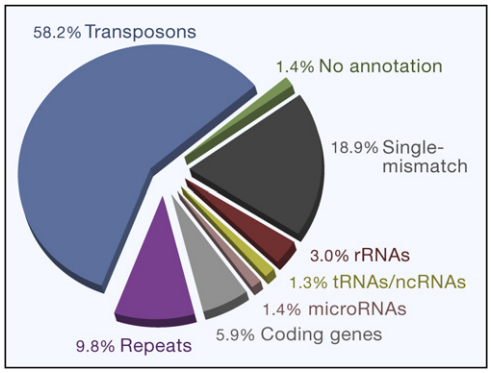
Molecular Biology Select

Cell

One of the first activities identified in the RNA interference (RNAi) pathway was siRNA-mediated endonucleolytic cleavage of target transcripts, referred to as the "slicer" function of Argonaute proteins. This activity was later shown to be mediated by Argonaute2 (Ago2) in both flies and mammals, and to be important for RNAi responses in *Drosophila* to exogenous double-stranded (ds)RNAs such as those generated by viruses during infection. Whether Ago2 has an important role in endogenous gene regulation, and, indeed, whether endogenous siRNAs exist in flies and mammals and what their biological functions could be have been open questions. A series of new studies now report the existence of endogenous siRNAs in both the germline and soma of flies, and in mouse oocytes, providing important insights into the biological roles of this class of small RNAs. The two mouse studies further reveal a role for pseudogenes in gene regulation via the RNAi pathway, and provide interesting insights into the evolutionary pressure for conservation of Ago2 catalytic activity.

siRNAs from Within



Endo-siRNAs overwhelmingly match transposon-derived sequences. Image courtesy of H. Siomi.

Argonaute proteins are essential components of the RNAi machinery that associate with distinct classes of small RNAs to exert their effector functions. One branch of the Argonaute family, the PIWI subfamily of proteins, form complexes with Piwi-interacting RNAs (piRNAs) and are essential for restricting the activity of transposons in the germline. Argonaute proteins are associated with small interfering RNAs (siRNAs) or microRNAs (miRNAs), and silence gene expression by either siRNA guided cleavage of the target mRNA transcript, or by miRNA-mediated posttranscriptional repression involving both translational inhibition and/or mRNA degradation.

In *Drosophila* there are three PIWI proteins and two proteins of the Argonaute family, AGO1 and AGO2. Genetic and biochemical evidence has demonstrated functional specialization in fly AGO proteins, with AGO1 binding to miRNAs and AGO2 being associated with siRNA-mediated gene silencing. Functional specialization extends to the biogenesis pathways associated with these small RNAs; miRNAs are processed from endogenous hairpin precursors by cleavage events involving the RNaseIII enzymes Drosha and Dicer1 (Dcr-1) with its partner Loquacious (Loqs). siRNAs loaded into AGO2 are processed from long dsRNAs by Dicer2 (Dcr-2) and its partner R2D2, but until recently only siRNAs from exogenous long dsRNAs had been reported in flies and mammals. A flurry of recent papers (Ghildiyal et al., 2008; Czech et al., 2008; Kawamura et al., 2008; Okamura et al., 2008) now report the isolation of endogenous siRNAs (endo-siRNAs) from both somatic and gonadal cells of *Drosophila* and provide insight into their biological functions. Interestingly, endo-siRNAs appear to be important in both genome protection and gene regulation, thus bridging roles normally associated with piRNAs and miRNAs. Also, by combining factors involved in the biogenesis of both exogenous siRNAs and miRNAs, they add complexity to our current view of the biogenesis pathways of small RNAs in *Drosophila*.

Structure and Function

Using a combination of biochemical and deep sequencing approaches, three of these groups (Ghildiyal et al., 2008; Czech et al., 2008; Kawamura et al., 2008) made libraries of total small RNAs or small RNAs enriched in AGO2 immunoprecipitates from either *Drosophila* cell lines or whole tissues (ovaries or fly heads). Okamura et al. (2008) took a computational approach, searching for potential long inverted repeat transcripts in the *Drosophila* genome that could provide a source of long dsRNAs, and further analyzing those regions for which small RNAs had been mapped by deep sequencing projects. In combination, these studies reveal the existence of a population of small RNAs that was slightly smaller than miRNAs (21 nucleotides versus the 22 nucleotide modal length of miRNAs), and bore the characteristic siRNA properties of a 5′ monophosphate and a 3′ terminal 2′-*O*-methylation. Endo-siRNAs appear to arise from three main genomic sources: transposons and other genomic repeats, loci that can form long dsRNAs directly (termed structured loci), and convergently transcribed loci. Strikingly, a population of endo-siRNAs arises from loci previously shown to be master generators of piRNAs, suggesting that these loci may serve a dual function as sources of small RNAs directed at transposon control. Despite similar functions, endo-siRNAs exhibit clear differences from piRNAs. For example, piRNAs are antisense biased whereas endo-siRNAs targeting transposons are not. It will be interesting to determine whether endo-siRNAs display a nucleotide bias at any particular position, and how this compares to characteristics of miRNAs and piRNAs.

Analyses of their functional roles suggest that endo-siRNAs bridge some of the activities normally associated with miRNAs and piRNAs, those of gene regulation and transposon control, respectively. Although the biological consequences of gene regulation by endo-siRNAs are not yet clear, expression of genes targeted by these small RNAs is increased in *ago2* and *dcr-2* mutant flies, indicating functional relevance. A large proportion of endo-siRNAs exhibit perfect complementarity to transposons, and increased expression of a subset of transposable elements was observed in the absence of AGO2 and Dcr-1 in both somatic tissues and in fly ovaries. This suggests that endo-siRNAs could be important for controlling transposons in both somatic and germline tissues of the fly. Small RNAs arising from converging transcripts are known to be involved in transcriptional

termination in yeast; it will be interesting to determine if this is also the case in the fly. In an interesting twist, these studies identify siRNAs that are complementary to *ago2* itself, suggesting the existence of a self-regulatory loop in the endo-siRNA pathway.

