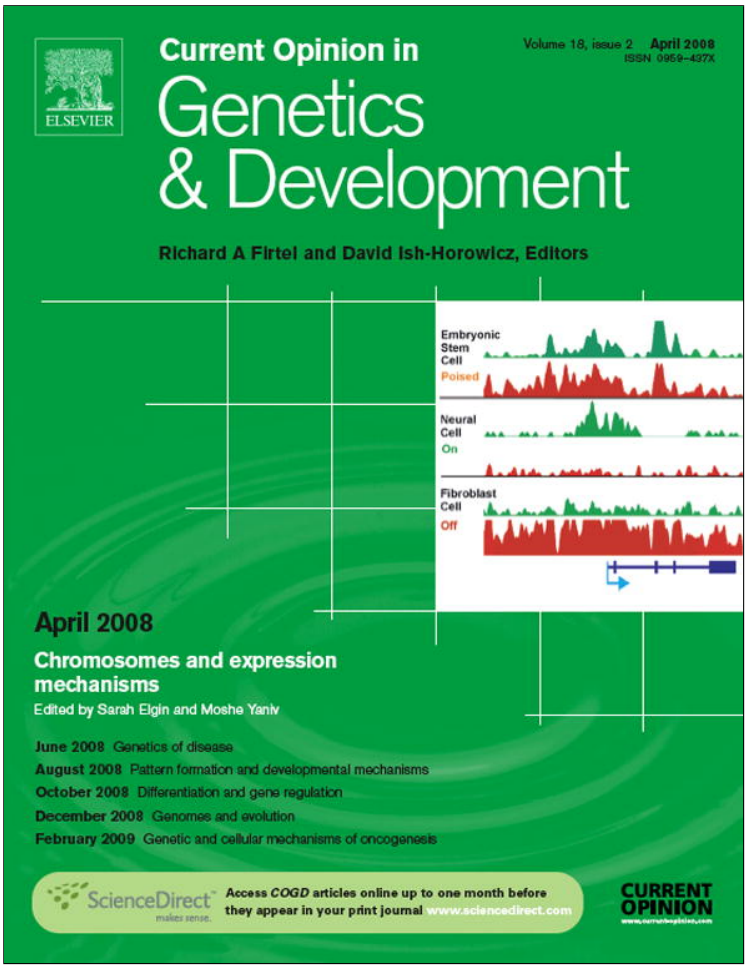

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Interactions between transposable elements and Argonautes have (probably) been shaping the *Drosophila* genome throughout evolution

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Transposable elements (TEs) are powerful mutagenic agents responsible for generating variation in the host genome. As TEs can be overtly deleterious, a variety of different mechanisms have evolved to keep their activities in check. In plants, fungi, and animals, RNA silencing has been implicated as a major defense against repetitive element transposition. This nucleic acid-based defense mechanism also appears to be directed at inherited silencing of TEs without altering the underlying DNA sequence. Complex interactions between TEs and RNA silencing machineries have been co-opted to regulate cellular genes.

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Introduction

'Selfish DNA – the large amounts of DNA in our chromosomes with no obvious function – may turn out to be part of another [tricks that are used to make for smoother and more rapid evolution]. It is entirely possible that this selfish DNA may play an essential role in the rapid evolution of some of the complex genetic control mechanisms essential for higher organisms.'

Francis Crick *What Mad Pursuit* (1988) [1].

It is remarkable to consider that more than 40% of the human genome is comprised of retroelements and their relics [2]. Retroelements were long thought of as either 'selfish' or 'parasitic' DNA elements that were there not for the sake of the host organism, but for their own sake in an evolutionary sense; thus they were considered to be either neutral or deleterious to their hosts. However, it is becoming increasingly clear that there are more complex interactions between retroelements and their hosts than strict parasitism; these elements produce changes that have a broad range of fitness values at an organismal level. Recent evidence indicates that these elements confer a fitness benefit to the host more frequently than previously recognized [3–5].

