

# PIWI-Interacting RNAs (piRNAs) – a Mouse Testis Perspective

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**Abstract**—Over the past decade, PIWI-interacting RNAs (piRNAs) have emerged as the most intriguing class of small RNAs. Almost every aspect of piRNA biology defies established rules of the RNA interference world while the scope of piRNA functional potential spans from transcriptional gene silencing to genome defense to transgenerational epigenetic phenomena. This review will focus on the genomic origins, biogenesis, and function of piRNAs in the mouse testis – an exceptionally robust experimental system amenable to genetic, cell-biological, molecular, and biochemical studies. Aided and frequently guided by knowledge obtained in insect, worm, and fish germ cells, mouse spermatogenesis has emerged as the primary model in understanding the role of this conserved pathway in mammals.

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First noted in fruit-flies [1, 2] and zebrafish [3], PIWI-interacting RNAs (piRNAs) emerged as a *de facto* novel class of small RNAs in a series of papers published in 2006 [4-9]. Stimulated by studies of RNA interference (RNAi) and related phenomena, and boosted by the incredible power of next generation sequencing, the field has moved briskly to reveal a broad picture of piRNA functioning in the protection of germ cell genomes from the danger posed by transposable elements (reviewed in [10-16]).

Prior research of piRNA biogenesis and function could be summarized as follows: (i) piRNAs are small (25-32 nt) single-stranded RNAs that are bound and function in effector complexes with PIWI-like proteins; (ii) piRNA biogenesis is distinct from that of miRNAs and endo-siRNAs and does not rely on the classical Droscha-Dicer pathway. Instead, piRNAs are produced from single-stranded precursor RNAs originating from transposable elements, dedicated piRNA-generating loci, and conventional protein-coding genes; (iii) piRNAs and PIWI-like proteins function almost exclusively in germ cells of metazoans. Abrogation of piRNA production or function results in derepression of transposable elements leading to a range of germ-cell phenotypes and infertility.

The focus of this review will be on the piRNA pathway (piRNAs and the molecular machinery involved in their biogenesis and function) in the male mouse germline. The review will first summarize the developmental and cellular context of the piRNA pathway and subsequently turn to genomic origins, mechanism of biogenesis, and functions of piRNAs.

## A BRIEF OVERVIEW OF THE MALE MOUSE GERMLINE

Mouse primordial germ cells (PGCs) become discernible around gastrulation, but the precise timing and, importantly, the mechanism of their specification are still unknown [17]. Sexual differentiation of mouse PGCs starts on day 12 of embryonic development (that normally lasts 20 days) [18]. Fetal prospermatogonia cease proliferation and remain cell cycle arrested until postnatal day P2 [18, 19]. Following the resumption of cell proliferation after birth, some prospermatogonia become spermatogonial stem cells, while others progress into a relatively synchronous first spermatogenic cycle at P9-10 [20, 21]. Spermatogenesis in adult mice is a cyclical process fueled by the continuous but asynchronous activity of spermatogonial stem cells. For this reason, the first spermatogenic wave is particularly convenient for the dynamic analysis of processes in the context of meiotic prophase I (P10-P19) and spermiogenesis (P19-P35). In meiosis, homologous chromosomes pair, assemble the synaptonemal complex, and recombine [22]. Spermatocytes that fail to accomplish proper chromosome pairing are eliminated in pachynema of prophase I, while those lacking crossovers are detected and apoptosed at the meiotic divisions [23-25]. Post-meiotic events of spermiogenesis are focused on the compaction of the haploid genome and the production of highly specialized cellular structures necessary for sperm formation and function [26]. Spermiogenesis requires precise temporal coordination of

gene expression, mRNA translation, formation of the acrosome and flagellum, histone/protamine exchange, and finally the shedding of the cytoplasm. Perturbations of any of these steps strongly affect fertility as even minor deviations compromise sperm viability, motility, or fertilization capability.

#### A BRIEF OVERVIEW OF THE EPIGENETIC REPROGRAMMING OF THE FETAL GERMLINE

While it appears that the most action in the male germline happens in the adult testes, a critical process known as epigenetic reprogramming of the germline genome occurs in fetal prospermatogonia [27-29]. Such reprogramming involves the removal of pre-existing biparental epigenetic information that was present in germ cell genomes from the onset of embryonic development. Mechanistically, this process is thought to involve remodeling of DNA methylation [27-29] and chromatin structure [30]. The rationale behind epigenetic reprogramming is the restoration of developmental potency of the germline and preparation of the genome for the establishment of male-specific DNA methylation patterns at imprinted genes. The latter class is characterized by their mono-allelic parent-of-origin mode of expression [31, 32]. Since diploid prospermatogonia eventually give rise to haploid sperm, the bi-parental epigenetic marks must first be erased and replaced with the “male exclusive” pattern.

