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Minireview

How selfish retrotransposons are silenced in *Drosophila* germline and somatic cells

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Abstract Transposable elements (TEs) are DNA elements found in the genomes of various organisms. TEs have been highly conserved during evolution, suggesting that they confer advantageous effects to their hosts. However, due to their ability to transpose into virtually any locus, TEs have the ability to generate deleterious mutations in the host genome. In response, a variety of different mechanisms have evolved to mitigate their activities. A main defense mechanism is RNA silencing, which is a gene silencing mechanism triggered by small RNAs. In this review, we address RNA silencing mechanisms that silence retrotransposons, a subset of TEs, and discuss how germline and somatic cells are equipped with different retrotransposon silencing mechanisms.

Keywords: Argonaute; Retrotransposon; siRNA; piRNA; RNA silencing; *Drosophila*

1. RNA silencing involving PIWI proteins

Biochemical and mutation analyses have revealed the existence of Dicer and Argonaute multigene families, members of which are the key factors in RNA silencing in *Drosophila* [1–4]. Dicer functions in maturing small RNAs [20–30 nucleotides (nt)] that trigger RNA silencing, whereas Argonaute functions in down-regulating gene expression post-transcriptionally by directly targeting particular mRNAs [5]. mRNA targets are selected by base-pairing with small RNAs, which have been processed by Dicer and loaded onto the Argonautes. Extensive studies at the molecular level have revealed that two members of the *Drosophila* Argonaute family, Argonaute1 (AGO1) and Argonaute2 (AGO2), specifically associate with miRNA and siRNA, respectively, and function in gene silencing mechanisms mediated by miRNAs and RNAi, respectively [3]. We have analyzed gene silencing in living cells using monoclonal antibodies against each of the Argonaute proteins to immunopurify endogenous antigens and specifically associated small RNAs from living cells [6]. AGO1 and AGO2 have been well studied, therefore, we focused on identifying small RNA binding partners, as well as investigating RNA silencing pathways of other *Drosophila* Argonaute members, namely AGO3, Aubergine (Aub), and Piwi. These three Argonautes are most likely expressed only in the germline [7], and are collectively called the PIWI proteins to distinguish them from ubiquitously expressed AGO1 and AGO2 [8].

Forward genetic approaches have shown that mutations introduced into the *Piwi* gene cause disruption to the germline and that *Piwi* is an essential factor in germline stem cell (GSC) self-renewal in both males and females [9–11]. It was also demonstrated that *Piwi* mutations impact retrotransposon mobility [12,13]; without functional *Piwi*, retrotransposons become abnormally active. These were the first studies to show the connection between *Piwi* function and regulation of retrotransposon activity. However, the molecular function of Piwi protein had yet to be determined.

Genetic studies have shown that *Aub* is required for pole cell formation [14] and for activating RNAi during *Drosophila* oocyte maturation [15]. *Aub* is also involved in silencing retrotransposons in the germline [16–18], and in silencing *Stellate* genes in the testis by targeting the *Suppressor of Stellate* [*Su(Ste)*] repeats on the Y chromosome, which are highly homologous to *Stellate*.

As mentioned above, AGO1 and AGO2 specific monoclonal antibodies were key reagents for the molecular investigation of Argonaute functions in RNA silencing. Thus, we endeavored to produce monoclonal antibodies against the PIWI proteins and were indeed successful in producing specific monoclonal antibodies that recognize each of the antigens and that do not cross-react with other Argonaute members [19–21].

2. The PIWI proteins associate with rasiRNAs in the germline

Not only Piwi and Aub proteins but also AGO3 was successfully purified from *Drosophila* ovary lysates. Small RNAs contained in the immunoprecipitates from about 200 ovaries were visible by silver staining ([21] and unpublished data), indicating their abundance. Identification and analysis of these small RNAs revealed that all three PIWI proteins preferentially associate with a particular set of small RNAs, previously termed as rasiRNAs (repeat-associated small interfering RNAs) [22]. This indicates that the PIWI proteins most likely

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function in pathways silencing retrotransposons in the germline since rasiRNAs are mainly derived from retrotransposons, remnants of ancient retrotransposons or from other repetitive sequences found in the genome [22]. Particular characteristics of small RNAs associated with the PIWI proteins are that (1) Piwi and Aub show a strong preference to bind to rasiRNAs originating from the antisense transcripts of retrotransposons, whereas AGO3 prefers to bind rasiRNAs from the sense strand and (2) the preferred lengths of rasiRNAs that associate with each PIWI protein differ. Piwi prefers to associate with longer rasiRNAs (24–30 nt), while AGO3 prefers to bind to relatively smaller rasiRNAs of approximately 23 nt [20]. rasiRNAs are longer in length than miRNAs and siRNAs, which function in gene silencing in association with AGO1 and AGO2 [22]. Currently, small RNAs binding to the PIWI proteins in other organisms, including mouse and fish, are collectively referred to as piRNAs (PIWI-interacting RNAs) [23]; thus, we will follow this terminology.