Retroelements, including retrotransposons (also called 'LTR retrotransposons'), retroposons (also called 'non-LTR retrotransposons'), mobile introns and related elements replicate through obligatory RNA intermediates using reverse transcriptase-dependent mechanisms [3]. Transposition of these elements may not only shape genomic landscapes by insertions, deletions and gene rearrangements but also create functional diversification of the host's genes on which natural selection can act. They modulate host gene expression in several ways. In some cases, a new insertion can disrupt an existing host regulatory element. In other cases, the retroelement may contribute its own *cis*-regulatory sequences (e.g. potential splice sites, polyadenylation signals, transcriptional promoters or enhancers) to a host gene, providing the potential for creating greater functional diversity of genes. Transcribed retroelements may also have a particular function in translation regulation [6]. As well, inserted sequences may be candidates for co-option or 'exaptation' as (or future evolution of) regulatory sequences. Thus retroelements could serve as dynamic reservoirs for new cellular functions [3–5].

It is clear that the unbridled activity of retroelements has the potential to cause deleterious insertions and re-

Glossary
AUB: Aubergine
dsRNA: double-stranded RNA
HP1: heterochromatin protein 1
miRNA: microRNAs
piRNA: Piwi-interacting RNA
PIWI: P element-induced wimpy testis
rasiRNA: repeat-associated small interfering RNA
RNAi: RNA interference
siRNA: small interfering RNA
TEs: transposable elements

arrangements in the host genome. Thus it is not surprising that multiple pathways have evolved to inhibit retrotransposition [3]. Since new insertions and rearrangements that happen in germ cells are likely to be passed to the next generation, these mechanisms are particularly important for genome surveillance in the germline. Conversely, these mechanisms are presumably also important for retroelements to modulate the extent of damage to the host genome upon which they depend. Recent evidence has linked RNA silencing with inhibition of expression and transposition of retrotransposons [4]. Here, we focus on recent work with the model animal *Drosophila melanogaster*, one that has contributed significantly to our understanding of RNA silencing and how interactions between retrotransposons and RNA silencing machineries have been co-opted to influence gene expression.

RNA silencing

RNA silencing is an evolutionarily conserved mechanism in which small RNAs trigger various forms of sequence specific gene silencing by guiding effecter complexes to target RNAs via base-pairing [7,8]. Biochemical and genetic analyses reveal the existence of multigene families encoding the two key proteins – Dicer and Argonaute – in RNA silencing. In RNA interference (RNAi) (see Glossary), small interfering RNAs (siRNAs) are produced from long exogenous double-stranded RNA (dsRNA) (see Glossary), whereas microRNAs (miRNAs) are excised in a stepwise process from RNA hairpins encoded by the genome. In flies, DICER-1 generates miRNAs while DICER-2 produces siRNAs [7,8]. These small RNAs presumably are complementary to target mRNA by strict Watson–Crick base pairing or according to miRNA (see Glossary) targeting 'seed' rules [7,8]. Single-stranded siRNAs or miRNAs are loaded onto RISCs (RNA induced silencing complexes) that participate in subsequent steps such as mRNA cleavage or mRNA translational repression.

Argonaute proteins are a defining component of RISC complexes [9]. They are a protein family conserved from fission yeast to humans; its name comes from its founding member in *Arabidopsis* [9]. Argonaute proteins are ~100 kD basic proteins and are composed of two principal domains; an amino-terminal PAZ domain, a single-stranded nucleic acid binding domain; and an carboxyl-terminal PIWI domain that is responsible for the RNase-H-like catalytic or 'Slicer' activity [10]. The PIWI domain contains two aspartates and a histidine that form a 'DDH' catalytic motif similar to the DDE catalytic triad (aspartate/aspartate/glutamate) motif seen in RNase H. Duplex formation between the guide small RNA and the target places the scissile phosphate of the target strand (located between the 10th and 11th nucleotides as measured from the 5′ end of the guide) adjacent to the Slicer catalytic site in the PIWI domain. If the catalytic site is functional and the guide-target duplex is complementary in the region of the scissile phosphate, the target strand can be cleaved. If the guide contains mismatches to the target sequences in the region of the scissile bond or the Argonaute does not contain the Slicer catalytic residues, slicing does not occur but the Argonaute-guide complex remains associated with the target and can induce translational repression or cleavage-independent mRNA decay.

