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piRNA Biogenesis in Drosophila Melanogaster

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Abstract

The PIWI-interacting RNA (piRNA) pathway is a conserved defense system that protects the genome integrity of the animal germline from deleterious transposable elements. Targets of silencing are recognized by small non-coding piRNAs that are processed from long precursor molecules. Though piRNAs and other classes of small non-coding RNAs, such as miRNAs and siRNAs, interact with members of the same family of Argonaute proteins and their function in target repression is similar, the biogenesis of piRNAs differs from those of the other two small RNAs. Recently, many aspects of piRNA biogenesis have been revealed in *Drosophila melanogaster*. In this review, we elaborate on piRNA biogenesis in *Drosophila* somatic and germline cells. We focus on the mechanisms by which piRNA precursor transcription is regulated and highlight recent work that advanced our understanding of piRNA precursor processing to mature piRNAs. We finish with discussing current models to the still unresolved question of how piRNA precursors are selected and channeled into the processing machinery.

Overview of piRNA function

The conserved family of Argonaute proteins interacts with small (19–33 nt) RNA guides in eukaryotic species. The guide enables the Ago complex to recognize RNA targets with a high level of specificity using complementary interactions. After recognition, the target is cleaved by the intrinsic endonuclease activity of the Argonaute protein [1–3]. Alternatively, suppression of the targets can be achieved without cleavage through the recruitment of additional effector proteins by Ago [4]. Three major classes of Ago-associated small RNAs are present in Metazoa: small interfering (si)RNA, micro (mi)RNA and piwi-interacting (pi)RNA (Box 1). In contrast to the ubiquitously expressed miRNA, piRNAs and their protein partners, members of the Piwi clade of the Argonaute protein family, are predominantly expressed in animal gonads [5–14]. Accordingly, Piwi mutatant flies and mice – two model organisms that were extensively used to understand this pathway – have normal somatic development, but show gametogenesis defects which result in sterility [7, 10, 15–17]. In flies piRNAs and Piwi proteins are required in germ cells for suppression of transposable elements, selfish genomic elements that are able to move in the genome [18–

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25]. The gametogenesis defects observed in flies that harbor mutations of Piwi proteins are likely to be the direct result of transposon activation and the associated double-stranded DNA breaks and concomitant activation of DNA damage checkpoint [26–28].

piRNAs have an amazing sequence diversity: deep sequencing of small RNA identified millions of unique piRNA reads that do not have much in common except for a bias in the first nucleotide at 5' end [20]. Attempts to classify piRNAs using the approach that was used for miRNA – giving every piRNA sequence its unique ID – were soon abandoned. The diversity of piRNAs reflects the difference in their biogenesis from that of both miRNA and siRNA. piRNA biogenesis can be divided into two stages. First, long RNA precursors are transcribed in the nucleus and exported into the cytoplasm. In the cytoplasm, piRNA precursors are further processed to generate mature piRNAs that get loaded into Piwi proteins.

Nuclear steps of piRNA biogenesis

To understand biogenesis of piRNAs, it is necessary to explore their genomic origin. Since the majority of piRNAs are derived from sequences of transposable elements that are present in many copies throughout the genome, this task is not as simple as it seems. The majority of piRNAs can't be mapped to a unique position in the fly genome, making conclusions about their origin ambiguous [20]. However, when only piRNAs that can be uniquely mapped are considered, it is apparent that a large fraction of piRNAs originate from a number of extended genomic loci, with sizes up to 200 kb in length. Such genomic regions were named piRNA clusters [20]. Each piRNA cluster produces thousands of piRNA sequences that are not arranged in any particular pattern and can even overlap with each other. With a few exceptions, major piRNA clusters do not overlap with protein-coding genes but harbor a diverse set of TE fragments.

Two types of piRNA clusters were described in flies: uni-strand, for which the vast majority of piRNAs map to one genomic strand, and dual-strand, for which piRNAs map to both genomic strands. The differences between the two cluster types extend far beyond the strand of transcription (Figure 1A). The transcription of uni-strand clusters seems to be similar to canonical mRNA transcription as these clusters have unique promoters and produce 5' capped and polyadenylated RNA that are sometimes spliced [29–31]. Dual-strand clusters produce non-polyadenylated RNA and the majority of dual-strand clusters does not have clear signatures of Pol II promoters, such as peaks of Pol II and H3K4me2 [31–33]. The absence of clear promoters led to the proposal that transcription of dual-strand clusters may be driven by the promoters of flanking protein-coding genes [31]. However, deletion of a putative promoter next to the major 42AB piRNA cluster did not affect piRNA production, arguing against this possibility [32]. It is possible that both transcription initiation and termination occur at multiple positions inside dual-strand clusters. As transcription of dual-strand clusters seems to be distinct from canonical Pol II transcription, study of dual-strand cluster transcription is not only important for a better understanding of piRNA biogenesis but might shed light on general mechanisms of Pol II transcriptional control.

Individual transposons – inserted in gene-dense euchromatic areas – also generate piRNAs [34, 35], but the precise quantification of the relative fractions of piRNAs derived from individual transposons versus piRNA clusters is impossible due to the significant fraction of piRNAs that map to both. In addition to individual transposon insertions and piRNA clusters, some protein-coding genes also produce piRNAs, predominantly from their 3'UTR region [36, 37].