Presently, the mechanism of DNA demethylation in the germline is unclear, with both passive and active mechanisms likely contributing to this process [33]. A prominent consequence of DNA demethylation, in addition to demethylation of imprinted genes, is activation of previously repressed mobile elements [29, 34]. This includes retrotransposons (RTs) such as LINE-1, non-LTR elements that utilize an RNA intermediate for their spreading about the genome [35]. Studies of mice lacking DNMT3L protein that participates in *de novo* DNA methylation demonstrated that RT silencing is critical for normal development and differentiation of the male germline [36, 37]. Loss of DNMT3L results in persistent expression of RTs, DNA damage, and meiotic defects in the postnatal testis, and, ultimately, infertility.

#### THE piRNA PATHWAY IN THE MOUSE MALE GERMLINE

At the core of the evolutionarily conserved piRNA pathway (piRNAs and the molecular machinery involved in their biogenesis and function) in male mice are three PIWI-like proteins encoded by the mouse genome – PIWIL1/MIWI, PIWIL2/MILI, and PIWIL4/MIWI2. These are the key proteins that form effector complexes

with piRNAs. First to be expressed during germline development is PIWIL2/MILI followed by PIWIL4/MIWI2. PIWIL2/MILI expression is activated in gonocytes and is linked to epigenetic reprogramming of the genome [38-40]. PIWIL4/MIWI2 is expressed in fetal prospermatogonia during the period of *de novo* DNA methylation but no longer detected soon after birth. PIWIL2/MILI continues to be expressed in adult testes throughout meiosis and at low levels in early round spermatids. PIWIL1/MIWI expression is first detected in pachytene spermatocytes but is particularly strong in round spermatids. Mouse PIWI-like proteins associate with piRNAs of characteristic size – 26 nt for PIWIL2/MILI, 28 nt for PIWIL4/MIWI2, and 30 nt for PIWIL1/MIWI. As will be described below, the size of mature piRNAs is determined by their respective PIWI proteins.

A second class of proteins with critical roles in the piRNA pathway is Tudor-domain repeat proteins (TDRDs) [41]. These proteins are characterized by the presence of at least one copy of the Tudor domain that is frequently accompanied by another functionally distinct domain. In the context of the piRNA pathway, Tudor domains bind symmetrically modified dimethylarginines (sDMAs) present on some PIWI-like proteins [42, 43]. An attractive idea is that TDRD proteins function as platforms that accommodate the binding of PIWI-like proteins and various other factors that participate in piRNA biogenesis and function [44]. In addition to PIWI-like and TDRD proteins, piRNA biogenesis and function requires other conserved factors including GASZ [45], MOV10L1 [46, 47], MitoPLD [48, 49], MVH/DDX4 [50], and MAEL [40, 51]. Precise biochemical functions and cellular roles of these proteins are presently being elucidated.

Using a targeted gene knock-out approach, early studies of PIWIL1/MIWI and PIWIL2/MILI proteins suggested their essential roles during spermatogenesis [52-54], consistent with *piwi* phenotypes in *Drosophila* [55, 56]. While the initial studies of piRNAs noted the presence of transposon-derived small RNAs, the first evidence implicating piRNAs in transposon control in mice arrived from analysis of piRNAs immunoprecipitated with PIWIL2/MILI protein [57]. Importantly, mice lacking *Piwil2/Mili* or *Piwil4/Miwi2* genes exhibited reduced DNA methylation and derepression of L1 and IAP elements in postnatal testes [57, 58]. These observations provided an important link between mammalian piRNAs with repeat-associated siRNAs in fruit flies [9].

#### COMPARTMENTALIZATION OF THE piRNA PATHWAY

Cytoplasmic granules are prominent features of germ cells throughout the animal kingdom [59, 60]. Commonly referred to as nuage (for *cloud* in French), the

most prominent types of these cytoplasmic structures have been observed for over a century [60, 61]. In mice, examples of nuage include intermitochondrial cement (IMC) [60] and chromatoid bodies [62-64]. Prior studies have suggested that these structures might contain RNAs, but their functions remained enigmatic [63, 65-71].