Argonaute proteins have proliferated and evolved a range of functions for endonucleolytic cleavage, translational repression, recruitment of chromatin-modification factors, and biogenesis of small RNAs (see below). Argonaute proteins fall into two classes [9]; the AGO subfamily based on *Arabidopsis thaliana* Ago1 and the PIWI subfamily based on *Drosophila melanogaster* PIWI. *Drosophila* has two AGO proteins (AGO1 and AGO2), that are ubiquitously expressed, and three PIWI proteins [AGO3, AUB (Aubergine), PIWI (P-element induced wimpy testis)], (see Glossary) whose expression is primarily observed in the germline [11]. Each has specialized functions. For instance, AGO1 is involved in the miRNA-dependent mRNA silencing pathway (mostly translation repression), whereas AGO2 functions in a pathway for siRNA-directed RNAi (see Glossary) [8]. AGO2 also promotes RISC assembly by slicing siRNA duplex precursors [7].

Genetic studies have indicated that PIWI is an essential factor in germline stem cell self-renewal while AUB is required for pole cell formation and spermatogenesis [12,13]. These PIWI proteins are also involved in silencing retrotransposons and repetitive sequences in the male and female germlines of *Drosophila*. PIWI proteins bind repeat-associated small interfering RNAs (rasiRNAs) [12,14•,15•,16••]. rasiRNAs are longer in length than siRNAs and miRNAs (24–27 nucleotides, as opposed to 20–22) and are mainly derived from transposons, their remnants or other repetitive elements in the genome. As well as their longer length, rasiRNAs are chemically different in that they lack one of the 3′-terminal hydroxyl groups characteristic of animal miRNAs; instead they are 2′-*O*-methylated at their 3′ ends [17,18]. Currently, small RNA binding partners for PIWI proteins (including rasiRNAs) are collectively referred to as PIWI-interacting RNAs (piRNAs); we will use the term hereafter. PIWI proteins are able to exhibit Slicer activity *in vitro* [12,14•,15•]. Thus, it can be postulated that these PIWI proteins function, at least in part, as a Slicer, to cleave transcripts from repetitive TEs (see Glossary) through their association with piRNAs. In this way, they silence active transposons, thereby protecting the genome from invasive elements. piRNAs map to discrete genomic clusters, suggesting that a limited number of master piRNA (see Glossary) loci might control germline transposon activity [16••].

piRNAs are distinct from both siRNAs and miRNAs not only in their length but also in their biogenesis. piRNA production appears not to require DICER-1 or DICER-2 [12]. Rather, piRNA biogenesis involves a Dicer-independent cycle that amplifies piRNAs [15•,16••]. piRNAs associated with PIWI proteins show a strong sequence bias. AUB and PIWI preferentially bind piRNAs derived from the antisense strand of reterotransposons, and the 5′ end is predominantly uridine (U), while AGO3 associated piRNAs arise mainly from the sense strand and show a strong preference for adenine (A) at nucleotide 10, but no preference for U at the 5′ end. As described above, Argonaute proteins exhibit Slicer activity that directs cleavage of its cognate RNA target across from nucleotides 10 and 11, measured from the 5′ end of the small RNA guide strand. These results together suggest that AGO3 associated with a rasiRNA (see Glossary) with A at nucleotide 10, can target a long RNA molecule by Watson–Crick base pairing and cleave the target RNA, resulting in sliced RNAs with U at the 5′ end. Similarly, when AUB or PIWI associated with rasiRNAs with U at the 5 end, slices its cognate RNA target, the resulting cleaved RNA will have an A at nucleotide 10. In this model, the Slicer activities of PIWI proteins serve a dual capacity; they degrade sense transposon transcripts and produce the antisense piRNAs. Thus, in piRNA biogenesis, sense and antisense transcripts from retrotransposons appear to interact through the action of PIWI proteins in a cycle that amplifies piRNAs (Figure 1). Therefore, retrotransposons are both a source gene for piRNAs and a target of piRNA-mediated silencing. Changes in the activity of the piRNA pathway in response to environmental stimuli or stress may allow a window of opportunity for expression or even transposition of some retrotransposons to occur. By creating new nodes in the piRNA biogenesis cycle or new inheritable insertions of retrotransposons, such transposons could influence end points of gene expression regulated by the piRNA pathway.