Independent of the genomic origin – piRNA clusters, individual transposons or genes – mature (23–30nt) piRNAs are processed from longer precursors. Genic piRNAs correspond to exonic sequences and are in sense orientation relative to the gene's mRNA [36, 37], suggesting that they are likely processed from spliced and processed mRNA. Currently it is not clear how - if at all - mRNAs destined for piRNA processing vs. translation are differentiated. Enrichment of piRNAs at the 3'UTRs suggests that the same mRNA can be used for both translation and piRNA processing and that active translation interferes with piRNA biogenesis.

piRNA clusters are transcribed as long non-coding RNAs by RNA polymerase II [30–33]. Despite this fact, the chromatin of piRNA clusters is enriched in the histone 3 lysine 9 trimethylation (H3K9me3) mark, which is usually found on silent, heterochromatic regions and is thought to be a mark that suppresses transcription [31, 33, 38–42]. Surprisingly, the presence of the H3K9me3 mark does not interfere with piRNA precursor transcription, but is in fact required for piRNA expression [31, 42, 43]. Depletion of one of the enzymes that installs the H3K9me3 mark, SetDB1/Egg, leads to a decrease in piRNA precursor expression [42]. The level of H3K9me3 signal also positively correlates with piRNA generation from clusters that are differentially expressed between two *D. virilis* strains [44].

possibile funzione di deadlock

In addition to the H3K9me mark, dual-strand clusters are decorated with a unique set of proteins, which are required for biogenesis of piRNAs from these regions, but not from uni-strand clusters and genic piRNAs [28, 45–47]. Three such proteins, specific to Diptera, Rhino (Rhi), Deadlock (Del) and Cutoff (Cuff) form the RDC complex [31, 32, 41, 43, 45]. Rhino is a paralog of the well-characterized heterochromatin protein HP1 [45] and binds the H3K9me3 mark through its chromodomain [31, 43, 48]. Two remaining key questions are how the RDC complex is recruited to piRNA-producing loci and by what mechanism it facilitates piRNA biogenesis from these regions. Currently it is not clear whether RDC is exclusively bound to chromatin of dual-strand piRNA clusters or whether it is also present at other genomic regions with high level of H3K9me3 [49]. The specificity to piRNA clusters could be achieved – in addition to binding of the H3K9me3 mark – by guiding the loading of RDC on its genomic targets by the nuclear Piwi protein. Piwi is necessary for installment of the H3K9me3 mark on genomic targets of the piRNA pathway, including piRNA clusters, while heterochromatic marks at other genomic regions that are not targeted by piRNAs are established in a Piwi-independent fashion [33, 38, 39]. It is attractive to speculate that recognition of piRNA targets by the Piwi/piRNA complex not only lead to deposition of the H3K9me3 mark but also recruits component(s) of the RDC complex to the locus; however, this hypothesis awaits experimental verification.

Three – not necessarily mutually exclusive – models were proposed for the role of the RDC complex in piRNA biogenesis: enabling transcription of piRNA precursors, suppressing their splicing and recruiting to nascent transcripts additional factors required for further processing of piRNA precursors (Figure 1B). Components of RDC were proposed to suppress splicing of piRNA precursors, probably by competing with the nuclear cap binding complex CBC for binding mRNAs [41]. However, the direct effect of RDC on CBC binding was not tested and it is not clear how the absence of splicing might be important for piRNA processing, as intronless mRNAs were not reported to be processed into piRNA more efficiently than intron-containing mRNAs.

RDC seems to be necessary for transcription of piRNA precursors, as transcription of piRNA precursors decreases notably upon knockdown or mutation of RDC components [31, 32]. RDC and associated proteins could enhance transcription of piRNA precursors through several mechanisms, including promotion of initiation and suppression of termination. As clusters are in genomic regions characterized by H3K9me3 enrichment and lack a canonical promoter, an important question is how transcription of clusters initiates. Genetic screens have identified Moonshiner to be required for piRNA biogenesis. Moonshiner physically interacts with both the RDC complex and with the TATA-box binding protein (TBP)-related factor TRF2, the component of core transcription machinery, and therefore might play a role in recruiting the transcription machinery to piRNA clusters and initiating transcription in the absence of proper promoter elements and in the presence of the repressive H3K9me3 mark, which would otherwise antagonize transcription initiation [50].

Another component of the RDC complex, Cutoff plays a role in transcription of piRNA precursors by suppressing premature termination [32]. Cuff suppresses termination by preventing cleavage by the CPSF complex at the polyA site and, in case cleavage does occur, Cuff prevents degradation of the cleaved 5'monophosphorylated RNA by the nuclear exonuclease Rat1/Xrn2. Since Cuff is homologous to the Rai1/Dxo protein, which has a binding pocket for the 5'end of RNA, Cuff might protect RNA degradation by preventing RNA recognition by Rat1. It was proposed that the anti-termination activity of Cuff allows dual-strand clusters to function as traps for transposon insertions: if transcription of piRNA precursors would be canonical, insertion of new TEs that carry their own polyA/termination signal would cause premature termination and collapse of piRNA biogenesis [32].

