Review

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Functional and structural insights into the piRNA factor Maelstrom

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Abstract

PIWI-interacting RNA (piRNA) is a germline-specific class of small non-coding RNAs that repress transposons in the gonads. Mael, which comprises a high mobility group box and a MAEL domain, is one of the key players in piRNA-mediated transposon silencing. However, the mechanism whereby Mael is involved in this pathway remains unknown. Recent biochemical and structural studies, along with bioinformatic analyses of Mael-associating RNAs *in vivo*, have shed light on the functional aspects of Mael in the piRNA pathway. We summarize the current understanding of Mael functions in the piRNA pathway, particularly in *Drosophila* and in mice.

Key words: piRNA; PIWI; Maelstrom; MTOC; transposon silencing

Introduction

Gene silencing mechanisms triggered by small, 20-33 nucleotide RNA are collectively called RNA silencing, which is involved in numerous biological events in a variety of organisms [1-6]. In RNA silencing, small RNAs associate with members of the Argonaute family of proteins to form RNA-induced silencing complexes (RISCs), which are guided to target transcripts through RNA-RNA base pairing [1-6]. Potent RNA silencing small RNAs include small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). Argonaute proteins are divided into AGO and PIWI subfamilies, depending on amino acid sequence similarity and tissue specificity [1-3]. Ubiquitous AGO proteins bind siRNAs and miRNAs to assemble siRISCs and miRISCs, respectively, whereas germline-specific PIWI proteins bind piRNAs to form piRISCs [6-8].

Like its protein partner PIWI, piRNA is germline specific. Deep sequencing of piRNAs in the germlines of various animals such as *Drosophila,* fish, frogs, mice, and marmosets showed that the majority of piRNAs have sequences that are complementary to transposons [9-15]. Transposons move around within the genome and therefore have the potential to cause genomic damage, which often induces harmful disorders including aberrant chromosomal rearrangements and developmental defects [6, 16]. Rerepression of transposons is therefore essential for living organisms, particularly in the germline; not surprisingly, the piRNA pathway is therefore highly conserved in animals with sexual reproduction systems.

piRNA precursors are transcribed from piRNA clusters, which are intergenic elements rich in transposon fragments and/or other types of repetitive sequences [9, 17]. piRNA precursor processing is extremely intricate and the underlying mechanism is not completely understood. After processing, mature piRNAs are specifically loaded onto PIWI proteins (although AGO proteins are also present in the germlines) [6, 18-25].

piRNA processing and piRISC formation are thought to occur at cytoplasmic loci termed nuage (French for “cloud”), chromatoid bodies, P-bodies or piP-bodies in germ cells, and Yb bodies in *Drosophila* ovarian somatic cells [6, 26-42]. This specific localization likely contributes to strict sorting of piRNAs onto PIWI proteins.

Based on high-throughput sequencing and bioinformatic analyses of piRNAs in various animals, two piRNA biogenesis pathways have been proposed, the primary processing pathway and the ping-pong amplification loop [6, 9, 10]. Germ cells in *Drosophila* ovaries express three PIWI proteins, Piwi, Aubergine (Aub), and AGO3; primary piRNAs produced through the primary processing pathway are loaded onto Piwi and Aub, but not onto AGO3 [6, 9, 10, 19, 23, 43]. Piwi-piRISC localizes to the nucleus upon its cytoplasmic assembly and triggers transcriptional silencing of transposons [6, 11]. Meanwhile, cytoplasmic Aub-piRISC initiates the ping-pong cycle with AGO3 by reciprocally cleaving target RNAs using an endonuclease (slicer) activity to produce secondary piRNAs in the germlines [6, 11, 43]. The ping-pong cycle, which consumes transposon transcripts to provide a secondary piRNA pool, is a coupled event consisting of post-transcriptional transposon silencing and piRNA biogenesis. Somatic follicle cells express Piwi, but not Aub and AGO3, and therefore primary piRNAs are solely loaded onto Piwi [6, 11, 12, 43]. As in germ cells, Piwi-piRISC in somatic cells silence transposons transcriptionally after assembling larger complexes with multiple co-factors in the nucleus [12, 44, 45].

