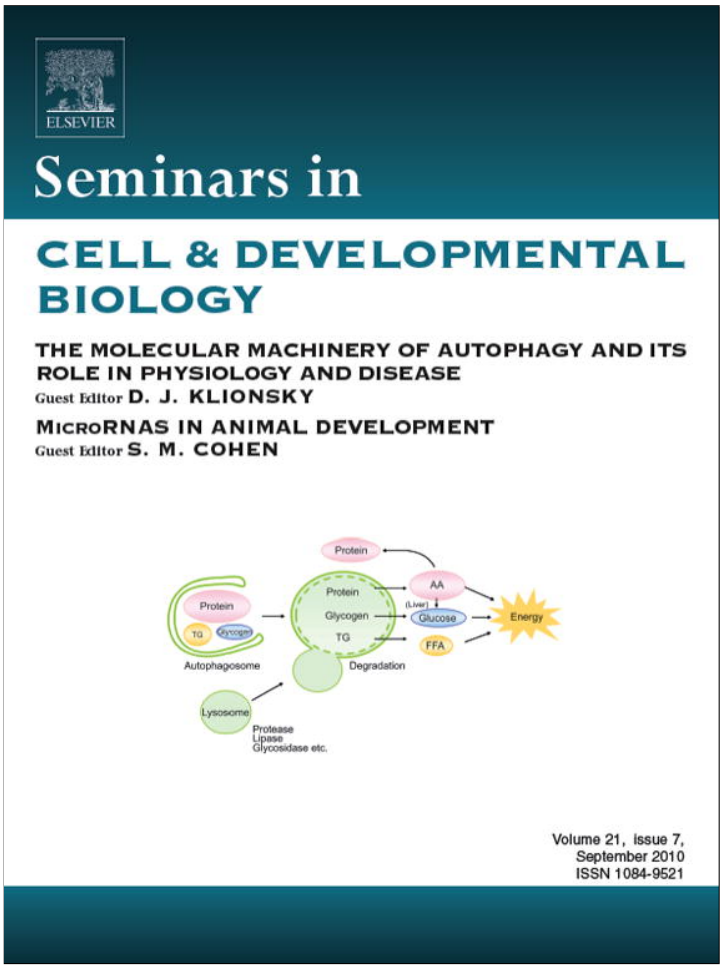

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Review

piRNA-mediated silencing in *Drosophila* germlines

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ABSTRACT

PIWI interacting RNAs (piRNAs) are a subset of small RNAs predominantly expressed in the germlines of various species. In *Drosophila*, the main sources of piRNAs are transcripts of mobile DNA elements, including active transposons and their wreckage, found on the genome. After maturing from the primary transcripts, piRNAs are specifically loaded onto germline-specific Argonaute proteins – Argonaute3, Aubergine and Piwi – collectively referred to as PIWI proteins. Loss of function of PIWI proteins and/or the piRNA loci on the genome lead to derepression of transposons and causes severe defects in gametogenesis and fertility. The necessity for both PIWI proteins and piRNAs in protecting the genome of the gametes from mischievous mobile genomic elements is thus obvious. There have been extensive biochemical and genetic studies on PIWI proteins and piRNAs. These have shed light not only on the molecular mechanisms of gene silencing mediated by piRNAs and PIWI proteins, but also on their intriguing relationship with cellular genes that have been shown to be important for gametogenesis and fertility.

Contents

1. Introduction

To date, the accumulated evidence clearly indicates that in *Drosophila* three sorts of small RNAs are expressed endogenously; endogenous small interfering RNAs (endo-siRNAs or esiRNAs), microRNAs (miRNAs) and piRNAs [1–3]. Although all these small RNAs are considered fundamentally separable, certain commonalities can be found in all. First, they are short ribonucleic acid molecules consisting of only 20–30 nucleotides (nt). Second, they all are not expressed 'as is'; rather, they are matured from much longer RNA precursors through processing pathways consisting of multiple, sequential RNA cleavage steps, which are accomplished by particular kinds of RNase enzymes [4]. Third, all these small RNAs function as important players in gene silencing machineries by associating directly with Argonaute proteins [1,5], which are another set of important players in the silencing mechanisms. The gene silencing pathways mediated particularly with small RNAs and Argonaute proteins are referred to collectively as RNA silencing [1]. The association between small RNAs and Argonaute proteins is direct and occurs stoichiometrically [6,7]. In addition, they do not cross-interact basically; namely, miRNAs and esiRNAs associate with the ubiquitously expressed Argonaute proteins, AGO1 and AGO2, respectively (although some minor miRNAs were recently shown to be associated with AGO2) [8–13], whereas piRNAs asso-

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ciate only with the PIWI proteins Piwi and Aubergine (Aub; also known as Sting) and Argonaute 3 (AGO3) [14–18]. The partnership between small RNAs and Argonaute proteins is well regulated.