The transcription initiation complex, TREX is also required for piRNA biogenesis from dual-strand, but not uni-strand clusters [51, 52]. In contrast to RDC, which is not conserved outside of Diptera, TREX is a conserved complex from yeast to humans and is co-transcriptionally loaded on many – if not all – nascent pre-mRNA transcripts [53–56]. However, TREX seems to be enriched on dual-strand piRNA precursors compared to other Pol II nascent transcripts. TREX might play two functions in piRNA biogenesis. In TREX mutants, transcription of piRNA cluster transcripts is reduced [51]. This effect might be mediated by the proposed ability of TREX to prevent formation of R-loops, hybrids between nascent RNA and DNA that inhibit transcription. Alternatively, proteins associated with nascent RNAs such as TREX might enhance transcription by preventing backtracking of Pol II, a role played by co-transcriptionally translating ribosomes in prokaryotes [57]. In addition, in yeasts and mammals TREX mediates nuclear export of RNA [53–56], therefore

it is plausible that TREX also promotes export of piRNA precursors, although this has not yet been tested.

Loading of TREX on mRNA – at least in mammals – is dependent on splicing[54, 56], which raises the question of how it can be enriched on dual-strand cluster transcripts that are not spliced. The genetic and physical interaction between RDC and TREX and their co-localization in nuclear foci suggests that chromatin-bound RDC might recruit TREX on nascent piRNA precursor transcripts (Figure 1B) [51]. Thus, the study of piRNA biogenesis revealed an alternative, splicing-independent, mechanism of TREX loading on RNA that depends on the H3K9me3 chromatin mark and the associated RDC complex. These results suggest that chromatin marks can reach beyond regulation of transcription and affect – through guiding the loading of a particular set of proteins onto RNA – the post-transcriptional fate of RNA.

Once transcribed, piRNA precursors are exported to the cytoplasm. Uni-stranded clusters, like *Flamenco*, behave similar to protein coding genes. *Flam* contains introns that are spliced leading to deposition of the exon-junction complex (EJC). Binding of the EJC and the exportin complex leads to efficient export of *Flam* [30, 58]. How dual-strand transcripts are exported is less clear. The clusters in the nucleus and the cytoplasmic processing granules in cytoplasm were reported to be juxtaposed to each other on opposite sides of the nuclear membrane suggesting that spatial proximity might enhance export of piRNA precursors and direct them to processing machinery [52].

It is remarkable how many unexpected insights were gained through studies of the piRNA pathway about the mechanisms of transcription and early RNA processing. Beyond revealing the role of chromatin in loading of RNA-binding proteins on nascent RNAs, these studies uncovered novel mechanisms of enabling transcription initiation in a hostile chromatin environment and of transcription termination control.

piRNA biogenesis in the cytoplasm

Once long piRNA precursors – transcripts from piRNA clusters, transposons and mRNA of genes – are exported across the nuclear envelope, further processing in the cytoplasm leads to generation of mature 23–29nt piRNA. The enzymatic machinery that processes piRNA precursors to generate the 5’ and 3’ ends of mature piRNAs is different from enzymes that process miRNA and siRNA (Dicer and Drosha) [25, 59].

Formation of the 5’-end of piRNAs can occur through two different mechanisms. In nurse and follicular cells of the fly ovary 5’-ends can be formed by cleavage of precursors by Zucchini (Zuc), an endonuclease anchored to mitochondria [60, 61]. The role of Zuc in piRNA processing is supported by genetic experiments – Piwi-loaded piRNAs are eliminated in Zuc mutant flies – as well as structural and biochemical studies that demonstrate the endoribonuclease activity of Zuc *in vitro* [60–63]. Despite these findings some questions about Zuc processing remain unanswered. *In vitro* Zuc does not show any preference for RNA cleavage at any specific nucleotide residue [60, 61], while the 5’ ends of Zuc-cleaved piRNAs show a strong bias for uridine. It is possible that the observed

preference to cleave in front of uridine is determined by a co-factor of Zuc. Alternatively, Zuc-mediated cleavage might be truly unspecific and the bias might be created by the selectivity of Piwi for 5'U RNA.

In contrast to follicular cells where Zuc-mediated cleavage is exclusively responsible for processing of piRNA 5' ends, in nurse cells an alternative mechanism, the so called ping-pong processing, is responsible for generation of piRNAs loaded into Aub and Ago3 [20, 23, 64]) (Figure 2A). The ping-pong mechanism seems to be a conserved feature of the piRNA pathway present in many organisms from Hydra to human [65–68]. This level of conservation suggests that ping-pong plays a crucial function: it was proposed that ping-pong enables the amplification of piRNAs that target actively expressed transposons [20, 64]. Upon recognition of the mRNA of active transposons by antisense piRNAs processed from cluster transcripts, ping-pong works as a cycle to generate more piRNAs from the cluster transcript to target the transposon. It was proposed that this mechanism, which can conceptually be compared to expansion of cells that produce antigen to pathogens during an immune response, allows for fine-tuning piRNA populations to fight active transposons [20]. In agreement with this, in mice transposon activation leads to increase in ping-pong and generation of piRNAs against the active transposon [69].