Mice express three PIWI proteins, MIWI, MILI, and MIWI2 in the testis in a spatiotemporal-specific manner [46]. In gonocytes, MILI and MIWI2, but not MILI2, are expressed, where MILI associates with primary piRNAs derived from prepachytene piRNA clusters, and cleaves target RNAs in the cytoplasm that produces secondary piRNAs for MIWI2. MIWI2 then localizes to the nucleus where it implements transcriptional transposon silencing through *de novo* DNA methylation. *De novo* DNA

methylation does not occur in *Drosophila* because the species lacks the enzyme responsible for the modification. At the pachytene stage, where MIWI2 is no longer expressed, MIWI and MIWI2 associate with primary piRNAs arising from pachytene piRNA clusters, and implement post-transcriptional silencing by cleaving target RNAs in the cytoplasm.

To date, a number of factors necessary for both the initiation (i.e., piRNA biogenesis) and effector (i.e., piRNA-mediated silencing) phases of the piRNA pathway, have been identified, many of which are evolutionarily conserved [42, 47-51]. Of those, Mael consists of a high mobility group (HMG) box and a MAEL domain at the N-terminal and central regions, respectively [44, 52, 53]. Mael was originally identified as a P-element-induced female sterile mutant that affects axis specification in *Drosophila* oocytes [52]. However, genetic studies have indicated that loss of Mael causes defects in piRNA accumulation and transposon silencing in *Drosophila* ovaries [29]. The link between oocyte axis specification and piRNA-mediated transposon silencing (both of which are dependent of Mael), as well as the mechanistic involvement of Mael in the piRNA pathway remains largely unclear. However, thanks to the insights into Mael function gained from recent studies, the model of piRNA pathway in animals has been improved.

Mael is a MTOC component in *Drosophila* ovaries

The localization of RNA transcripts (including *Bicoid* (*bcd*), *Oskar* (*osk*), and a ligand for the *Drosophila* Egf receptor *Gurken* (*grk*)) to the posterior end of developing oocytes plays a pivotal role in embryogenesis. The process depends on polarization of the microtubule cytoskeleton and correlates with the formation of a microtubule-organizing center (MTOC), a structure from which most microtubules grow [54-62]. Meanwhile, mutations in *Mael* disturb the localization of mRNAs in developing oocytes because of

improper placement and/or anchoring of the MTOC, because the MTOC fails to move from the anterior to the posterior end of the oocytes in *Mael* mutants [63]. Indeed, mutations in *Mael* prevent clustering of centrioles and posterior localization of the MTOC in stage 2-6 oocytes, indicating the involvement of Mael, directly or indirectly, in MTOC formation [63]. Centrosomes act as MTOCs that nucleate microtubules with uniform polarity and consist of a pair of centrioles that recruit a large number of proteins, collectively referred to as pericentriolar materials. Of these, six proteins, Centrosomin (Cnn), *Drosophila* transforming acidic coiled-coil protein (D-TACC), Mini spindles (Msps), Short spindle 2 (Ssp2), dNAT1, and No circadian temperature entrainment (Nocte), were identified as major Mael-interacting proteins [64-74]. A clear link therefore exists between Mael and the MTOC.

Mael also co-localizes with alpha- and gamma-tubulin in both dividing cysts in the germarium and in dividing follicle cells in ovarioles, indicating that Mael preferentially localizes to microtubule spindles [64]. In *Mael* mutant ovaries, abnormal egg chambers with reduced cell numbers are generated because cytoplasmic mitotic divisions are arrested [64]. Moreover, gamma-tubulin and Cnn are mis-localized or abnormally accumulated in *Mael* mutant egg chambers, which suggests functional involvement of Mael in proper localization and expression of MTOC proteins [64]. Thus, Mael nucleates other MTOC components to form a functional MTOC during *Drosophila* oogenesis.