Regardless of the commonalities described above, the three small RNAs are considered distinct because each shows specific features. Thus, piRNAs are germline-specific whereas miRNAs and esiRNAs are ubiquitous. Whereas miRNAs originate mainly from their own miRNA coding genes [4], esiRNAs do not have their own genes but are derived mainly from various transposons, their wreckage and other intergenic repetitive elements [2]. In this regard, esiRNAs are considered relatively closer to piRNAs than are miRNAs.

Although their origins are barely distinguishable, esiRNAs and piRNAs are considered distinct groups. One of the main reasons is because esiRNAs and piRNAs are loaded onto different Argonaute proteins [1–3]. Factors required for the biogenesis of esiRNAs and piRNAs also differ from one another. Recent studies have shown that esiRNAs are processed from long RNA precursors by an RNase III enzyme, Dicer2 [8–10,19]. In the esiRNA processing pathway, Dicer2 is associated with one particular isoform of Loquacious (Loqs), Loqs-PD (the *loqs* gene gives rise to four isoforms, Loqs-PA to Loqs-PD) [20–22]. Dicer2 was originally discovered as a factor excising siRNA duplexes from exogenous long double-stranded (ds) RNAs [23]. In this exogenous siRNA (exo-siRNA) excision, Dicer2 is known to associate with R2D2 [24]. The most recent understanding regarding siRNA processing is that R2D2 and Loqs-PD associate with Dicer2 simultaneously, in part [22] and act sequentially in both esiRNA and exo-siRNA processing pathways [25]. The two isoforms of Loqs, Loqs-PA and Loqs-PB, function in the miRNA pathway by associating specifically with Dicer1 [22,26,27].

piRNA biogenesis does not require *dicer* genes [28]. It is therefore obvious that esiRNAs and piRNAs are produced through individual pathways, although their precursors cannot be classified scrupulously. This individuality of their processing is also supported by the notion that the size distribution of esiRNAs and piRNAs clearly differs; esiRNAs are about 21 nt while piRNAs range from 24 to 30 nt [1–3]. The esiRNA pathway is ubiquitous while the piRNA pathway is germline-specific. It is peculiar that only germlines possess both the pathways. The two mechanisms do not seem to be redundant in germlines; this is because loss of the piRNA pathway causes severe defects in gametogenesis [29,30] while loss of the esiRNA pathway showed some mild defects in embryogenesis [31].

In this review, we summarize findings obtained so far about *Drosophila* piRNAs and PIWI proteins. This article includes biogenesis and the 3′-terminal modification of piRNAs, expressional regulation and post-translational modification of PIWI proteins and the mode of function of the PIWI–piRNA complexes in silencing transposable genomic elements and non-transposable genes in ovaries and testes.

2. piRNA biogenesis; the amplification loop

Analyses of the piRNAs that associate with PIWI proteins in *Drosophila* germlines have led to two models for piRNA biogenesis; the amplification loop pathway (also known as the Ping–Pong pathway) and the primary processing pathway [1,4].

In *Drosophila* ovaries, Aub is associated mainly with antisense piRNAs that predominantly contain uracil at their 5′ end (1st-U) [15,16]. By contrast, AGO3 is associated mainly with sense piRNAs that show a strong bias for adenosine at the 10th nucleotide from the 5′ end (10th-A) [15,16]. In fact, piRNA sequences found in the Aub- and AGO3-associated small RNA libraries often form ‘pairs’ through 10 nucleotides from their 5′ ends. Unique characteristics shown by these piRNA sequences prompted an intriguing idea for piRNA production; namely, an involvement of the target RNA cleavage activity of Aub and AGO3 in the processing pathway [15,16]. This new idea correlated well with the earlier observation that *dicer* genes are not required for piRNA production [28]. The basic concept of this model is that reciprocal target RNA cleavage reactions by Aub and AGO3 circulate and constantly bear abundant piRNAs in germline cells. The potentiality that Aub and AGO3 possess a target RNA cleavage activity, as in the case of other Argonaute proteins – another fundamental concept required for instituting this model – was demonstrated experimentally using *in vitro* assay systems [16,18]. Based on these observations, the amplification loop model was constructed (Fig. 1). In parallel with piRNA production, transposons should be silenced efficiently because the majority of piRNA precursors are transposon transcripts. This concept further strengthened the plausibility of the amplification loop model. A recent study shows that the *rhino* gene, encoding a nuclear heterochromatin protein 1 (HP1) homolog, might also be involved in the amplification loop [32], but the detailed mechanism remains unknown.