Historically, Zuc-dependent piRNA processing was called the primary biogenesis pathway, as it was believed not to require pre-existing piRNAs and – at least in follicular cells – can function independently of ping-pong. In contrast, slicer-dependent (ping-pong) biogenesis was initially dubbed secondary pathway, as it requires pre-existing piRNAs and it was proposed that piRNAs generated by primary processing feed into the ping-pong cycle [20, 21]. However, recent research suggests that the interaction between the two biogenesis pathways is more complicated and raises the question whether true 'primary' piRNA biogenesis that is independent of pre-existing piRNAs and slicer-cleavage exists in nurse cells. First, it was found that piRNA biogenesis in germ cells requires maternally inherited piRNAs [44, 59, 70, 71]. In addition to initiating installment of the H3K9me3 mark on piRNA clusters (described above), they can start the ping-pong cycle, eliminating the need for 'starter' piRNAs formed by a different mechanism. Second it was reported that elimination of the ping-pong cycle in germ cells in Aub/Ago3 double mutants leads to loss of Piwi-loaded piRNA, which were thought to be generated by ping-pong-independent primary biogenesis [72–75]. Furthermore, slicer cleavage by Aub or Ago3 not only generates one ping-pong piRNA from the cleaved precursor, but leads to Zuc-dependent processing of the downstream fragment, which generates Piwi-bound piRNAs. Thus, the ping-pong pathway, which was thought to be secondary, in fact initiates the Zuc-dependent 'primary' pathway. Simply swapping the names of the two pathways is, however, not fixing the problem, as – at least in follicular cells where ping-pong does not work – the Zuc-dependent pathway can be truly independent of ping-pong. Therefore, our deeper understanding of piRNA biogenesis calls for abandoning the terms of 'primary' and 'secondary' biogenesis. Instead we propose to base the nomenclature on the nature of the enzymatic machinery that generates the 5' end of piRNA: slicer-mediated (or ping-pong) and Zuc-mediated processing (Figure 2B).

The mechanisms responsible for 3' end formation are even more diverse than those that lead to generation of the 5' end of piRNAs (Figure 2A). The 3' end can be generated through cleavage by Zuc [72, 73]. Zuc-mediated piRNAs exhibit a 'phasing' signature, i.e. when mapped to the sequence of the precursor one piRNA is immediately followed by another piRNA suggesting that a single Zuc cleavage can simultaneously generate the 3' end of an upstream and the 5' end of a downstream piRNA [72–74, 76]. 3' end formation can also be induced by slicer (ping-pong) cleavage. In this case, the mature piRNA is generated by two closely spaced slicer cleavages [77]. Finally, 3' end formation might require processing by exonuclease(s). In this case, a 3'-to-5' exonuclease trims a longer precursor formed by cleavage by an endonuclease (slicer or Zuc) to make mature piRNAs of the correct size. Such 'Trimmer' activity was first observed *in vitro* using lysate from a silkworm cell line [78] and later associated with the exonuclease Nibbler (Nbr) [77, 79, 80]. Independently of the mechanism by which the 3'end of piRNAs is generated, the last step is the 2'OMe-modification of the last nucleotide by Hen1, **which is thought to** increase the stability of piRNAs [80–82].

In addition to the above described proteins – Aub, Ago3, Zuc, Nbr and Hen1 – piRNA biogenesis requires many other proteins of which the functions are less understood [47, 83, 84]. As only the Zuc-dependent pathway operates in follicular cells, fewer proteins are involved in piRNA biogenesis in these cells. Many factors required for piRNA biogenesis in follicular cells such as the putative RNA helicase Armitage (Armi), the Tudor-domain protein Vreteno (Vret) and Yb co-localize in perinuclear foci that were termed Yb bodies [85–90]. Though at steady-state Piwi is present exclusively in the nucleus of follicular cells, deletion of its nuclear localization signal leads to Piwi localization to Yb bodies [40]. Therefore, it is plausible that Yb bodies represent a site of piRNA processing and loading into Piwi before the Piwi/piRNA complex relocates to the nucleus. In agreement with this, transcripts from the somatic piRNA cluster *flamenco* were reported to localize close to Yb body [89]. The molecular functions of protein components of Yb bodies remain largely unknown. Additionally, Zuc, as well as other proteins with unknown functions required for piRNA biogenesis, such as GASZ and Minotaur (mino), are anchored on the mitochondrial surface [91–93], suggesting that mitochondria also play a key role in piRNA precursor processing. The interplay between Yb bodies and mitochondria merits further investigation.

In the germ cells, proteins involved in piRNA biogenesis also localize to a distinct cytoplasmic compartment, called nuage, which surrounds the nuclei of nurse cells [94, 95]. Krimp, one of the most stable components of nuage that is able to form granules in the absence of other nuage proteins, recruits piRNA-loaded Aub and unloaded Ago3 to form a complex [96]. The spatial proximity of Aub and Ago3 in such complex was proposed to facilitate delivery of Aub-cleaved product into Ago3 during ping-pong [96]. Qin - another Tudor domain protein - has a role in maintaining heterotypic ping-pong by preventing the loading of Aub cleavage products into Piwi or Aub [74, 97]. Qin forms a complex with Vasa [98, 99], a member of the DEAD box helicase family, with ATPase, RNA binding and RNA unwinding activity [100–102]. Two roles were proposed for Vasa in piRNA processing. First, the RNA-unwinding activity of Vasa helps to release cleaved products from the piRNA-protein complex to facilitate the ping-pong cycle [99]. Second, Vasa was proposed to

participate in assembly of the ping-pong complex [98]. The molecular function of numerous other nuage factors remains unknown, despite the fact that they genetically and physically interact with piwi proteins and other nuage components. In the future, the challenge will be to elucidate their functions and the molecular interplay between these factors.