Mutations in genes belonging to the *Drosophila* piRNA pathway may lead to germline DNA damage and disruption of embryonic axis specification through activation of ATM/Chk2, which functions in DNA damage response signalling [75-78]. During oogenesis, DNA damages (such as double-strand DNA breaks) caused by de novo transposon insertions, are met with activation of *Mei-41* and *Mnk* that encode ATR and Chk2 kinases, respectively; Chk2 then blocks axis specification by disrupting microtubule organization and phosphorylating Vasa [78-80]. Vasa is a DEAD-box RNA

helicase required for axis specification that has been implicated in *Grk* mRNA translation [78]. Embryonic axis specification defects (which have been linked to several piRNA pathway mutations) are genetically suppressed by a loss-of-function mutation in *Mnk*, which is required for DNA damage signaling [78-80]. Loss of Mael function disrupts the posterior localization of Osk and the dorsal anterior localization of Grk in the oocyte [64]. However, unlike other piRNA pathway mutations, the localization of Osk and Grk is not restored in *Mnk;Mael* double mutants [64]. Therefore, defects in microtubule organization observed in the *Mael* oocyte may have distinct origins comparable with DNA damage caused by defects in the piRNA pathway.

The role of Mael in transcriptional silencing in *Drosophila* ovarian somatic cells

Piwi functions in transcriptional transposon silencing in both germ and ovarian somatic cells in *Drosophila* [9-12, 25, 44]. Piwi-mediated transposon silencing depends on Piwi's nuclear localization and piRNA loading, but not on its slicer activity [32, 44, 81, 82]. Transposons targeted by Piwi in ovarian somatic cells are classified into groups I, II, and III [44]. The expression of group I transposons are greatly affected by loss of Piwi, while that of group II transposons are only mildly affected, and group III transposons exhibit little change upon loss of Piwi [44]. Piwi-bound antisense piRNA levels are typically high for transposons in groups I and II, but little for group III transposons [44]. Thus, Piwi-mediated silencing depends on its piRNA pool.

In Piwi-depleted cells, RNA Polymerase II (Pol II) occupancy increases significantly at the genomic loci of group I and II transposons, but not in group III transposons. Further, group I transposons exhibit low levels of histone H3 lysine 9 trimethylation (H3K9me3), a major repressive histone mark [44]. These results provide sufficient proof that Piwi-mediated silencing occurs at the transcriptional, but not the post-transcriptional

level [44]. Loss of Piwi also leads to up-regulation of neighboring genes, which suggests that Piwi-piRISCs trigger local H3K9me3 spreading [44].

In *Mael* mutant ovaries, piRNA levels are reduced, and as a consequence, transposons are derepressed [29]. By contrast, in cultured *Drosophila* ovarian somatic cells (OSCs) [12, 83], *Mael* deletion by RNAi does not affect piRNA biogenesis, but results in derepression of transposons [7, 32, 44]. Thus, Mael acts during the effector phase of the somatic piRNA pathway [84]. Indeed, knocking down Mael results in derepression of group I and II transposons similar to Piwi knockdowns [44]. Likewise, Piwi and Mael double knockdowns exhibit transposon derepression similar to Piwi single knockdowns, further supporting the idea that both proteins act in the same pathway [44]. Although Piwi and Mael depletion increases transcription and Pol II occupancy of transposons in a very similar manner, changes in H3K9me3 patterns differ significantly in cells where Piwi and Mael were depleted. H3K9me3 at transposon loci is slightly reduced in *Mael* knockdown cells. Thus, Mael apparently acts downstream of Piwi and the establishment of H3K9me3 [44].