Work from several groups has now provided compelling evidence of a functional link between the piRNA pathway and nuage structures [40, 72-77]. Immunofluorescence and immuno-gold electron microscopy studies demonstrated that PIWIL2/MILI and its main Tudor domain partner, TDRD1, are concentrated in IMC of fetal prospermatogonia [39, 40, 73]. Ablation of *Piwil2/Mili* or *Tdrd1* genes prevents IMC assembly [39, 42]. Based on this evidence of its functional specialization, morphologically defined IMC structures have been coined pi-bodies [40].

Two other proteins are known to localize to IMC/pi-bodies: GASZ [45] and MVH/DDX4 [40]. The former is a conserved protein of unknown function that possesses several types of protein interaction domains and is required for IMC/pi-body assembly [45]. MVH/DDX4 is the mouse homolog of *Drosophila* DEAD-box helicase Vasa, a broadly conserved marker of germ and stem cells throughout the animal kingdom [50, 78-80]. Deletion of MVH/DDX4 also perturbs the formation of IMC/pi-bodies and normal production of piRNAs [50]. Initially, numerous IMC/pi-bodies are present in a polar fashion in the cytoplasm forming a cap-like structure over the nucleus of fetal prospermatogonia but quickly spread throughout the cytoplasm. This transition depends on a mitochondria surface-localized MitoPLD protein, a proposed RNA endonuclease strongly implicated in piRNA biogenesis [49, 81, 82].

A second PIWI-like protein expressed in fetal prospermatogonia, PIWIL4/MIWI2, and its Tudor domain partner, TDRD9, concentrate in nuage structures that are noticeably larger but significantly less abundant than IMC/pi-bodies. These second type of bodies are formed from the association of PIWIL4/MIWI2 with components of processing bodies (P-bodies) such as DCP1a, XRN1, GW182/TNRC6a, and DDX6/p54 [40, 75]. P-Bodies are cytoplasmic structures that function as mRNA degradation and/or storage sites and are ubiquitously present in all types of somatic cells [83, 84]. Accumulation of PIWIL4/MIWI2 protein in P-bodies is quite remarkable given the fact that this PIWI-like protein is predominantly loaded with anti-sense piRNAs and, therefore, could be surveying the mRNA content of P-bodies in search for suitable targets. The resulting hybrid structures, coined piP-bodies, possess distinctive architecture where PIWIL4/MIWI2, TDRD9, and MAEL, a protein of unknown biochemical function reside in the core, while the outer shell is formed by P-body proteins [40, 51]. Prevention of PIWIL4/MIWI2-TDRD9 localization to piP-bodies (such as in the *Mael*

mutant mice [51]) results in a noticeable transition of their piP-body appearance (but not complete disassembly), suggesting that activity of piRNA pathway proteins contributes to the characteristic appearance of these nuage structures. Failure of PIWIL4/MIWI2 to associate with piP-bodies results in reduced levels of 28 nt piRNAs associated with the protein and in reduced RT silencing [40].

Another prominent nuage structure of mammalian male germ cells is the chromatoid body that first appears in pachytene spermatocytes in meiotic prophase I and, like IMC/pi-bodies, contains PIWIL2/MILI and TDRD1 proteins [42, 85]. Following meiotic divisions, the chromatoid body incorporates components of the IMC/pi-body and piP-bodies in a TDRD7-dependent manner [85]. Fully developed chromatoid bodies of round spermatids are large hybrid structures that harbor two PIWI-like proteins (PIWIL1/MIWI and PIWIL2/MILI), several Tudor-domain proteins, accessory proteins of the piRNA pathway (DDX4/MVH, MAEL), and resident proteins of P-bodies and stress granules [85].

In addition to localizing to various cytoplasmic nuage structures, some piRNA pathway components are also present in germ cell nuclei. Of particular interest is nuclear localization of PIWIL4/MIWI2 protein that predominantly binds anti-sense piRNAs that are thought to guide an effector complex to sites of actively transcribed RT loci. Lack of RT DNA methylation in *Piwil4/Miwi2* mutant germ cells further suggests that *de novo* DNA methylation machinery is guided by piRNA-PIWIL4/MIWI2 complexes to genomic sites marked by nascent RT RNAs. It remains to be determined if this guidance is mediated by direct interactions of the PIWIL4/MIWI2 and its associated proteins with *de novo* DNA methylation machinery or by an indirect mechanism that requires chromatin remodeling at sites of RT expression. A Tudor domain-containing partner of PIWIL4/MIWI2, TDRD9 protein with a DExH-box helicase domain, is also found in piP-bodies and the nucleus [75]. Interestingly, unlike its cytoplasmic accumulation in piP-bodies, nuclear localization of TDRD9 appears to be unaffected in piRNA pathway mutants including *Tdrd1* and *Piwil4/Miwi2* [40, 75]. Similarly, MAEL protein is also detectable in the nuclei of fetal prospermatogonia and spermatocytes [51]. Interestingly, while PIWIL4/MIWI2 localization to piP-body absolutely requires MAEL, its nuclear localization is delayed by a couple of days but eventually occurs resulting in *de novo* DNA methylation of LINE-1 [40].