Generation of *Drosophila* piRNAs is also distinct from biogenesis of plant siRNAs and *Caenorhabditis elegans* secondary siRNAs, both of which also involve amplification steps [7]. Plant siRNAs might prime dsRNA synthesis by RNA-dependent RNA polymerase (RdRP), leading to dicing and amplification of siRNAs as long as the target RNA continues to be produced. By contrast, each secondary siRNA in *C. elegans* is synthesized individually by RdRP [19,20]. However, the *Drosophila* genome lacks genes encoding RdRP. The biogenesis mechanism of piRNAs appears conserved in fish and mammals [21,22]. A key difference between transposon management in *Drosophila* and in mammals is the role of cytosine methylation in maintaining stable repression. Recent evidence indicates that mammalian PIWI–piRNA complexes may function in an RNA-dependent DNA methylation pathway [22].

How then is the piRNA pathway connected with germline maintenance? One possibility is that loss of the piRNA pathway affects germline integrity through derepression of transposones, whose activity can lead to DNA damage [23]. Another possibility, not mutually exclusively, is that loss of the piRNA pathway affects expression of specific genes that might be important for germline development. Since the biogenesis of piRNAs appears to involve a long single-stranded precursor [15•,16••], transcripts from cellular genes (whose product benefits the cell) in the germline, in principal, could participate in an amplification cycle as nodes if they have perfect or near perfect piRNA target sites. Cellular transcripts containing a stretch of sequences that are sufficiently complementary to a piRNA could also be targets of piRNA. In the latter case, piRNAs could guide PIWI proteins either to cleave the target with low turnover rates or to repress translation of the target in a fashion similar to miRNA mediated control. In this regard, it is interesting that many AUB-associated piRNAs in testis arise from the *Suppressor of Stellate* [*Su*(*Ste*)] repeats on chromosome Y which are required to silence *Stellate* genes on the X chromosome [14•]. In addition, some AUB-associated piRNAs in testis also show significant complementarity to part of a *vasa* transcript, a germline specific transcript involved in oocyte differentiation and cyst development. An immunopurified AUB-piRNA complex from testes displays Slicer activity to cleave target RNA containing the *Stellate* sequences or the *vasa* sequence [14•]. Therefore, piRNAs have the potential to trigger RNA silencing *in cis* (at the locus from which they are derived) and *in trans* (at a locus from which they do not arise). It is also interesting to note that a fraction of mouse PIWI (Miwi) comigrates with polysomes on density gradients [24]. A recent computer-based pattern discovery search revealed that short blocks (~16 nucleotides) termed 'pyknons' are dispersed at multiple sites in eukaryotic genomes; the great majority of them are found both inside repeat TEs and in repeat-free regions [25,26], suggesting that many cellular genes may have piRNA target sites. In support of the view that piRNAs could regulate expression of cellular genes as well, we note that many miRNAs appear to have evolved from transposable elements, and their involvement in gene regulation appears to be an outcome of the antagonistic relationship between transposons and the host genome [5,27].

Epigenetic silencing

Retroelements have been estimated to constitute ~27% (retrotransposons: 22%; retroposons; 5%) of the *Drosophila melanogaster* genome [3]. These repetitive sequences account for the majority of all heterochromatic DNA in *D. melanogaster*, whose genome is ~30% heterochromatic [28], suggesting that they play a central role in the assembly of heterochromatin.

Historically, heterochromatin has been regarded as transcriptionally inert. Recent evidence in fission yeast *S. pombe* indicates that components of the RNAi system are involved in heterochromatin formation. While it seems counterintuitive, the data suggest that transcription of silenced loci by RNA pol II and processing into siRNA are essential for the maintenance of the heterochromatic state [29]. Thus, heterochromatin must be expressed to maintain 'silence.'