3. piRNAs are methylated

In *Drosophila* piRNAs are chemically different from miRNAs [18,20,24,25]. As opposed to miRNAs expressed in plants, miRNAs in animals show sensitivity to peridate ($NaIO_4$) oxidation and beta-elimination treatments since they contain a 2′, 3′-*cis*-diol at their 3′ ends [26]. On the other hand, piRNAs are resistant to these chemical treatments, indicating that piRNAs lack one of the terminal hydroxyl groups [18,20,24,25]. Further investigation using the mutant fly, *piggyBac*^{f00810}, in which *CG12367* is disrupted by an element called *piggyBac*, revealed that piRNAs expressed in fly ovaries and those associated with the PIWI proteins are 2′-*O*-methylated at their 3′ ends [24,25]. The gene responsible for this modification is *DmHen1*/*Pimet* (piRNA methyltransferase) (dubbed *CG12367* in FlyBase), which is the *Drosophila* orthologue of the *A. thaliana* gene, *Hen1*, identified as a source of miRNA methyltransferase activity by Yu et al. [27]. Under conditions where the PIWI proteins physically associate with DmHen1/Pimet, the miRNA binding partner, AGO1, is not able to associate with the enzyme [24]. This might explain why miRNAs are not methylated in *Drosophila*. Phil Zamore and his colleagues showed that siRNAs exogenously introduced in S2 cells are also methylated [25]. Recently, we performed periodate oxidation and beta-elimination on siRNAs associated with AGO2 in S2 cell lysates that had been pre-incubated with siRNA duplexes, and we confirmed that exogenous siRNAs could indeed serve as substrates for DmHen1/Pimet (unpublished observations).

4. Association of AGO2 with endogenous siRNAs

Flies utilize the RNAi mechanisms to defend against viral infection [28]. siRNAs originating from infecting viruses that produce double-stranded RNAs (dsRNAs) as part of their replication cycle associate with AGO2 [28]. Mutations in *Ago2* and *Dicer2* caused loss of viral infection resistance, which strengthens the idea that the RNAi system is involved in virus defense.

A question we then raised was whether AGO2 could exist simply to lie in wait for exogenous small RNAs, such as siRNAs originating from the viral dsRNAs, or does AGO2 have its own endogenous partner(s) in naïve cells and organs. To address this question, we specifically immunoprecipitated endogenous AGO2 from naïve S2 cells that were grown under normal conditions, and visualized small RNAs associated with the protein [29]. We found that in these cells AGO2 existed in association with endogenous small RNAs of around 21 nt. Interestingly, the size peak was 1 nt shorter than that of miRNAs associated with AGO1 in S2 cells. Since this was the first demonstration that AGO2 has its own endogenous small RNA partners, as do other Argonautes in *Drosophila*, we referred to them as endogenous short interfering RNA, esiRNA. Identification and analysis of esiRNAs revealed that they are mainly derived from retrotransposons and other genomic repetitive elements. This property of esiRNAs resembled that of piRNAs. However, piRNAs and esiRNAs are clearly distinct classes of small RNAs in respect to their sizes and their protein partners, as piRNAs bind to the PIWI proteins and their sizes are about 24–30 nt in length. In contrast, esiRNAs are approximately 21 nt and associate specifically with AGO2. The expression profiles of piRNAs and esiRNAs through development are also different; piRNAs are found in principle only in the germline, while esiRNAs are likely to be expressed ubiquitously, based on the observation that esiRNAs could be detected, not only in S2 cells, but also in adult bodies devoid of germline, as well as in embryos (unpublished data) where most of the cells are somatic.

5. piRNA and esiRNA biogenesis

piRNAs associated with Piwi and Aub in ovaries, show a strong preference for uracil (U) at the 5′ ends, while AGO3-associated piRNAs show a strong preference for adenosine (A) at the 10th nucleotide from the 5′ ends. By contrast, esiRNAs show little or no bias for nucleotides at any position, indicating that the processing mechanisms of piRNAs and esiRNAs might be different. In early 2007, the Hannon group and ourselves proposed a model for "piRNA biogenesis" [20,30]. In agreement with the observation of Vagin et al. [18], in which piRNAs were produced Dicer-independently, our model also excludes Dicer activities. Although it is not yet entirely understood, it is generally accepted that the Slicer activities of PIWI proteins [19–21] contribute to the generation of piRNAs, at least in determining and producing their 5′ ends.

How, then, are esiRNAs produced in vivo? Using bioinformatic analyses of esiRNAs, we summed the number of unique small RNAs in a 5 kb sliding window and plotted this against the *Drosophila* draft genome assembly. Clusters of small RNA production were observed, from which we estimated that most esiRNAs could be divided into two types: those that matched retrotransposons and those that arose from long stem–loop structures from repetitive sequences located on the X chromosome. Plotting of the esiRNAs derived from retrotransposons against the *Drosophila* draft genome assembly revealed a distinct "hotspot" that produces a number of esiRNAs and that the hotspot expresses esiRNAs from both sense and antisense strands. By looking at the plotting pattern, we postulated that esiRNAs must be largely derived from dsRNAs arising from the bi-directional transcripts of retrotransposons in a Dicer2-dependent manner. By contrast, esiRNAs arising from stem–