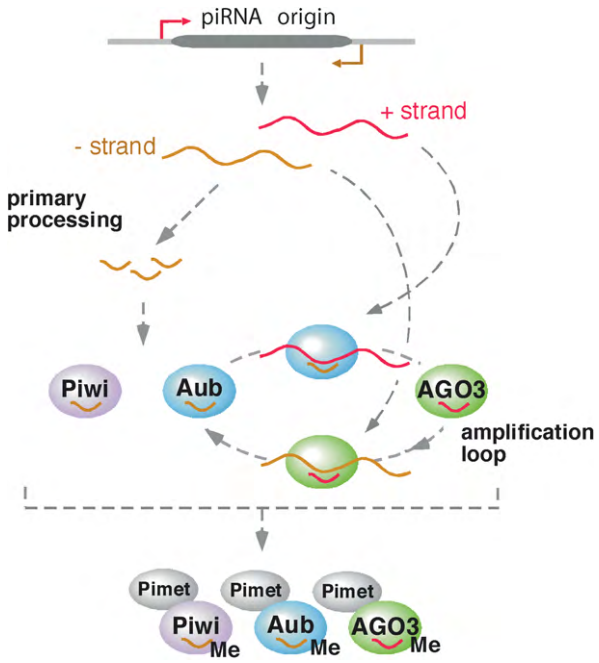

Fig. 1. Amplification loop and primary processing pathways for piRNA production. Transcripts of transposons in antisense orientation are processed to piRNAs through primary processing and loaded onto Piwi and Aub. piRNAs loaded onto Aub are amplified through the amplification loop. However, piRNAs loaded onto AGO3 are thought to be produced only through the amplification loop. Pimet/dmHEN1 associates with PIWI proteins while 2′-*O*-methylates piRNAs that are already loaded onto proteins.

3. piRNA biogenesis; the primary processing pathway

piRNAs derived from the *flamenco* (*flam*) locus on the X chromosome are exclusively loaded onto Piwi in *Drosophila* ovaries [33]. Expression of *flam* has been shown to occur only in ovarian follicle cells where the expressions of Aub and AGO3 are undetectable [15,16,34]. From all these results, it was inferred that there should be another system for the piRNA production, besides the amplification loop pathway [15,35]. This piRNA biogenesis system is referred to as the ‘primary processing pathway’ (Fig. 1). Recently, thorough piRNA sequence analyses have been carried out using various mutants of the piRNA pathway genes, including *ago3* and others. These revealed that the primary piRNA processing does not require AGO3 and that requirements for the piRNA pathway genes in the primary and secondary piRNA processing pathways differ from one

another [36,37]. For instance, it was found that *zucchini* (*zuc*), a putative cytoplasmic nuclease, is likely involved predominantly in the primary piRNA processing while *spindle-E* (*spn-E*; also known as *homeless*), a protein containing Tudor and RNA helicase domains, might be involved predominantly in the amplification loop [37]. Exclusion of Aub and AGO3 from the primary processing pathway was further supported by the notion that, even in established cell lines, piRNAs are abundantly expressed in ovarian somatic sheets (OSSs) [35] and ovarian somatic cells (OSCs) [38], where the expressions of Aub and AGO3 are undetectable. The requirement of *zuc* for primary piRNA processing was confirmed by RNA interference (RNAi)-mediated knockdown of *zuc* and following deep sequencing analysis of piRNAs in OSCs [38]. To date, other factors necessary for the primary processing remain undetermined. A Piwi mutant that lacks its putative nuclear localization signal and thus is now localized exclusively in the cytoplasm, still associates with piRNAs to the same extent as the full-length Piwi protein [38]. This clearly indicates that factors required for primary piRNA processing might exist in the cytoplasm and that Piwi is imported into the nucleus after being loaded with mature piRNAs in the cytoplasm. In Schneider 2 (S2) cells, Piwi expressed by transfection is localized in the nucleus, although Piwi in the environment is not loaded with piRNAs (personal communication with K. Saito). It seems that Piwi does not have to be bound with piRNAs to be imported into the nucleus, at least in S2 cells. The *vasa* gene (see below) might be unnecessary for primary processing because OSSs and OSCs do not express Vasa at a level detectable by immunoblotting [38,39].