Licensing of piRNA precursors for processing

One of the most important remaining unresolved questions is how piRNA precursors are discriminated from other cellular RNA and directed for processing into piRNA. In other examples of RNA processing, such as splicing or processing of CRISPR RNA, specific sequence and/or structure motifs in precursor RNA are recognized by the processing machinery. Similarly, Drosha and Dicer, the two key enzymes in miRNA processing, recognize the secondary structure of pre-miRNA[103–105]. To date, no common sequence or structural motifs that are shared by all piRNA precursors were identified. Inserting an extended artificial sequence into natural piRNA precursors results in its processing into piRNAs arguing against the requirement of local sequence or structural motifs for processing [33, 106]. viene processato a piRNA ugualmente

In follicular cells some piRNA precursors – such as the mRNA of *traffic jam (tj)* and transcripts from the uni-strand *flamenco* piRNA cluster – contain sequences that target them for processing [75, 107]. Inserting a fragment of the *tj* 3'UTR or *flamenco* into an unrelated RNA transcript is sufficient to trigger production of piRNAs from this transcript [75, 107]. The *tj*- and *flamenco*- derived sequences that trigger piRNA generation associate with the RNA-binding protein Yb, although a specific motif that is recognized by Yb has not been determined [107]. Whether recruitment of Yb to RNA is sufficient to trigger its processing into piRNAs has not yet been directly tested. Nor is it known whether other somatic piRNA precursors also harbor sequence motifs that are bound by Yb. Nonetheless, the above results suggest that Yb may recognize specific sequence motifs in transcripts and recruit them to the processing machinery (Figure 3).

The mechanism of piRNA precursor selection seems to be different in germ cells and somatic follicular cells as no sequences that would trigger processing were identified in piRNA precursors expressed in germline. Two models were proposed to explain how piRNA precursors can be selected in the absence of any sequence motifs (Figure 3). The first – which can be the called 'persistent nuclear mark' - model suggests that a specific protein (or proteins) tightly associates with piRNA precursors in the nucleus and remains associated in the cytoplasm where it activates the processing machinery. The model relies on the fact that piRNA processing in germ cells depends on the RDC complex, which is enriched on chromatin of piRNA-generating loci [31, 32, 43, 45], and the TREX complex, which co-transcriptionally binds piRNA precursors in an RDC-dependent fashion [51, 52]. It was proposed that components of either the RDC or the TREX complex might constitute the mark that triggers cytoplasmic piRNA processing [41, 52]. As localization of the RDC complex seems to be specific to genomic regions that generate piRNA, this model explains how piRNA precursors can be discriminated and targeted to processing in the absence of sequence motifs. However, both the nature of the mark and the mechanism by which it engages the processing machinery remain unclear. Evidence that components of RDC or

TREX remain associated with piRNA precursors after their export to the cytoplasm is also lacking. Finally, tethering of Rhino, a component of the RDC complex, to a single-stranded transgene does not trigger piRNA biogenesis [41], arguing against the idea that binding of RDC by itself is sufficient to specify piRNA precursors.

An alternative model, which we will call 'selection by pre-existing piRNA', suggests that precursors are specified in the cytoplasm by complementary piRNAs associated with the cytoplasmic Piwi proteins. The model relies on the observation that a transcript that is recognized by complementary piRNAs residing in Aub or Ago3 is first cleaved by their slicer activities to generate a single responder piRNA followed by Zuc-dependent processing of the remainder of the transcript to multiple piRNAs [72, 73]. Insertion of a single piRNA target sequence into a heterologous transcript leads to efficient processing of the transcript into piRNAs [73, 77, 108]. This model raises the obvious question of how the very first piRNAs – which subsequently recognize piRNA precursors - are made. The answer seems to be inheritance of piRNAs from the previous generation. Maternally expressed piRNAs are present in the early Drosophila embryo [70]. Furthermore, trans-generational inheritance of piRNAs is necessary for piRNA biogenesis in germ cells of the new generation [33, 38, 43, 59], suggesting that maternally provided piRNAs initiate piRNA biogenesis.

Concluding Remarks

The above models suggest that the mechanism of precursor selection in the soma and the germline is radically different: in the soma selection relies on recognition of sequence motifs in precursor RNA, while in the germline selection is sequence-independent. However, the core piRNA processing machinery composed of Zuc and a number of other proteins operates in both cell types, suggesting that a common principle for precursor selection should exist. We propose that sequestration of RNA into a distinct cellular compartment might be such a central principle that is shared by both cell types. The central postulate of this proposal is that any RNA that is localized to the processing compartment will be processed to piRNAs in a sequence-independent fashion. Recruitment of RNA to this compartment might be achieved by different mechanisms including recognition of sequence motifs either by RNA-binding proteins or by complementary piRNAs associated with the cytoplasmic Piwi proteins. Both Yb and the cytoplasmic Piwi proteins Aub and Ago3 localize in a distinct compartment – the Yb body in follicular cells or nuage granules in the germline – and therefore might be able to recruit RNA to these structures. The sequestration hypothesis puts the fact that Zucdependent piRNA biogenesis in germ cells requires Aub and Ago3 in new light. While originally this result was interpreted as a requirement for slicer cleavage to trigger Zuc-dependent processing [72, 73], it is possible that piRNA-loaded Aub and Ago3 are necessary not (only) to slice the precursor, but to recruit the substrate into the compartment. This model is supported by the finding that expression of catalytically-impaired Ago3 and Aub can at least partially rescue Zuc-dependent processing of Piwi-associated piRNAs [74]. We propose that this alternative, slicer-independent, mechanism to initiate Zuc-processing is due to recruitment of precursor RNAs to the processing machinery by the slicer-impaired Aub and Ago3.