Recent studies also suggested that PIWIL1/MIWI, PIWIL2/MILI, and piRNAs localize to the dense body of early spermatocytes, a compartment of unknown function positioned in the vicinity of sex chromosomes [86]. PIWIL2/MILI is also reported to localize sex chromosome-associated peri-chromocenter in nuclei of round spermatids [86]. In addition, a single Tudor/Piwi-con-

taining nuclear body harboring PIWIL1/MIWI, PIWIL2/MILI, TDRD1, TDRD5, TDRD6, TDRD7, and MVH/DDX4 has been described in mid-to-late pachytene spermatocytes [87]. What functional roles performed by these nuclear bodies are remains to be determined.

### GENOMIC ORIGINS OF MOUSE piRNAs

The discovery of piRNAs shattered the vision of the world of small RNAs that was built around the Dicer-dependent mechanism of miRNA biogenesis [88]. Besides being longer than miRNAs, piRNAs do not originate from miRNA loci and in fact do not require Dicer [9, 89]. Instead, piRNAs are processed from RNAs originating from intact and mutated transposable elements, dedicated piRNA-generating loci, and even conventional protein-coding genes. The individual contribution of these classes of RNAs varies in the course of germ cell development and differentiation.

In mice, RTs (non-LTR elements such as LINE-1 and LTR-containing endogenous retroviruses such as IAP) are the major source of piRNAs, particularly in fetal prospermatogonia where up to 76% of PIWIL4/MIWI2 piRNAs derive from RTs [39, 90]. Due to the high abundance of repeat sequences including transposons in the mouse genome, roughly 50% of piRNAs in fetal prospermatogonia map to more than one position in the genome. Uniquely mapping prenatal piRNAs derived from non-coding RNAs and mRNAs are also present in fetal prospermatogonia, although at lower levels.

As the male germline transits from the quiescent population of fetal prospermatogonia to stem cell-driven adult spermatogenesis, the composition of piRNA populations in pre-meiotic, meiotic, and post-meiotic cells drifts away from predominantly transposon-associated piRNAs to sequences with unique positions in the mouse genome. piRNAs sequenced from P10 testes (also known as pre-pachytene piRNAs) and even more so from P15 and P21 (pachytene piRNAs) exhibit a progressive reduction of transposon piRNAs in favor of sequences mapping uniquely to dedicated piRNA-generating loci. Although criteria used to define piRNA clusters differ among individual studies, these genomic regions are obvious hotspots for precursor piRNA transcripts. In *Drosophila*, 90% of all unique piRNAs associated with fruit fly Piwi and Piwi-like proteins map to just 142 of such loci [91]. No less than 250 prominent piRNA clusters encompassing fetal, neonate, and adult stages of spermatogenesis have been identified in mice [90, 92, 93]. Some piRNA clusters are extremely large, such as a 240-kb-long cluster 42AB on chromosome 2R in *Drosophila* that is estimated to contribute ~21% of uniquely mapping piRNAs [91]. Most mouse piRNAs whose expression is induced in pachynema of meiotic prophase I are also produced from

prominent clusters exceeding 100 kb [4-6]. On the other hand, recent studies in mice revealed, in addition to large piRNA clusters, numerous smaller-sized piRNA hotspots throughout the genome [92, 93]. In all likelihood, future refinement of computational mapping strategies and deeper sequencing of small RNAs will uncover additional examples of piRNA clusters [92-94].

Clustered mapping of piRNAs to distinct genomic loci, frequently in a strict strand-strand specific manner, is highly consistent with their derivation from long precursor RNA molecules. The existence of long piRNA precursors is supported by deletion and P-element insertion studies in *Drosophila* [91] and expressed sequence tags (ESTs) and RT-PCR data of mouse piRNA clusters [7, 8]. However, the density of piRNAs mapped along these putative precursor RNAs could vary quite significantly in what was described as a “quasi-random” fashion [5, 94]. Therefore, it will be important to determine if this distribution reflects on the mechanism of processing of precursors into mature piRNAs or indicates the production of shorter piRNA precursors that span large chromosome territories giving the impression of existence of large clusters. For example, despite being clustered in two vast regions on chromosome IV, 21U piRNA-like RNAs of *C. elegans* are transcribed from individual mini genes controlled by Forkhead family (Fkh) transcription factors [95, 96].

