNAi effector complex that is functionally and physically linked to the Dcr-2/R2D2 initiator complex. These authors present an ordered pathway for RISC assembly; native gel electrophoresis revealed the presence of three distinct complexes that assemble on siRNAs in *Drosophila* cell extracts. Over time, two intermediate complexes, including an initiating complex containing Dicer associated with siRNAs, end up in a large holo-complex that still contains Dicers and R2D2 and display many (if not all) features known to be associated with RISC, including conversion of a double-strand siRNA into a single-stranded guide molecule. Importantly, whereas known RISC-associated factors copurify with "holo-RISC" in the absence of siRNAs (which might hint at miRNA-triggered RISC), the Dcr-2/R2D2 complex is only present when extracts are triggered by siRNAs.

Could this mean that the apparent substrate specificity of Dcr-1 and Dcr-2 reflect different RISCs: Dcr-2 builds siRISC onto siRNAs whereas Dcr-1 assembles miRISC from miRNAs (see Figure 1)? Perhaps, but it should be noted that other animals have only one Dicer that can perform both jobs, so the specialization seen here is not universal. Also, miRNAs can act identically to siRNAs when perfectly matched mRNAs are offered (Hutvagner and Zamore, 2002) and actually do so in vivo in *Arabidopsis* (Llave et al., 2002). Conversely, siRNAs, when modified to include centered nonmatching nucleotides, can replace miRNAs in imposing a block for translation (Doench et al., 2003), arguing that siRISC is functionally very similar (if not identical) to miRISC. At present, holo-RISC appears awfully large, perhaps as a result of ribosomal association. Smaller, functional siRISC complexes have been identified but perhaps for miRNA-induced translational interference, this colocalization is essential.

Thus in the field of RNAi research, the ying and yang of biochemistry will remain, on the one hand the desire to find underlying similarities between systems (siRNA and miRNA; silencing in plants, animals, and fungi) and on the other hand the recognition that differences do exist.

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Molecular and Cellular Cognition: The Unraveling of Memory Retrieval

While the study of molecular and cellular cognition has begun to elucidate the mechanisms of acquisition, consolidation, and storage of memories, the understanding of retrieval has lagged behind. In this issue of *Cell*, Murchison et al. (2004) use molecular genetic approaches combined with pharmacology to demonstrate that β-adrenergic receptor function regulates retrieval of certain forms of memory.

Although acquisition, consolidation, storage, and retrieval are the bedrock of studies of memory, retrieval has always been a poor fourth cousin. The visionary Richard Semon coined the words "engram" to reflect the physical changes in brain that encode the memory trace and "ecphory" to refer specifically to events during retrieval that are engaged in the recovery of a specific memory, rather than events that are required to organize memory retrieval itself. It is important to realize that the complex events underlying retrieval are not static; neurobiological and psychological data indicate that the processes that access and reactivate stored engrams are dynamic and can result in dramatic changes in the information stored (see for example, Nader, 2003). The study by Murchison et al. (2004) in this issue reinforces emerging evidence that retrieval has tractable molecular components and that it can be studied separately from acquisition, consolidation, or storage (Mansuy et al., 1998).

Norepinephrine is not a stranger to the study of molecular and cellular cognition. A prominent theory of norepinephrine function in learning and memory has held that the memory strengthening effect of emotion is due to noradrenergic action at β-receptors in the basolateral amygdala. (Cahill and McGaugh, 1998). In this model, the peripheral release of epinephrine during emotionally charged events, leads to the activation of β-noradrenergic receptors in the amygdala and subsequent strengthening of memory in multiple brain regions.

However, norepinephrine plays other roles in the brain. For example, noradrenergic activity is required for LTP in certain brain regions (Stanton and Sarvey, 1985), a model of the synaptic changes required for learning. Thus, noradrenergic function is thought to have a role in the acquisition of new information. The source of hippocampal norepinephrine is the locus coeruleus, which responds to novelty by releasing norepinephrine throughout the forebrain (Sara et al., 1994). The β-receptor is coupled to G proteins and its activation results among other things, in increases in cAMP, activation of PKA and phosphorylation of CREB, events that have previously been implicated in memory consolidation (Matynia et al., 2002). However, the elegant studies by Murchison et al. (2004) show that norepinephrine function is also involved in memory retrieval.

The authors used a knockout of the gene encoding *dopamine β-hydroxylase* (*Dbh*), the enzyme responsible for converting dopamine to norepinephrine (an intermediate in the synthesis of epinephrine). The knockout mice release dopamine from cells that would normally release norepinephrine or epinephrine. Behavioral analysis showed that the $Dbh^{-/-}$ mice have specific deficits in fear conditioning. In this memory task, animals learn to associate a cue (or conditioned stimulus) with a foot-shock. Interestingly, the $Dbh^{-/-}$ mice show normal tone conditioning, in which the conditioned stimulus is a tone paired with foot-shock, but impaired contextual conditioning. In this type of conditioning, the conditioned stimulus is the context (i.e., the conditioning chamber) in which the animal receives the foot-shock. A popular hypothesis proposes that the hippocampus processes information about the context and then feeds this into the amygdala where it is associated with foot-shock. Since the mutants show normal tone conditioning, their contextual conditioning deficits could not be due to abnormalities in either shock sensitivity, release of norepinephrine from the periphery, motor responses to conditioning, or general amygdala deficits. Abnormalities in these phenomena would have affected both tone and contextual conditioning. These and other results demonstrated that the $Dbh^{-/-}$ mutation affected hippocampal memory. But, is this deficit due to impairments in acquisition, consolidation, storage, or retrieval?

Remarkably, Murchison et al. (2004) showed that the contextual conditioning deficits of the mutants could be rescued by injection of L-DOPS (a *Dbh*-independent synthetic amino acid precursor of norepinephrine) prior to testing, but not by injection prior to training. This unexpected result demonstrated that the contextual memory deficit of the mutants was not due to deficits in either acquisition, consolidation, or storage, but was rather caused by faulty retrieval. Providing L-DOPS immediately before retrieval restores noradrenergic function and rescues this process, demonstrating that in the absence of norepinephrine the $Dbh^{-/-}$ mice can acquire, consolidate, and store contextual information normally. This result is a departure from previous molecular studies of retrieval, where involvement in this phenomenon was always inferred from loss of function studies (see for example, Mansuy et al., 1998). Thus, it has been difficult to determine whether failures in retrieval are the result of temporary alterations of the engram caused by the molecular manipulations used, or by deficits in retrieval per se.

Convergent evidence from genetic and pharmacological approaches is an emerging gold standard in molecular and cellular cognition studies. Accordingly, Murchison et al. (2004) show that β-receptor antagonists delivered specifically into the hippocampus prior to testing could also impair contextual memory retrieval (see also Barros et al., 2001). In contrast, infusions in cortex or into the lateral ventricles, which contact many other