It should be noted that our hypothesis goes beyond simply stating the fact that compartmentalization is important for piRNA processing. Compartmentalization plays an important role in almost all RNA processing pathways such as splicing, rRNA maturation etc. However, these processes still depend on the presence of sequence motifs in the RNA substrates. In other words, in these pathways localization of RNA substrates to the processing compartments is necessary but not sufficient to trigger processing. We propose that the piRNA pathway operates differently and localization of RNA into nuage/Yb granules is both necessary and sufficient to initiate piRNA processing. Importantly, this hypothesis is testable as it suggests that piRNA biogenesis can be triggered in both cell types in a sequence-independent fashion by recruiting RNA into the processing compartment (See Outstanding Questions).

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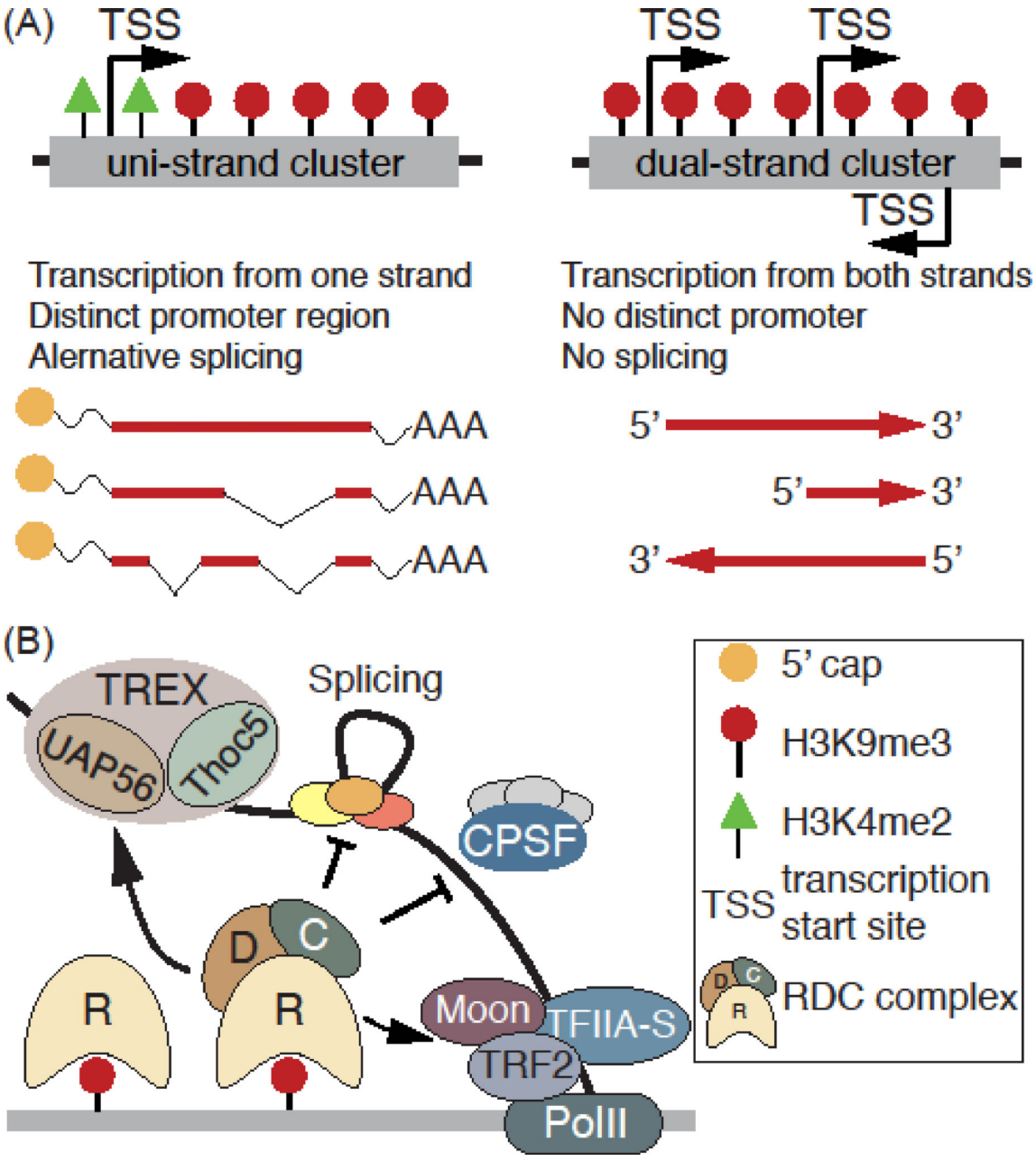