The MAEL domain in Mael exhibits endoribonuclease activity

The MAEL domain is predicted to adopt an RNase H-like fold [53]. Recently, the X-ray crystal structure of the MAEL domain of *Drosophila* and *Bombyx* Mael has been solved (Figure 1). The *Drosophila* MAEL domain consists of a twisted five-stranded mixed β-sheet surrounded by 13 helices, with a zinc ion coordinated by the ECHC motif [85]. The structure of *Bombyx* MAEL domain shows a high similarity to that of *Drosophila* Mael [86]. The ECHC motif is highly conserved in MAEL domains in animals [85]. Mutations in the ECHC motif destabilize the Mael protein, which demonstrates that zinc ion coordination by the ECHC amino acid tetrad is indispensable for proper functioning of Mael [85]. Interestingly, the MAEL domain of Mael lacks canonical catalytic residues

DEDDh conserved among RNase H-like superfamily members [85]. However, unexpectedly, it exhibits a single-stranded (ss) endoribonuclease (RNase) activity *in vitro* [85]. The cleavage pattern of ssRNA substrates by the MAEL domain is similar to that of RNase T1, a well-studied endonuclease that specifically cleaves ssRNA at the 3' side of guanine residues [87]. However, unlike RNase T1, the ssRNase activity of the MAEL domain does not require divalent metal ions, but is inhibited by high salt [85]. The RNA cleavage mechanism of the MAEL domain is distinct from that of RNase T1. Notably, the ssRNase activity is also observed in the MAEL domain of Bombyx and mouse Mael proteins *in vitro*, at least under condition of a low salt [85].

Mutations in several conserved residues among orthologs of the MAEL domain severely reduced, but did not abolish its ssRNase activity, implying that those residues might be involved in ssRNA binding, but not catalysis. Neither the catalytic center nor the mechanism of catalysis has been uncovered to date. One could argue that the MAEL domain would have evolved from a DEDDh exonuclease by switching the catalytic residues from DEDDh to ECHC (Figure 1) [53, 85]. The ECHC tetrad is highly conserved among Mael in various species, which suggest that this tetrad might be the active center. However, because the tetrad is responsible for maintaining both protein structure and nuclease activity, this would be too difficult to prove experimentally.

The MAEL domain of *Drosophila* Mael is able to restore silencing defects caused by depletion of endogenous Mael in OSC cells, which means that the HMG box is dispensable for nuclear silencing of transposons in the cells (Figure 2) [7, 44, 85]. However, MAEL domain mutants that reduce the ssRNase activity of Mael have also been shown to rescue the silencing defects in OSCs [85]. The relevance of Mael's ssRNase activity in piRNA-mediated silencing therefore remains unclear. Whether the ssRNase activity of the MAEL domain is required for piRNA biogenesis in *Drosophila* ovarian somatic cells is not yet known.

Mael in adult mouse testis binds pachytene piRNA precursors

Based on microarray-based expression profiling in mice, Mael is necessary for early spermatogenesis [88]. Mael is highly expressed in the testis, but its cellular localization changes through spermatogenesis; in spermatocytes it is cytoplasmic while it localizes in the nucleus in spermatids [87]. MAEL is concentrated at the sex body in the nucleus and chromatoid body/nuage in the cytoplasm [88-90]. The sex body is a specialized nuclear area where both transcription and homologous recombination are restricted during meiosis, whereas the chromatoid body/nuage is a unique cloud-like dense structure found in male germ cells, where various piRNA factors accumulate [31, 33, 34, 38, 39, 41, 46]. Unlike Mael in *Drosophila*, Mael in mice interacts with Miwi and Mili, two of three murine PIWI proteins, in the adult mouse testis [88]. MIWI and Mili (also known as PIWIL1 and PIWIL2), but not Miwi2 (PIWIL4), are expressed in the adult mouse testis [6, 31, 34, 46]. The *Mael* knockout mouse testis appears normal anatomically, with the exception of smaller testes [89]. In the absence of Mael, post-meiotic germ cells (round and elongated spermatids) were completely absent, demonstrating the necessity of Mael in spermatogenesis [89]. In addition, long interspersed nucleotide element (LINE)-1 and intracisternal A particle (IAP) element, non-LTR and LTR retrotransposons, respectively, are derepressed, and CpG DNA methylation levels associated with LINE-1 genomic regions are significantly reduced in loss of Mael testes [89]. Overall, *Mael* knockout mice phenocopy *MILI* and *MIWI2* knockout mice: a close functional relationship of MAEL with these two PIWI proteins was suggested. Recently, analysis of the Mael-associated proteome revealed that Mael is strongly associated with MIWI and a Tudor domain-containing protein TDRD6 in adult mouse testes (Figure 2) [91, 92]. Three other Tudor proteins TDRD1, TDRD4/RNF17, and TDRD9 also appeared in the Mael-associated proteome. MILI was detected in the adult mouse testis, although, at a