Genome mapping of pachytene piRNAs suggests that most prominent piRNA clusters are expressed from divergent bidirectional promoters. Presently, details of the transcriptional control of these loci are unknown but, in light of the meiotic context of their expression, elucidation of their regulation could provide insights into functional roles of pachytene piRNAs. In addition to the identification of transcription factors regulating these loci, it would be interesting to determine whether these bidirectional piRNA loci simultaneously expresses both precursor piRNAs and whether this expression is mono- or bi-allelic. We also cannot exclude a possibility of expression of different precursor piRNAs from individual homologous chromosomes or even differential assortment of expressed piRNA precursors between individual spermatocytes.

Overall, developmental patterns of piRNA cluster organization and expression in mice are consistent with existence of a continuously operating defensive program that relies on transcriptional control mechanisms specific to individual stages of male germ cell development. In the fetus, genome-wide DNA demethylation results in activation of individual RTs, which become the major source of piRNAs. Following *de novo* DNA methylation and repression of RT expression, the “piRNA hotspot” identity shifts from individual elements to piRNA clusters whose expression is controlled by transcription factors specific to individual stages of spermatogenesis and whose primary function is likely to trap RTs expressed during corresponding stages of spermatogenesis. A rela-

tively low RT content of pachytene piRNA clusters present in mammalian genomes is likely the reflection of reduced RT expression and, hence, retrotransposition activity during later stages of spermatogenesis. At the same time, we can predict that emergence of a new RT capable of expression in meiotic or post-meiotic cells can be efficiently regulated by capturing such an element in a piRNA cluster. This prediction is supported by experiments showing that an irrelevant (from the germ cell perspective) fragment of DNA will be efficiently turned into piRNAs [97-99].

#### A SPECIAL CASE OF piRNA-GENERATING mRNAs

The mechanism that selects piRNA precursors from all cellular RNAs and directs them for piRNA processing is unknown. In principle, all possible mechanisms fall into two broad categories – random transcriptome sampling or selective targeting. In the former, a small but representative pool of all cellular RNAs exported from the nucleus could be processed into piRNAs (either directly or following partial degradation by conventional machinery). This hypothetical mechanism predicts that expressed RNAs must contribute to the population of mature piRNAs at levels proportional to expression levels. A somewhat related mechanism of transcriptome surveillance by means of Dicer-independent primal small RNAs (priRNA) was proposed to exist recently in fission yeast [100]. Alternatively, RNAs could be selected based on characteristic features, be that sequence motif, secondary structure, nucleotide modifications, or a combination of the above. These features could be recognized directly by the piRNA machinery or mediated by proteins that are normally bound to RNA sequences or structured regions. In addition, RNA selection might operate in specific sub-cellular compartments, such as piP-bodies, where only a subset of RNAs, selected previously on the basis of their other features, are screened for piRNA substrates.

One clue to a possible mechanism of RNA funneling into the piRNA pathway comes from analyses of protein-coding mRNAs that end up processed into genic piRNAs. In fruit fly cultured cells, over 2300 protein-coding genes produced more than 50 genic piRNAs [101]. In zebrafish, sense-strand transcripts of 1900 genes give rise to 18% of piRNAs [102]. In mice, 18% of pre-pachytene piRNAs derive from mRNAs [39]. These genic piRNAs allow examination of the relationship between mRNA expression levels and piRNA production. In *Drosophila* cultured cells, piRNA biogenesis is more active on highly abundant transcripts, yet a large group of highly expressed transcripts failed to yield piRNAs [101]. Likewise, mouse testicular piRNA-producing transcripts showed only slight skewing towards highly expressed genes with house-keeping genes underrepresented [93, 101]. These obser-

vations argue against random sampling of the transcriptome but are consistent with a selective mechanism of genic piRNA production.

What is the basis for the preferential targeting of some mRNAs for piRNA production? One possibility is the presence of transposon-derived sequences in 5' or 3' UTRs of piRNA-producing mRNAs. Such sequences could act as decoys and provide either sequence or structural elements sufficient for the recognition and targeting of mRNAs to piRNA processing sites. However, this is unlikely to be a sole or even a wide-spread mechanism as numerous piRNA-generating mRNAs do not contain well conserved transposon-derived sequences [101]. Another possibility is suggested by results of Gene Ontology analysis of piRNA-producing genes that reveals selective overrepresentation of those with regulatory or developmental function, suggesting that the piRNA machinery favors regulated over house-keeping mRNAs [93, 101]. This observation is consistent with participation of additional features of mRNPs in their targeting for piRNA processing.