Figure 1. Transcription of piRNA clusters

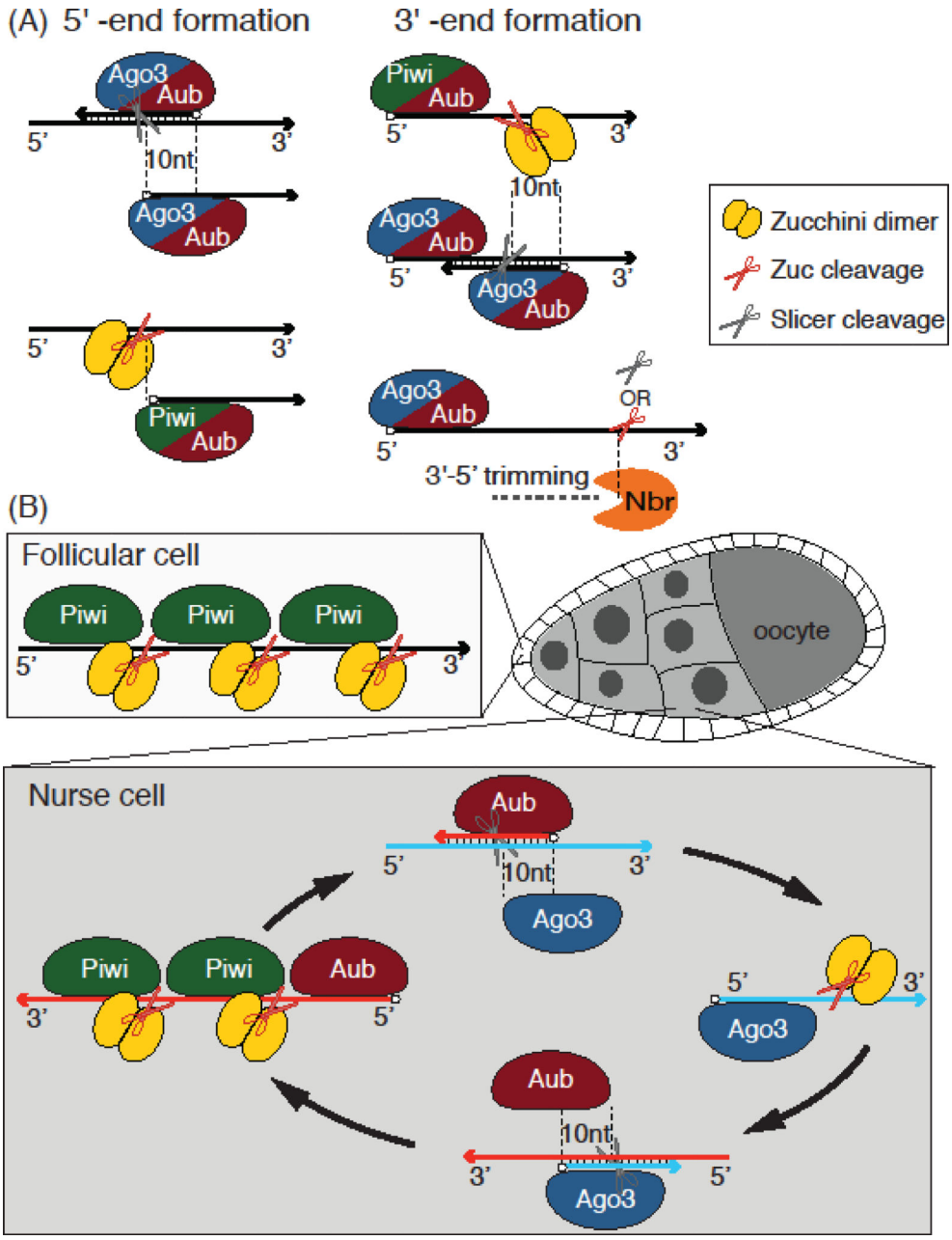

Figure 2. Processing of the 5'- and the 3'-end of piRNA

(A) The 5'-end of piRNAs can be formed either through the endonuclease (slicer) activity of the cytoplasmic Piwi proteins, Aub and Ago3, or through cleavage by the endonuclease Zucchini. Slicer cleavage of piRNA precursors is guided by complementary piRNA. The 5'-end of a slicer product is shifted by exactly 10 nt relative to the 5' end of the guide piRNA. Cleavage mediated by Zuc is independent of guide piRNA. piRNAs formed through slicer-dependent mechanism are loaded into Aub and Ago3, while piRNAs formed by Zuc are loaded into Piwi and Aub. The 3'-end of piRNAs can be formed by three mechanisms:

through endonucleolytic 25 cleavage by Zuc or slicer or by 3' to 5' trimming of longer precursors by the exonuclease Nibbler.

(B) piRNA processing in somatic follicular and germline cells of Drosophila ovary. Only Piwi, but not Aub and Ago3, is expressed in follicular cells. Therefore, in these cells both ends of mature piRNAs are formed exclusively through Zuc-mediated processing with possible contribution of a 3'-end trimming activity. The single Zuc cleavage can simultaneously generate the 5'-end of a downstream and the 3'-end of an upstream RNA resulting in a characteristic phased pattern of piRNAs.