lower percent coverage compared with MIWI. An earlier report showed that a DEAD-box RNA helicase Mouse Vasa homolog (MVH) associates with MIWI and TDTD6 in adult testes [93, 94]. However, Mael complexes rich in MIWI and TDTD6 appeared to be absent of MVH [91]. Mael interaction with MIWI and Tdrd6 is likely RNA independent. Examination of RNA components in the Mael complex revealed pachytene piRNA precursors (Figure 2) [91]. In addition, the Mael complex is also enriched with transcripts of known and hypothetical protein-coding genes, pseudogenes, as well as non-coding RNAs. Because the Mael complex is devoid of mature piRNAs, Mael may play an important role in the pachytene piRNA processing step. Indeed, loss of Mael in mouse testes reduces the level of pachytene piRNAs in the testis. A recent study showed that the MAEL domain of mouse and *Drosophila* Mael exhibits an ssRNase activity *in vitro* [85]. Further studies are needed to determine whether Mael is responsible for pachytene piRNA processing in the adult mouse testis. The HMG box in mouse Mael preferentially binds structured RNAs [95]. Given that mouse Mael is found in both the nucleus and the cytoplasm [88], and that Mael is a shuttling protein [28], Mael may bind pachytene piRNA primary precursors in the nucleus through its HMG box and export them to the cytoplasm, where the precursors accumulate prior to being processed into mature piRNAs (Figure 2) [92].

Perspective

Mael is a conserved piRNA factor in animals, consisting of two domains, a HMG box and a MAEL domain. The MAEL domain of Mael has ssRNase activity. In *Drosophila* ovarian somatic cells, Mael acts as an effector of the Piwi-mediated transcriptional silencing of transposons; only the MAEL domain, and not the HMG box of Mael is required for this silencing. In addition, its ssRNase activity is not required for silencing transposons in somatic cells. These observations imply that the MAEL domain acts as a scaffold for

Piwi-nuclear complexes to effectively silence transposons in *Drosophila* soma. In contrast, Mael is involved in the piRNA production in germ cells. Moreover, the HMG box of Mael is dispensable for silencing in somatic cells but is required for the piRNA production in germ cells. The cell-specific dual functions of Mael in the piRNA pathway is a unique aspect of Mael.

Functional analyses of mouse Mael may provide good clues to understanding the molecular functions of Mael in piRNA production in *Drosophila* germ cells. The mouse Mael complex, which contains MIWI and TDRD6, is enriched with pachytene piRNA primary precursors, where precursors are undergoing fragmentation/processing into mature piRNAs. Because the mouse HMG box has binding activity to structured RNAs, it seems likely that pachytene piRNA primary precursors may be anchored by the HMG box and then fragmented by the MAEL domain. In *Drosophila* germ cells, Mael is involved in the production of piRNAs, and the HMG box of Mael is required for germline-specific transposon silencing. These results imply that *Drosophila* Mael also performs similar functions to mouse Mael. Further biochemical studies are needed to shed light on the molecular roles of Mael in piRNA pathways.

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Figure legends

Figure 1. Crystal structure of the MAEL domain of *Drosophila* Mael.

Overall structures of *Drosophila* MAEL domain and a DEDDh exonuclease VASV NP [96]. The bound dsRNA is omitted for clarity. VASV NP has the DEDDh catalytic residues in the catalytic groove, but *Drosophila* MAEL domain lacks them in the central groove.