#### piRNA BIOGENESIS

piRNAs are produced by two mechanisms, both of which are independent of Dicer [9, 89]. In the primary mechanism, piRNA are produced from single-stranded piRNA precursors including transposon RNAs, long noncoding RNAs expressed from piRNA clusters, and mRNAs. Recent data suggest that the initial cleavage of piRNA precursors is accomplished by the phospholipase D superfamily member MitoPLD [81, 82] whose *Drosophila* homolog *zucchini* was implicated in piRNA biogenesis previously [103-105]. MitoPLD is reported to localize to the outer surface of mitochondria in cultured cells and to be involved in the production of phosphatidic acid [48]. MitoPLD localization to the mitochondrial outer surface is of particular interest since IMC/pi-bodies occupy spaces between adjacent mitochondria in the germ cell cytoplasm [60, 73, 74]. Genetic ablation of MitoPLD function results in male germ cell phenotypes reminiscent of those observed in mice lacking *Piwil2/Mili* [53, 54, 90, 106] and *Piwil4/Miwi2* [58] genes involved in piRNA-dependent RT silencing [48, 49]. However, as the initial studies failed to detect any appreciable nuclease activity associated with recombinant MitoPLD protein, it was proposed to participate in piRNA biogenesis indirectly [48, 49]. New data, however, suggest that recombinant MitoPLD protein has weak endonuclease activity towards single-stranded nucleic acids and produces ssRNA/DNA fragments with 5' monophosphate characteristic of mature piRNAs [81, 82].

A hallmark feature of primary piRNAs bound by their respective PIWI-like proteins, such as PIWIL1/MIWI and PIWIL2/MILI in mice, is the presence of 5'

uracil (1U) [4, 6, 39, 91]. Previously, it was not known whether this bias is a result of primary processing of piRNA precursors or a consequence of preferential binding of 1U-containing piRNAs by PIWI proteins. The combination of an apparent lack of sequence specificity of the endonuclease activity of MitoPLD and evidence of the selectivity of some PIWI-like proteins toward 1U piRNAs *in vitro* suggest that PIWIs have a major role in defining the 5' 1U bias of primary piRNAs [107].

Once bound by a PIWI-like protein, the primary piRNA precursor is subsequently trimmed down on its 3'-end to the final size. The enzyme responsible for this reaction is presently unknown, but a Mg<sup>2+</sup>-dependent 3'-to 5'-exonuclease "Trimmer" activity from a silkworm ovary-derived cultured cell line has been characterized [107]. The final length of mature piRNA appears to be determined exclusively by the associated PIWI protein. Following trimming, mature piRNAs are subject to 3'-end 2'-O-methylation carried out by the RNA methyltransferase HEN1 [89, 108-111].

In addition to the primary mechanism, there is a secondary mechanism for piRNA biogenesis. Secondary piRNAs could be processed from practically any cellular target RNA that is complementary (at the minimum) to nucleotides 2 through 22 of the primary piRNA associated with a PIWI-like protein [112]. The 5'-end of the secondary piRNAs is produced by the "slicing" activity of the PIWI-like protein itself, with the cleavage of the target RNA happening opposite to the bond between nucleotides 10 and 11 of the primary piRNA. As a result, the primary piRNA and the cleaved target RNA overlap in a characteristic 10-bp-long region of perfect complementarity at their 5'-ends [57, 91, 113, 114]. piRNA sequencing studies further suggest that at least some of the cleaved target RNAs re-enter the pool of piRNA precursor and associate with available PIWI-like proteins and are processed into mature piRNA at their 3'-end as described for primary piRNAs.

Initially deduced from features of piRNA sequences and preferential association of piRNAs of opposing strandedness with different PIWI-like proteins, the above mechanism is further supported by the demonstration of the requirement of the slicing activity for the formation of secondary piRNAs and the discovery of target RNA-derived byproducts of secondary piRNA biogenesis [77, 115, 116]. These sequences correspond to the remainder of the target RNA sequence complementary to the primary piRNA starting at the 11th nucleotide and imply existence of an additional 5'-nuclease activity involved in secondary piRNA biogenesis [77]. Finally, it also appears that the production of secondary piRNAs requires the co-chaperone FKBP6 and chaperone HSP90 proteins, perhaps to facilitate ejection of the byproduct of secondary piRNA biogenesis from the PIWI-like protein [77].