The first indication that components of RNA silencing pathways function in assembly of heterochromatin-like domains came from studies of gene silencing in *Drosophila*. Genetic studies revealed that repeat-induced gene silencing in *Drosophila* requires components of the Polycomb group proteins and PIWI [30,31]. The chromatin context of a gene can determine its expression, as illustrated by position-effect variegation (PEV) in *Drosophila*, in which the mosaic silencing of a normally active gene occurs when it is located in or near heterochromatin. *piwi* and *aub* mutants can suppress PEV of a *white* reporter, and appear to result in some delocalization of the heterochromatin proteins HP1 (see Glossary) and HP2, proteins that bind normally at silenced loci [32]. Collectively, these observations suggest that PIWI proteins (and presumably their associated piRNAs) are involved in setting chromatin states. This also implies that the factors which confer silencing status in heterochromatin can spread into nearby genes that would otherwise be active. Therefore, insertion of transposons into or near genic regions could affect expression of nearby genes, on which natural selection can act. A recent study indicates that a new insertion of a P transposable element carrying a 1360 repetitious element can indeed suppress expression of an adjacent reporter gene in the same element, given an appropriate chromosomal environment [33•]. The *piwi* gene also interacts genetically with the regulation of a Polycomb response element and Polycomb protein function, suggesting a role influencing cellular identity during embryonic development through a chromatin-dependent mechanism [34]. Furthermore, recent studies have shown that PIWI appears to interact directly with HP1, which also implicates PIWI in heterochromatin formation [35]. Surprisingly, the protein is also implicated in activation of transcription in some cases, notably at telomeres [36•].

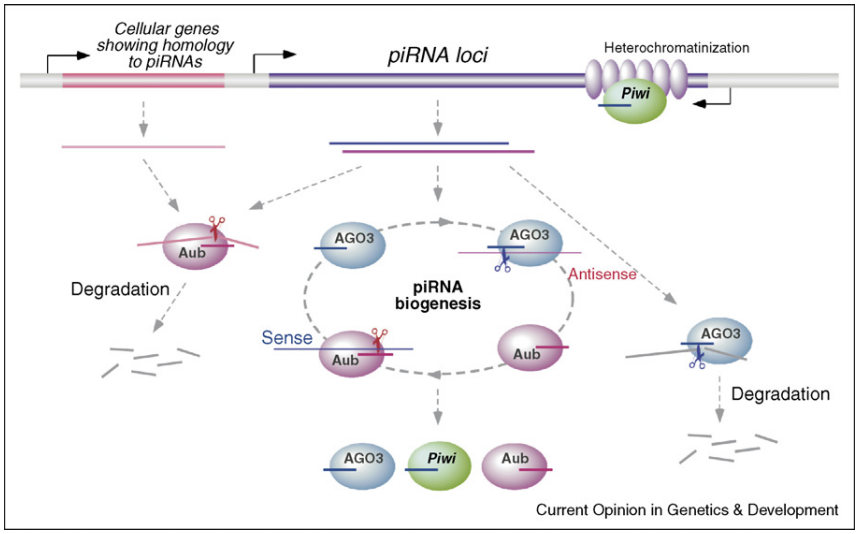
Given the biogenesis model of piRNAs, the potential for RNA silencing machineries in *Drosophila* to have an epigenetic characteristic appears to involve both heterochromatin formation and a second mechanism at the RNA level. In the former mechanism, piRNAs may home in on homologous nascent RNAs traversing the silencing loci and recruit, together with PIWI proteins, chromatin-modification factors to these regions. In the latter mechanism, RNA transcripts from silenced loci would be expressed continuously [37•] and enter into a piRNA amplification cycle in concert with the Slicer activity of PIWI proteins to initiate epigenetic silencing. In this cycle, piRNAs would have the potential to target further 'maintenance' rounds of piRNA production on the original transcripts and on homologous transcripts. This would result in the degradation of transposon transcripts, thereby leading to the suppression of transposon activity. In effect, these maintenance rounds of silencing provide both a genetic memory of transposons to which the population has been exposed and a molecular memory of primary piRNAs.

Implications of RNA mediated inheritance

Since expression of PIWI proteins is largely restricted to the germline, the manifestation of heterochromatic states, PEV and in some cases the control of gene expression in somatic cells, mediated by PIWI proteins [30,31,34,38] should be preprogrammed during very early ontogeny and therefore must involve chromatin memory. How could this be achieved?