Figure 2. Possible models of Mael function in piRNA biogenesis and transposon silencing. Left: In *Drosophila* ovarian somatic cells, Mael is involved in Piwi-mediated transcriptional transposon silencing and acts downstream of H3K9me3 establishment by Piwi. The MAEL domain, but not its ssRNase activity and HMG box, is indispensable for silencing, which suggests that Mael may act as a scaffold of nuclear-effector complexes to silence transposons. Right: In mouse adult testes, Mael interacts with Miwi and TDRD6, and is required for pachytene piRNA biogenesis. During piRNA biogenesis, Mael may bind to pachytene piRNA primary precursors through the HMG box and fragment it by the MAEL domain.

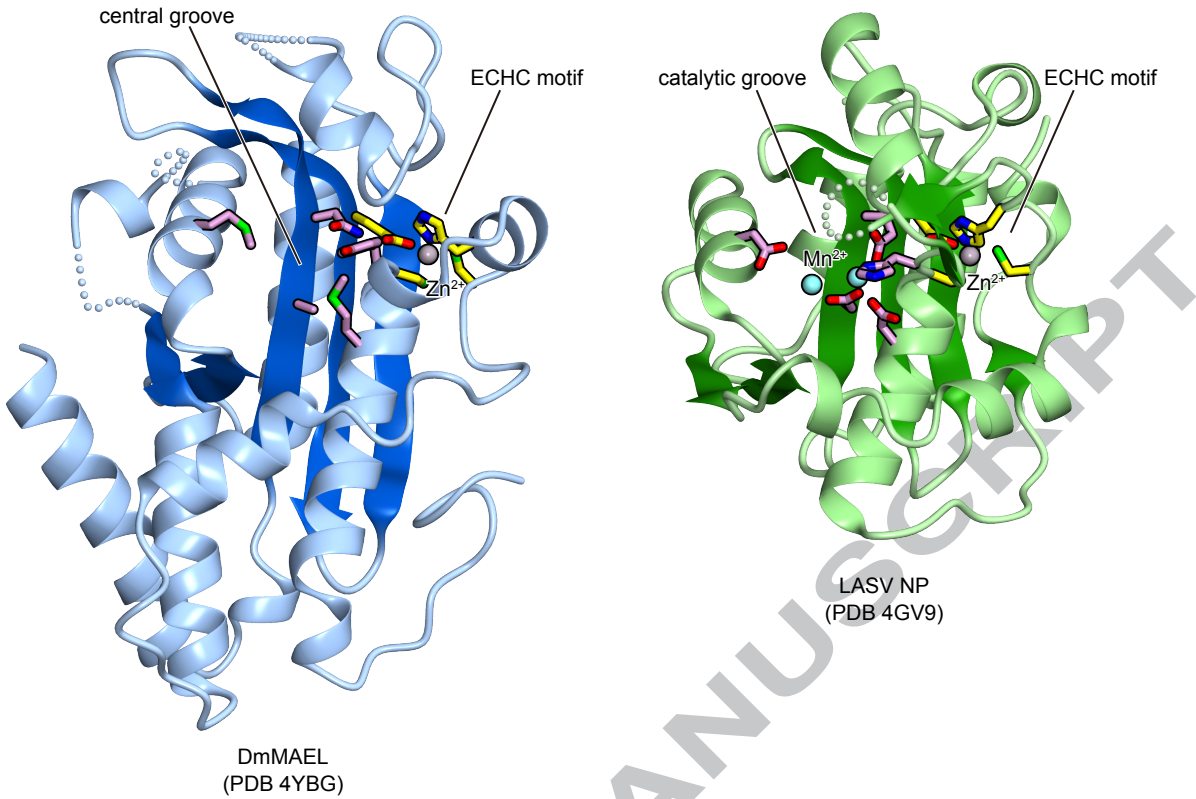

Figure 2_sato and siomi