4. piRNA modification

Drosophila piRNAs but not miRNAs show resistance to particular chemical reactions; periodate oxidation and β elimination [28,40]. This infers that either the 2′ or 3′ terminal hydroxyl group on the ribose at the 3′ end of piRNAs is chemically modified. Moreover, as in the case of piRNAs in mice, *Drosophila* piRNAs contain 2′-*O*-methyl groups at their 3′ ends [40,41] (Fig. 1). The enzyme responsible for this modification was determined to be dmHEN1/Pimet, the *Drosophila* homolog of *Arabidopsis* HEN1 that catalyzes the 2′-*O*-methyl modification for miRNAs [40,41] (Fig. 1). HEN1 in *Arabidopsis* and in other plants such as rice, contains two functional domains: dsRNA-binding and methyltransferase domains [42,43]. In contrast, dmHEN1/Pimet has methyltransferase domain but lacks dsRNA-binding domain. Thus, dmHEN1/Pimet is just about half the size of *Arabidopsis* HEN1. In plants, miRNAs become 2′-*O*-methyl modified when they are still in a duplex form with miRNA* molecules in the nucleus [42]. *Drosophila* piRNAs are 2′-*O*-methyl modified after being loaded onto PIWI proteins via the direct interaction of dmHEN1/Pimet with PIWI proteins [40,41]. It could then be speculated that dmHEN1/Pimet discarded the dsRNA-binding domain through evolution because it became unnecessary to be retained for its function. In plants, miRNAs are stabilized by the 2′-*O*-methyl modification [42,44]. However, the physiological significance of 2′-*O*-methyl modification of piRNAs in *Drosophila* germlines remains obscure [40].

5. Regulation of PIWI expression

The extent of piRNA accumulation in germlines is largely influenced by the expression levels of parental DNA elements (*i.e.*, transposons, other intergenic repetitive elements and the piRNA loci such as *flam*). The availability of these piRNA processing factors might also influence the piRNA level in germlines. How, then, is the expression of PIWI proteins regulated? Studies in the OSC line revealed that the expression of Piwi protein – one of the PIWI proteins – in ovarian somatic cells must be tightly regulated by the *traffic jam* (*tj*) gene that encodes a large Maf transcriptional factor, Traffic jam [38] (Fig. 2). This upregulation of *piwi* expression by *tj* should also take place in ovaries because in *tj* mutants only somatic cells but not germ cells (ovarian stem cells and their developing cells) lose *piwi* expression [38]. This observation regarding the cell type-dependent expression of *piwi* correlates well with the fact that the *tj* gene is exclusively expressed in somatic cells in ovaries [45]. The factors required for driving the expression of Piwi – as well as Aub and AGO3 – in ovarian germ cells remains undetermined. It should be noted that Piwi expression in somatic niche cells is controlled *Yb*, encoding a protein containing Tudor and RNA helicase domains [46]. The mechanisms through which the expressions of Aub and AGO3 are repressed in the ovarian somatic cells also remain unknown. Depletion of HP1, a nuclear protein involved in heterochromatinization, upregulates Aub expression but not Piwi expression in S2 cells (M.C.S., unpublished data). Therefore, a direct involvement of HP1 in *aub* repression is likely.

6. Post-translational modification of PIWI proteins

PIWI proteins in *Drosophila* have been determined to contain symmetric dimethyl arginines (sDMAs) [47,48]. The factor mediating this post-translational modification was also determined as a methyltransferase, PRMT5 [47] (Fig. 2). Earlier studies showed that Sm proteins in U snRNP complexes, such as SmB/B', contain sDMAs and that sDMAs of Sm proteins are recognized by and bound with a Tudor domain-containing protein, Survival Motor Neuron (SMN) [49,50]. This association of Sm with SMN promotes the recruitment of U snRNA and leads to the efficient assembly of U snRNP. Through this sequential system, the quality of U snRNP is maintained [49]. It has thus been inferred that functions of PIWI proteins, as well as piRNA loading onto PIWI proteins, would be controlled by an sDMA-dependent association of Tudor domain-containing protein(s) with PIWI proteins. In fact, it was revealed that Tudor, one of 19 *Drosophila* Tudor family members, associates with Aub and AGO3 specifically thorough their sDMAs and that the sDMA-specific association maintains the population of piRNAs associated with Aub and AGO3 in ovaries [48]. A specific fraction of Aub that was associated with Tudor was loaded with far less mature piRNAs but instead held piRNA precursor like RNA molecules within the complex, compared with the Tudor-free state of Aub. Thus, Tudor in the piRNA pathway might act like SMN in U snRNP assembly. SMN contains one Tudor domain and binds with the Sm protein. This means that one Tudor domain might be sufficient to associate with single or multiple sDMAs on a target protein. Tudor contains eleven Tudor domains and thus it is plausible that one Tudor molecule can associate with both Aub and AGO3 at the same time. Indeed, the Tudor complex immunoisolated from ovary lysates contains both Aub and AGO3. Aub and AGO3 could dimerize and this association does not require Tudor. Dimerization of Aub is not detectable. Together these results suggest that Tudor might function as a platform for the amplification loop of piRNA production by recruiting the piRNA precursors to the Aub–AGO3 complex efficiently (Fig. 2). Whether Tudor can be associated with Piwi in an sDMA-specific manner and/or if Tudor is also involved in the primary piRNA processing remains unclear and further investigations will be needed to answer these questions.