In *C. elegans*, silencing induced by RNAi can be transmitted for multiple generations [39], and chromatin-modifying factors appear to play a role in this inheritance mechanism [40]. In *Drosophila*, misregulation of normally silenced sequences occurs when males carrying certain transposable elements are crossed with females lacking them [4,12]. This results in transposon activation with detrimental consequences that induce hybrid sterility. The reciprocal cross of a female carrying a repressed transposon with a male lacking it does not result in sterility associated with high rates of germline transposition in the offspring. These phenotypes are collectively referred to as hybrid dysgenesis. The maternal suppression of hybrid dysgenesis suggests the involvement of a cytoplasmic factor, termed cytotype, that suppresses the activity of transposable elements, and that the repressive signal is passed from females to their offspring. Recent evidence indicates that maternal suppression of hybrid dysgenesis depends on PIWI proteins and piRNAs [4,41]. AUB and PIWI proteins are deposited in the developing germline during oogenesis through germline transmission [12,42]. Thus, the maternal contribution of PIWI proteins and presumably their associated piRNAs, as well as the sequences of piRNA themselves, provide a physical basis for the hypothesis that the transfer of silencing signals and chromatin memory to off-springs is mediated in the form of PIWI–piRNA complexes. This also implies that *Drosophila* may possess the capacity to store information at numerous genetic loci in the form of PIWI–piRNA complexes and so pass them on from generation to generation. These genetic loci not only include transposons and other repetitive elements but may also include their homologous sequences that can be part of cellular genes. The maternal transmission of PIWI–piRNA complexes may also imply that primary piRNAs that initiate an amplification cycle of piRNA biogenesis are supplied through

Figure 1



The biogenesis and functions of piRNAs in *Drosophila*. piRNAs bind the PIWI subfamily of Argonaute proteins – AGO3, Aubergine (AUB) and PIWI – and target homologous transposons scattered around the genome for silencing. PIWI–piRNA complex might also target cellular transcripts, if a part of the transcript is homologous to piRNA. The nuclear localization of PIWI [14•,16••,42] may indicate that this protein directly participates in heterochromatin formation. In piRNA biogenesis, the Slicer activities of PIWI proteins serve a dual capacity: cleaving sense transposon transcripts and producing antisense piRNAs. PIWI-mediated cleavage events generate new piRNAs, thereby setting up a self-reinforcing cycle. It is not yet known how AGO3, AUB, PIWI are able to specifically recognize the respective sense and antisense strands of repetitive elements.

germline transmission; thus the cycle could operate between generations.

Conclusions

RNA silencing is thought to have evolved as a form of nucleic acid-based, and thus sequence-directed, immunity to block the action of viruses and TEs [43]. Host–parasite interactions are typically associated with rapid evolution because of a permanent antagonistic relationship resembling an 'arms race' in which parasite adaptations are countered by host adaptations [44]. Consistent with this, recent evidence indicates that components of RNA silencing pathways are among the fastest evolving immune-related genes [45]. It is becoming increasingly clear that the 'arms race' between transposons and hosts leads to positive selection for cellular defense mechanisms, part of which are co-opted or 'borrowed' for evolving new regulatory circuits, thus enabling the integration and networking of complex suites of gene activity. However, there are numerous gaps in our understanding of the biochemical events that transpire in this 'arms race', which has the potential to create diversification of gene expression in hosts. Many questions remain unanswered; some examples follow. How are *Drosophila* AGO3, PIWI, AUB able to specifically recognize the respective sense and antisense strands of retrotransposones and repetitive elements? What might be the mechanism that specifies the 3′ ends of piRNAs? How are antisense transcripts of retrotransposons produced and how are they regulated? How can a *de novo* piRNA biogenesis cycle be initiated? Precisely how do piRNAs mediate epigenetic regulation of gene expression? What are the developmental roles of piRNAs? As well as silencing transposons in the germline, can piRNAs regulate germline development by affecting cellular genes directly? Components of piRNA pathways are also implicated in activation of transcription and translation in some cases [36•,42,46]. So, what can turn normally repressors of gene expression into activators? Recent studies have shown that TEs may also actively transpose in some somatic cells, creating somatic mosaicism in animals [47]. Some retrotransposons are transcribed in *Drosophila* somatic cells and they are not repressed by the piRNA pathway [48]. Thus, how is transposon activity in somatic cells controlled? Since small RNA triggers guide Argonaute proteins to ensure that they specifically silence transcripts homologous to the small RNA, deciphering how these partner Argonautes contribute to the function of small RNAs is integral to understanding their biological roles. Studies that catalog interacting small RNA partners of all of the *Drosophila* Argonaute proteins in the germline and various somatic tissues by high-throughput sequencing should yield some insight into these issues. We look forward to the day when these and other important mysteries are solved and when other, for now unexpected, roles for Argonautes and transposons are uncovered.

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