Being related to Argonaute proteins, the RNA "slicers" in RNAi, most PIWI proteins possess the cat-

alytic DDH triad in their Piwi domains and, by analogy with AGO [117-119] proteins, are anticipated to cleave RNA. All three mouse PIWI-like proteins possess the catalytic DDH triad and were duly anticipated to require their slicer activities for normal piRNA production and function. For example, based on the preferential association of primary piRNAs with PIWIL2/MILI and secondary anti-sense piRNAs with PIWIL4/MIWI2, the two proteins were proposed to function together in a feed forward loop of piRNA production [39, 113]. Indeed, recombinant and immunopurified PIWIL2/MILI [92] as well as PIWIL1/MIWI [92, 112] enzymes have been shown to possess weak slicing activity. Furthermore, replacement of aspartate residues in DDH triads of PIWIL2/MILI and PIWIL1/MIWI to alanine (generating DAH triads anticipated to be catalytically inactive by analogy with AGO2 protein) resulted in male germ cell-deficient phenotypes reminiscent of those observed in their respective null mutants [112, 120]. By contrast, the catalytic triad of PIWIL4/MIWI2 was found to be dispensable for its function in RT silencing and piRNA biogenesis [120]. This finding implies that the slicing activity of PIWIL2/MILI alone is capable of sustaining the feed-forward mechanism of piRNA production. In addition, these findings imply that the nuclear function of the PIWIL4/MIWI2-piRNA effector complex does not require slicing of the nascent RT RNAs for the efficient targeting of chromatin modifying or *de novo* DNA methylation machinery. Finally, this work illuminates our present lack of understanding of the trafficking of piRNA intermediates between pi- and piP-bodies and necessitates additional studies employing fluorescently labeled proteins or piRNA precursors to reveal the real time dynamics of piRNA biogenesis in germ cells.

## FUNCTIONS OF piRNAs IN MICE

The central question, of course, is that of piRNA functions in mouse germ cells. From the genetic perspective, piRNAs are clearly required for normal development and differentiation of the male germ cell lineage. Most prominently, genetic ablation of factors of the piRNA pathway in mice leads to arrest of spermatogenesis either in meiotic prophase I [45-47, 53, 58, 75] and/or in spermiogenesis [52, 73, 85, 87]. In addition, aging males lacking PIWIL4/MIWI2, exhibit depletion of the spermatogonial compartment, although it remains to be demonstrated if this is a direct effect of piRNA deficiency [58]. Remarkably, and for reasons yet entirely unknown, none of the mouse piRNA mutants with severe defects in male germ cells have been reported to affect oogenesis even though some factors are expressed in oocytes [39, 73, 75].

At the molecular level, the piRNA pathway plays critical roles in the silencing of RTs. Loss of piRNAs

results in significantly lower levels of *de novo* DNA methylation and increased RT RNA and protein levels in fetal prospermatogonia. Interestingly, despite this massive upregulation of RTs in fetal prospermatogonia, male germ cells appear normal until meiotic prophase I. It seems that non-cycling fetal prospermatogonia are resistant to the deleterious effects of RT overexpression. Even more surprising is that undifferentiated and differentiating spermatogonia of adult testes lacking full activity of the piRNA pathway appear morphologically normal, with exception for *Piwil4/Miwi2* mutant mice that exhibit progressive depletion of the stem cell compartment with age [58]. PIWI4/MIWI2 is expressed precisely at the time of *de novo* DNA methylation and localizes to the nucleus [58]. Delaying nuclear entry of PIWI4/MIWI2 (such as in *Macl* mutant mice) prevents timely *de novo* methylation of RTs [39, 40]. Perhaps not surprisingly, *Piwil4/Miwi2* phenotypes are similar to that of *Dnmt3l* mutant mice [36].

Despite this initial apparent resistance to RT activation, in the majority of piRNA pathway mutants (*Piwil2/Mili*, *Piwil4/Miwi2*, *Tdrd1*, *Tdrd9*, *Gasz*, *Macl*, *MitoPld*, *Mov10l1*) meiotic entry is followed by apoptosis of spermatocytes before they reach the meiotic divisions. piRNA mutant spermatocytes fail to synapse homologous chromosomes, and they accumulate massive amounts of DNA damage [121]. As suggested by results of the analysis of double-mutant spermatocytes lacking MAEL, a conserved piRNA pathway factor [122, 123], and SPO11, the sole enzyme for generating meiotic DNA breaks [124, 125], the observed massive DNA damage is unrelated to the meiotic program and is likely to arise from aborted retrotransposition attempts [51, 126]. Therefore, in contrast to fetal prospermatogonia and adult pre-meiotic spermatogonia, spermatocytes appear to be particularly susceptible to RT activity. Perhaps, in light of the danger posed by unrepaired DNA breaks to the integrity of the meiotic genome, DNA repair and checkpoint mechanisms operating in meiotic cells have evolved to efficiently detect and eliminate germ cells with increased RT activity.