7. Mode of function of the PIWI–piRNA complex in gene silencing

The PIWI–piRNA complexes target transposon transcripts to silence them and to protect the genomes of gametes from their invasion. However, targets of the PIWI–piRNA complexes are not

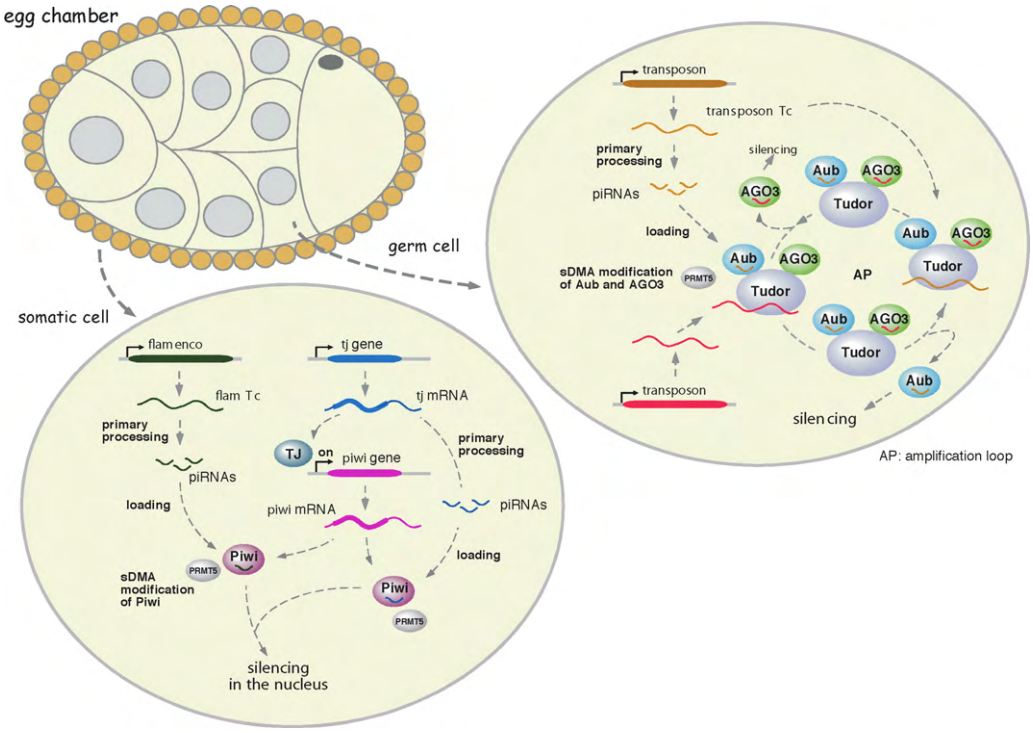

Fig. 2. Schematic of RNA silencing in somatic and germline cells in ovaries. In somatic cells, Piwi protein expression is driven by the *tj* gene. piRNAs derived from the 3′ UTR of protein-coding genes including *tj* are loaded onto Piwi protein. This complex of Piwi and piRNA functions in silencing target genes such as *fasIII*. Transposon silencing mediated by Piwi also occurs in somatic cells. In ovarian germline cells, Piwi is expressed but may be unnecessary for silencing [58]; thus Piwi is omitted here. Tudor can associate with Aub and AGO3 at the same time in germline cells, putting the two proteins in a very proximal region. By doing this, Tudor might promote piRNA production through the amplification loop pathway. There is evidence that Tudor may also functions to recruit piRNAs precursors to the complex.

limited to transposons. Studies in testes showed that piRNAs can also target protein-coding genes, *stellate* (*ste*) and *vasa* (Fig. 3). The *ste* gene encodes Stellate protein that is homologous to Casein kinase β subunit [50,51]. Once expressed in testes under conditions without any silencing regulation, the Stellate protein forms needle-shaped aggregates and these aggregates interfere with spermatogenesis by unknown mechanisms [51,52]. To overcome this defect, *Drosophila* has, through evolution, acquired a silencing system for the *ste* gene that relies on piRNAs derived from the repetitive *suppressor of stellate* [*su*(*ste*)] elements that are highly homologous to the *ste* gene [28,53]. The *su*(*ste*)-derived piRNAs are loaded onto Aub and block *ste* expression, most likely by cleaving its transcripts. This was inferred from the observation that the Aub–piRNA complex immunopurified from testes could cleave the *ste* transcript *in vitro* [18]. Notably, *su*(*ste*)-piRNAs are the most abundant forms associated with Aub in testes. It is very unlikely that ovaries express *su*(*ste*)-piRNAs because they have not been found in the Aub small RNA library and other libraries made from total ovarian small RNAs.