Slicer-dependent and Zuc-dependent processing co-exists and the two pathways cooperate in germline nurse cells. When a new piRNA is formed by the slicer-dependent mechanism and loaded into Aub or Ago3, it can guide formation of the next piRNA giving rise to the so-called ping-pong cycle. Products of Aub-guided cleavage are predominantly loaded into Ago3, while products of Ago3-guided cleavage are loaded onto Aub. Slicer-dependent cleavage by Aub or Ago3 also directs Zuc-dependent substrate RNA processing.

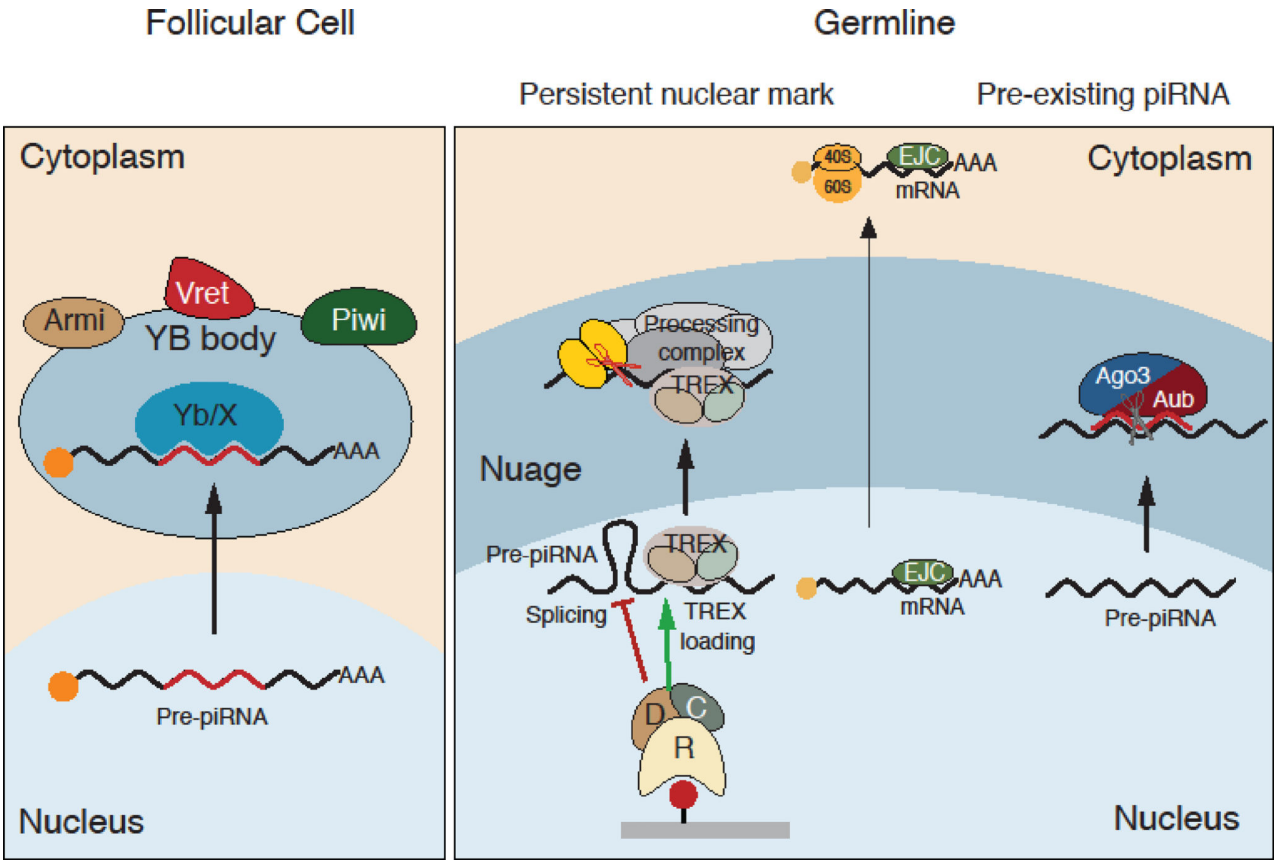

Figure 3. Selection of piRNA precursors for processing

The selection mechanisms of piRNA precursors seems to differ between somatic (follicular) and germline cells. In follicular cells, specific sequences in piRNA precursors are recognized in the cytoplasm through binding by Yb or other yet-to-be-identified RNA-binding proteins (X). It was proposed that Yb, which forms cytoplasmic granules called Yb-bodies, recruit other factors necessary for piRNA processing such as Zuc, Vret and Armi. Two models were proposed for selection of piRNA precursors in germline cells. According to the 'persistent nuclear mark' model piRNA precursors are marked and licensed for processing in the nucleus. The mark – probably an RNA-binding protein – was proposed to shuttle with the piRNA precursor into the cytoplasm and activate the processing machinery. The identity of the mark is not known, although its deposition was proposed to depend on the RDC complex, which is present on chromatin of piRNA-generating loci. Normal mRNAs – that are destined for translation – are bound by the exon-junction complex (EJC) loaded on mRNA as a result of productive splicing. It was proposed that stalled splicing of piRNA precursors might induce marking of piRNA precursors and trigger their processing in the cytoplasm. The TREX complex, which is loaded on piRNA precursors in an RDC-dependent fashion is a candidate for such nuclear mark. According to the second model, selection of RNA for processing only happens in the cytoplasm and is governed by complementary piRNAs that recognize potential precursors and trigger their processing through slicer- and zuc-dependent mechanisms.