As mentioned earlier, a central goal of epigenetic reprogramming in the male germline is the erasure of biparental and establishment of mono-parental, paternal epigenetic marks on imprinted genes. Imprinting and the role of *de novo* DNA methylation machinery in this process are important areas of research because of their link to diseases including cancers [127-129]. In this regard, it is interesting to note that studies of the piRNA pathway in mice have not provided any evidence in support of piRNAs participating in the process of imprinting. A notable exception is *Rasgrf1* that is always inherited in the inactive, DNA methylated state from the paternal germline [75, 130]. The silencing of *Rasgrf1* is accomplished by targeting of *de novo* DNA methylation machinery by piRNAs (derived from RMER4B solo LTR

RT) to “piRNA-targeted non-coding RNA” (pit-RNA) expressed from the *Rasgrf1* locus [130-132]. While being an unusual imprinted gene, *Rasgrf1* is a powerful experimental system that has a great chance of providing important insights into the mechanism of piRNA action, transgenerational inheritance of piRNA-mediated epigenetic modifications of the germline, and the evolutionary origins of imprinting.

Deletion mutations of *Piwil1/Mili* and several Tudor domain-encoding genes arrest spermatogenesis at the round spermatid stage [52, 85, 87, 92, 133, 134]. Interestingly, this arrest is not accompanied by activation of RTs in round spermatids. In fact, instead of abrogating the production of pachytene piRNAs, these mutants appear to have lost normal regulation of the post-meiotic gene expression program that is heavily dependent on the transcription factor CREM [52]. These observations raise a possibility that post-meiotic functions of proteins implicated in the piRNA pathway in the fetal germline, stem cells, and spermatocytes focus not on piRNAs but on regulation of mRNA transcription and translation in spermatogenesis. This view is supported by a recent study that suggested that PIWIL1/MIWI also has a remarkable ability to bind spermiogenic mRNAs directly and protect them from premature degradation or translation until necessary later in spermiogenesis [92]. This idea is a stark departure from a generally accepted mechanism of action of PIWI-like proteins and should be examined critically in the future. Despite its novelty, this mechanism echoes other studies linking PIWI-like proteins to translational control [6, 135, 136]. It also has been proposed that since the vast majority of PIWIL1/MIWI- and PIWIL2/MILI-associated pachytene piRNAs have no legitimate targets among protein-coding mRNAs, their functions (if any) are unlikely to have anything in common with regulation of spermiogenic mRNAs [92]. It should be noted, however, that despite the low level of RT content of pachytene piRNA clusters, inactivation of the catalytic triad of PIWIL1/MIWI results in increased L1 mRNA and protein levels, and DNA damage in early round spermatids [112]. This implies that PIWIL1/MIWI utilizes transposon-targeting pachytene piRNAs to degrade RT mRNAs starting in the latter half of meiotic prophase I and possibly throughout the meiotic divisions.

## FUTURE DIRECTIONS

What lies ahead for mouse piRNAs? With the field being so young, the list of open questions is long but not limited to the following:

1. *What is the mechanism of piRNA precursor selection?* We need to understand which sequence motifs or other features define piRNA precursors.
2. *What is the complete catalogue of piRNA clusters and what controls their expression at different stages of sper-*

*matogenesis?* We need to characterize the piRNA-encoding landscape of the mouse genome and to elucidate the logic of the transcriptional control during spermatogenesis.

3. *Could piRNA biogenesis and function be visualized in germ cells?* We need to further understand the dynamic aspects of subcellular compartmentalization of the piRNA pathway, its interaction with other RNA-centered molecular pathways such as mRNA storage, degradation, and translation.

4. *What is the mechanism of piRNA-mediated guidance of de novo DNA methylation of RTs?* What happens once the PIWI4/MIWI2-piRNA effector complex enters the nucleus of prospermatogonia [137, 138]?

5. *Do piRNAs orchestrate or participate in transgenerational epigenetic phenomena in mammals?*

6. *Could the power of a piRNA system be utilized to regulate gene expression in a directed manner in germ cells as well as in somatic cells?*

7. *What about recent evidence of PIWI and piRNA expression in normal somatic cells and in tumor cells?* Mice lacking PIWI-like proteins exhibit no apparent deficiencies in organs and tissues outside gonads. Could the reported expression and even activity of PIWI-like proteins in mouse and *Aplysia* neurons [139, 140], and in tumors [141-145], lead to new directions of piRNA research?

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