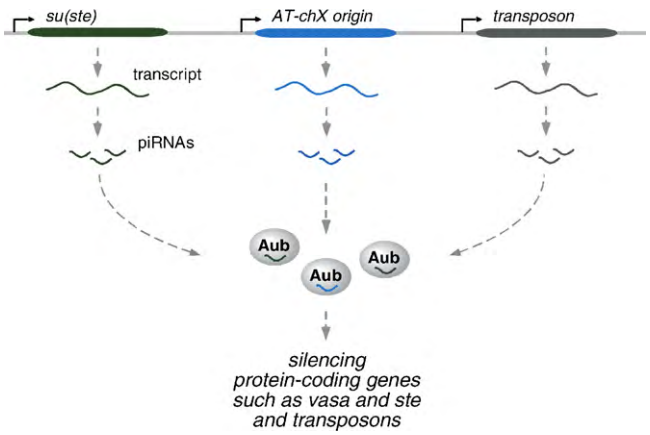

Fig. 3. RNA silencing mediated by Aub in fly testes. There is clear evidence that Aub in testes, by associating with piRNAs derived from non-transposable DNA elements, functions for silencing of not only transposons but also protein-coding genes such as *vasa* and *ste*.

The *vasa* gene encodes a putative RNA helicase, Vasa [54]. Genetic studies revealed that loss of *vasa* function caused severe defects in oogenesis [55]. However, any importance for Vasa functions in testes was not reported. However, it was later reported that the protein level of Vasa in *aub* mutant testes was increased by about twofold compared with those in wild type testes and that *aub* mutants resulted in oversized apexes in the testes, where the spermatogonial cells and spermatocytes are located [18]. The same phenomenon was also observed by *vasa* overexpression [18]. Aub-associated piRNAs in testes included AT-chX-piRNAs derived from two tiny intergenic elements on chromosome X, both of which showed strong complementarities to *vasa* transcripts. The biological consequences of *vasa* gene silencing by the Aub–piRNA complex in the testis remain unknown, but it seems likely that Aub regulates *vasa* gene expression in concert with AT-chX-piRNAs in testes, most likely at the post-transcriptional level because the *vasa* mRNA level was not changed in *aub* mutant testes.

In ovaries, in addition to transposon transcripts, piRNAs are also derived from the 3′ UTR (untranslated region) of *tj* transcripts and loaded onto Piwi in OSC and OSS lines [38,56]. It seems that the Piwi-*tj*-derived piRNA complex functions in downregulating particular protein-coding genes in ovarian somatic cells, such as *fascicline III* (*fasIII*) [38]. FasIII is a cell adhesion molecule necessary for gametogenesis [57]. Thus, the *tj* gene in ovarian somatic cells has at least dual roles regulating *piwi* functions; first as a transcriptional factor that drives the *piwi* expression; and second as a

precursor of piRNAs that are specifically loaded onto Piwi protein to silence specific target genes including *fasIII*. It should be noted that the 3′ UTRs of many other genes also act as sources for piRNAs in OSC and OSS lines [38,56].

8. Conclusions

Extensive studies on *Drosophila* piRNAs and PIWI proteins have so far revealed many unique and interesting aspects of how the molecules function in RNA silencing mechanisms occurring specifically in germlines. On the other hands we also realize that many other aspects of PIWI proteins and piRNAs molecules remain unclear and therefore investigation on this particular topic needs to be continued. The remaining important questions include; what are the factors necessary for piRNA production? Candidate genes include *armitage*, *spn-E*, *maelstrom*, *krimper*, *rhino*, *vasa* and *squash*. Deficient fly lines of each of these genes and/or OSC and OSS lines from which each gene function is depleted by RNAi will be very useful tools to address this particular question and related questions. sDMA modification of PIWI proteins is another important issue in this germline-specific RNA silencing field. What are the dynamics of sDMA modification through the piRNA pathway? Is there any enzyme that removes the dimethyl groups from the sDMAs of PIWI proteins? When does conversion of sDMA to regular arginines – if it occurs – take place during the development? What is the physiological meaning of this reaction? Expectations for the accomplishment of future research are endless.