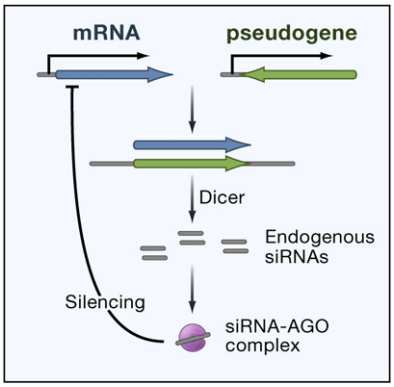
Biogenesis

These studies also provide initial insight into the biogenesis pathways of endo-siRNAs in the fly. Interestingly, endo-siRNA biogenesis appears to blur the distinction between the canonical miRNA biogenesis pathway involving AGO1, Dcr-1, and Loqs, and the exogenous siRNA biogenesis pathway involving AGO2, Dcr-1/R2D2, and the RNA methyltransferase Hen1. In fact, the generation of endo-siRNAs is dependent on AGO2 and Dcr-1, but paradoxically also involves Loqs. Loss of Loqs appears to have the greatest impact on those endo-siRNAs that arise from structured loci. Endo-siRNAs from these loci are also comparatively overrepresented in AGO1 immunoprecipitates, suggesting that different endo-siRNAs may rely on distinct biogenesis and/or loading mechanisms. Finally, the generation of endo-siRNAs and piRNAs from the same loci in both flies and mammals (see below) begs the question of whether the biogenesis of these two small RNAs may be somehow linked.

M. Ghildiyal et al. (2008). Science. Published online April 10, 2008. 10.1126/science.1157396.
B. Czech et al. (2008) Nature. Published online May 7, 2008. 10.1038/nature07007.
Y. Kawamura et al. (2008). Nature. Published online May 7, 2008. 10.1038/nature06938.
K. Okamura et al. (2008). Nature. Published online May 7, 2008. 10.1038/nature07015.

Pseudogenes Move Front and Center

In a pair of related studies, Tam et al. (2008) and Watanabe et al. (2008) address the question of whether endogenous siRNAs exist in mammals. In mice, deletion of any of the PIWI family members results in male sterility characterized by germ cell loss. This loss correlates with the activation of transposons that would normally be silenced by germline piRNAs. Paradoxically, although protection from transposon overactivity is clearly important in both the female and male germline, female mice bearing homozygous deletions of individual *Piwi* genes are phenotypically normal and fertile. Tam et al. (2008) asked whether another small RNA pathway similar to piRNAs could be at work in oocytes. Watanabe et al. (2008) had previously reported endogenous siRNAs in mouse oocytes, but the number identified was small, leaving open the question of the potential functional relevance of these small RNAs. Both groups undertook a similar approach and analyzed libraries of small RNAs from mouse oocytes by deep sequencing. As expected, piRNAs arising from discrete genomic loci formed a substantial portion of the small RNAs sequenced in oocytes, pointing to a conserved role for the Piwi pathway in transposon control in the female germline. However, analysis of the libraries also revealed a population of small RNAs ~21 nucleotides in length that mapped primarily to retrotransposons and to structured loci in the genome. Production of these endo-siRNAs was dependent on Dicer, and in line with a role for these endo-siRNAs in transposon control, Dicer-deficient oocytes expressed a greater number of transposons. Strikingly, endo-siRNAs are not detected in the male germline, providing a potential explanation for the difference between male and female mice bearing mutations in Piwi family members.



Regulation of the founding source gene by a pseudogene via an RNAi mechanism. Image courtesy of H. Sasaki.

A salient aspect of their findings is that in oocytes, endo-siRNAs can be generated from dsRNAs formed by pairing of sense protein-coding transcripts with antisense transcripts from pseudogenes. The investigators identified siRNAs exclusively from overlapping regions between pseudogene and gene transcripts. Some of these siRNAs exhibited perfect complementarity to their putative target transcripts, suggesting an Ago2-mediated slicer effector mechanism. The abundance of the target transcripts of these endo-siRNAs increases in Dicer-deficient cells and Ago2 conditional knockout oocytes, suggesting that regulation of transcript levels by endo-siRNAs may be important physiologically. Together, these studies point to a role for pseudogenes in the regulation of their "founding source genes" (the genes from which the pseudogenes are derived) via an RNAi mechanism. Previously, endo-siRNAs had only been found in organisms that have RNA-dependent RNA polymerase (RdRP) activities such as plants and worms. These new studies indicate that endo-siRNAs with regulatory roles are present in organisms such as flies and mice that lack RdRP activity. The next step will be to query the existence and potential roles of endo-siRNAs in other organisms. In addition, endo-siRNAs in mouse so far appear to be restricted to oocytes; it will be very interesting to uncover whether this is indeed the case, and, if so, how this tissue-specific expression is regulated. Finally, these endo-siRNAs are enriched in Ago2 immunoprecipitates and most likely exert their functions via slicer cleavage of their target transcripts. In mammals, the slicer activity of only one Argonaute protein, Ago2, has been maintained through evolution. The discovery of endo-siRNAs now provides a potential explanation for the evolutionary pressure to maintain the catalytic activity of one of the Argonaute proteins.

O.H. Tam et al. (2008). Nature. Published online April 13, 2008. 10.1038/nature06904.
T. Watanabe (2008). Nature. Published online April 10, 2008. 10.1038/nature06908.

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