Acknowledgments

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References

[1] Siomi H, Siomi MC. On the road to reading the RNA-interference code. Nature 2009;457:396–404.
[2] Okamura K, Lai EC. Endogenous small interfering RNAs in animals. Nat Rev Mol Cell Biol 2008;9:673–8.
[3] Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nat Rev Genet 2009;10:94–108.
[4] Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009;10:126–39.
[5] Hutvagner G, Simard MJ. Argonaute proteins: key players in RNA silencing. Nat Rev Mol Cell Biol 2008;9:22–32.
[6] Wang Y, Sheng G, Juranek S, Tuschl T, Patel DJ. Structure of the guide-strand-containing argonaute silencing complex. Nature 2008;456:209–13.
[7] Wang Y, Juranek S, Li H, Sheng G, Tuschl T, Patel DJ. Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. Nature 2008;456:921–6.
[8] Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, Dus M, et al. An endogenous small interfering RNA pathway in *Drosophila*. Nature 2008;453:798–802.
[9] Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, et al. *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 2008;453:793–7.
[10] Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, et al. Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. Science 2008;320:1077–81.
[11] Förstemann K, Horwich MD, Wee L, Tomari Y, Zamore PD. *Drosophila* microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. Cell 2007;130:287–97.
[12] Okamura K, Liu N, Lai EC. Distinct mechanisms for microRNA strand selection by *Drosophila* Argonautes. Mol Cell 2009;36:431–44.
[13] Czech B, Zhou R, Erlich Y, Brennecke J, Binari R, Villalta C, et al. Hierarchical rules for Argonaute loading in *Drosophila*. Mol Cell 2009;36:445–56.
[14] Saito K, Nishida KM, Mori T, Kawamura Y, Miyoshi K, Nagami T, et al. Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. Genes Dev 2006;20:2214–22.
[15] Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, et al. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell 2007;128:1089–103.
[16] Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, et al. A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in *Drosophila*. Science 2007;315:1587–90.
[17] Yin H, Lin H. An epigenetic activation role of Piwi and a Piwi-associated piRNA in *Drosophila melanogaster*. Nature 2007;450:304–8.
[18] Nishida KM, Saito K, Mori T, Kawamura Y, Nagami-Okada T, Inagaki S, et al. Gene silencing mechanisms mediated by Aubergine piRNA complexes in *Drosophila* male gonad. RNA 2007;13:1911–22.
[19] Okamura K, Chung WJ, Ruby JG, Guo H, Bartel DP, Lai EC. The *Drosophila* hairpin RNA pathway generates endogenous short interfering RNAs. Nature 2008;453:803–6.
[20] Hartig JV, Esslinger S, Böttcher R, Saito K, Förstemann K. Endo-siRNAs depend on a new isoform of *loquacious* and target artificially introduced, high-copy sequences. EMBO J 2009;28:2932–44.
[21] Zhou R, Czech B, Brennecke J, Sachidanandam R, Wohlschlegel JA, Perrimon N, et al. Processing of *Drosophila* endo-siRNAs depends on a specific Loquacious isoform. RNA 2009;15:1886–95.
[22] Miyoshi K, Miyoshi T, Hartig JV, Siomi H, Siomi MC. Molecular mechanisms that funnel RNA precursors into endogenous small-interfering RNA and micro RNA biogenesis pathways in *Drosophila*. RNA, 2010, doi:10.1261/rna.1952110.
[23] Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001;409:363–6.
[24] Liu Q, Rand TA, Kalidas S, Du F, Kim HE, Smith DP, et al. R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science 2003;301:1921–5.
[25] Marques JT, Kim K, Wu PH, Alleyne TM, Jafari N, Carthew RW. Loqs and R2D2 act sequentially in the siRNA pathway in *Drosophila*. Nat Struct Mol Biol 2009:1.
[26] Förstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, et al. Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 2005;3:e236.
[27] Saito K, Ishizuka A, Siomi H, Siomi MC. Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. PLoS Biol 2005;3:e235.
[28] Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD. A distinct small RNA pathway silences selfish genetic elements in the germline. Science 2006;313:320–4.
[29] Lin H, Spradling AC. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. Development 1997;124:2463–76.
[30] Schüpbach T, Wieschaus E. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. Genetics 1991;129:1119–36.
[31] Deshpande G, Calhoun G, Schedl P. *Drosophila* argonaute-2 is required early in embryogenesis for the assembly of centric/centromeric heterochromatin, nuclear division, nuclear migration, and germ-cell formation. Genes Dev 2005;19:1680–5.
[32] Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, et al. The *Drosophila* HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell 2009;138:1137–49.
[33] Sarot E, Payen-Groschêne G, Bucheton A, Pélisson A. Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster flamenco* gene. Genetics 2004;166:1313–21.
[34] Pélisson A, Song SU, Prud'homme N, Smith PA, Bucheton A, Corces VG. Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila flamenco* gene. EMBO J 1994;13:4401–11.
[35] Lau NC, Robine N, Martin R, Chung WJ, Niki Y, Berezikov E, et al. Abundant primary piRNAs, endo-siRNAs, and microRNAs in a *Drosophila* ovary cell line. Genome Res 2009;19:1776–85.
[36] Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, et al. Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. Cell 2009;137:509–21.
[37] Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, et al. Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. Cell 2009;137:522–35.
[38] Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, et al. A regulatory circuit for *piwi* by the large Maf gene *traffic jam* in *Drosophila*. Nature 2009;461:1296–9.
[39] Niki Y, Yamaguchi T, Mahowald AP. Establishment of stable cell lines of *Drosophila* germ-line stem cells. Proc Natl Acad Sci USA 2006;103:16325–30.
[40] Saito K, Sakaguchi Y, Suzuki T, Suzuki T, Siomi H, Siomi MC. Pimet, the *Drosophila* homolog of HEN1, mediates 2'-O-methylation of Piwi-interacting RNAs at their 3′ ends. Genes Dev 2007;21:1603–8.
[41] Horwich MD, Li C, Matranga C, Vagin V, Farley G, Wang P, et al. The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. Curr Biol 2007;17:1265–72.
[42] Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, et al. Methylation as a crucial step in plant microRNA biogenesis. Science 2005;307:932–5.
[43] Chen X. MicroRNA biogenesis and function in plants. FEBS Lett 2005;579:5923–31.
[44] Li J, Yang Z, Yu B, Liu J, Chen X. Methylation protects miRNAs and siRNAs from a 3′-end uridylation activity in *Arabidopsis*. Curr Biol 2005;15:1501–7.
[45] Li MA, Alls JD, Avancini RM, Koo K, Godt D. The large Maf factor Traffic Jam controls gonad morphogenesis in *Drosophila*. Nat Cell Biol 2003;5:994–1000.
[46] Szakmary A, Reedy M, Qi H, Lin H. The Yb protein defines a novel organelle and regulates male germline stem cell self-renewal in *Drosophila melanogaster*. J Cell Biol 2009;185:613–27.

[47] Kirino Y, Kim N, de Planell-Saguer M, Khandros E, Chiorean S, Klein PS, et al. Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability. Nat Cell Biol 2009;11:652–8.
[48] Nishida KM, Okada TN, Kawamura T, Mituyama T, Kawamura Y, Inagaki S, et al. Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in *Drosophila* germlines. EMBO J 2009;28:3820–31.
[49] Meister G, Eggert C, Fischer U. SMN-mediated assembly of RNPs: a complex story. Trends Cell Biol 2002;12:472–8.
[50] Battle DJ, Kasim M, Yong J, Lotti F, Lau CK, Mouaikel J, et al. The SMN complex: an assembly machine for RNPs. Cold Spring Harb Symp Quant Biol 2006;71:313–20.
[51] Bozzetti MP, Massari S, Finelli P, Meggio F, Pinna LA, Boldyreff B, et al. The Ste locus, a component of the parasitic cry-Ste system of *Drosophila melanogaster*, encodes a protein that forms crystals in primary spermatocytes and mimics properties of the beta subunit of casein kinase 2. Proc Natl Acad Sci USA 1995;92:6067–71.
[52] Palumbo G, Bonaccorsi S, Robbins LG, Pimpinelli S. Genetic analysis of Stellate elements of *Drosophila melanogaster*. Genetics 1994;138:1181–97.
[53] Aravin AA, Naumova NM, Tulin AV, Vagin VV, Rozovsky YM, Gvozdev VA. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. Curr Biol 2001;11:1017–27.
[54] Lasko PF, Ashburner M. The product of the *Drosophila* gene vasa is very similar to eukaryotic initiation factor-4. Nature 1988;335:611–7.
[55] Styhler S, Nakamura A, Swan A, Suter B, Lasko P. vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. Development 1998;125:1569–78.
[56] Robine N, Lau NC, Balla S, Jin Z, Okamura K, Kuramochi-Miyagawa S, et al. A broadly conserved pathway generates 3′UTR-directed primary piRNAs. Curr Biol 2009.
[57] Snow PM, Bieber AJ, Goodman CS. Fasciclin III: a novel homophilic adhesion molecule in *Drosophila*. Cell 1989;59:313–23.
[58] Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H. A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. Genes Dev 1998;12:3715–27.