

Germ granules in spermatogenesis of *Drosophila*

Evidences of contribution to the piRNA silencing

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Abbreviations: CB, chromatoid body; IMC, intermitochondrial cement; *Su(Ste)*, *Suppressor of Stellate*; piRNA, PIWI-interacting RNA

Ribonucleoprotein-containing granules in the cytoplasm of germinal cells are known to be a common attribute of eukaryotic organisms. Germ granules appear to ensure the posttranscriptional regulation of germline mRNAs. Recent studies specify the participation of the germ granules in genome integrity maintenance by mechanisms involving short piRNAs. PIWI clade proteins and associated piRNAs are considered as key participants of the germline-specific piRNA pathway. Proteins of the PIWI clade, Aub and AGO3, concentrated in the germline-specific perinuclear granules called nuage, are involved in silencing of retrotransposons and other selfish repetitive elements in the *Drosophila* genome. In *Drosophila* testes, two types of perinuclear nuage granules are found: a large amount of small particles around the nuclei and significantly larger structures, the piNG-bodies. In this mini-review, we analyze the recent published data about structure and functions of *Drosophila* male germ granules, and especially their involvement in the piRNA silencing pathway.

Cytoplasmic RNA-rich non-membranous structures, which are remarkably conserved in germinal tissues of many eukaryotic species, are currently designated as germ granules. More than a century ago, a perinuclear granule named chromatoid body (CB) was found in the cytoplasm of mammalian male germ cells from late spermatocytes to round spermatids.¹ Germ granules differ significantly in their morphology and functions in various species, for example, polar granules or pole plasm in *D. melanogaster*, *C. elegans*, *X. laevis*, nuage and sponge body in *D. melanogaster*, P granules in *C. elegans*, CB and intermitochondrial cement (IMC or pi-body) in mammals.^{1,2} CB is thought to be cognate to the germ cell specification structure, nuage, in *Drosophila*. It has been proposed that germ granules contain proteins and mRNAs needed for germline development and gametogenesis. Germ granules also shared some protein components with cytoplasmic granules of somatic cells known as P-bodies or processing bodies.³ However, their molecular functions remain mysterious to this day. Recent studies suggest the involvement of germ granules in defense of the germline genome from active endogenous elements, such as

transposons.⁴ Maintaining genome integrity during spermatogenesis and oogenesis is critical for a species viability. Discoveries of the past ten years revealed the essential role of gonad-specific piRNAs (small RNAs of 25–30 nt that associate with proteins of PIWI clade) in the silencing of transposons.^{5–10} This is now considered as a general mechanism of genome defense in the germlines of plants, fungi, worms, insects and mammals.⁴ piRNAs are the largest and most complicated class of small RNAs. Biogenesis of piRNAs and piRNA-mediated posttranscriptional silencing appear to take place mainly in the cytoplasmic germ granules.^{11–14} In this mini-review we focus on the recent investigations of male germ granules from the well-known eukaryotic model organism, *Drosophila*, and their involvement to the piRNA silencing pathway.

Nuage as an Organelle Composed of Germ Granules

Perinuclear granules in germ cells of *Drosophila* have been named nuage (which means “cloud” in French). Nuage was described at first in nurse cells of the *Drosophila* ovaries.^{11,15–17} These structures are visible as discontinuous rings around the nuclei on confocal slices. Similar structures were also detected in spermatogonial cells and primary spermatocytes in the testes.^{17–19} The main marker and essential component of nuage in both sexes is RNA-helicase Vasa.^{16,17,20} Proteomic content of ovarian nuage granules includes: proteins of the PIWI subfamily, Aubergine and Argonaute 3; RNA-helicases, Vasa, Spindle E and Belle; Tudor domain-containing proteins, Tudor, Spindle E, Krimper, Tejas; proteins known as components of somatic P-bodies taking part in mRNA degradation, DCP1, Me31B and Pacman; and other proteins, such as putative nuclease Squash and high mobility box group protein Maelstrom.^{11,12,15–17,21–24} Nuage is thought to be involved in the selection and translational control of mRNAs transported from the nucleus.^{12,16,17} Recent studies revealed the association of nuage with the piRNA biogenesis and piRNA-dependent silencing of transposons.^{11,12}

Proteins of the PIWI subfamily, Piwi, Aubergine (Aub) and Argonaute 3 (AGO3), form complexes with piRNAs. These complexes recognize mRNAs complementary to guide piRNAs and perform RNA slicing, causing target degradation. PIWI clade proteins are considered the main players of the piRNA pathway.^{5–8,10,25} Two main models of piRNA processing have

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been suggested based on deep-sequencing analysis of ovarian piRNAs. In germinal cells of the ovaries, piRNAs complementary to transposon transcripts undergo amplification via the “ping-pong” cycle.^{7,8} Aub interacts mainly with antisense piRNAs derived from specialized genomic “master” loci, and AGO3 preferentially associates with the sense ones.^{7,8,10,25} Sense transposon transcripts are targeted by the Aub-containing antisense silencing complexes, which cause transcript cleavage. The cleavage act results in the production of 5'-termini of sense piRNAs. The details of 3'-processing of piRNAs are unknown up to now. These sense piRNAs are subsequently loaded into the AGO3-containing sense silencing complexes, which presumably recognize long antisense transcripts and generate 5'-ends of antisense piRNAs, which are involved in the next round of the cycle.^{7,8} The amplification cycle produces pairs of sense and antisense piRNAs which have a ten nucleotide overlap of complementary sequence in their 5'-ends. Nuage in nurse cells of the *Drosophila* ovaries is considered to be a site for both the piRNA maturation and the piRNA-guided silencing.^{10-12,25} The third member of PIWI subfamily, nuclear protein Piwi, is expressed in both germline and somatic cells of the ovaries. In somatic cells in the absence of germline-specific Aub and AGO3 Piwi-associated piRNAs are produced by another mechanism, presumably via direct cleavage of long transcripts (primary processing). The ping-pong cycle is shown to be independent from Piwi.^{7,10} However, Piwi is essential for transposon silencing in the germline,^{6,10,25} and, according to recent data, is involved in co-transcriptional repression of telomeric retrotransposons.²⁶

piRNA Pathway in the Testes

The first case of natural piRNA-mediated silencing of endogenous genes was discovered in the *Drosophila* testes.⁵ However, the majority of papers published since then is devoted to the piRNA silencing in the ovaries, where powerful transposon activity is easily detectable in piRNA pathway mutant flies. Here we summarize the known peculiarities of the piRNA pathway in the testes.

Transcripts of the X-linked endogenous tandemly repeated *Stellate* genes, but not of transposons, are shown to be the main targets of the piRNA silencing in the testes of *D. melanogaster*.^{18,27} *Stellate* gene expression is strongly repressed by the piRNA machinery in wild type males. In case of a deletion of the homologous Y-linked *crystal* or *Suppressor of Stellate* (*Su(Ste)*) locus, abnormally high-level *Stellate* expression occurs in primary spermatocytes and leads to the accumulation of protein crystals, disturbances in meiotic chromosome condensation and segregation and essentially reduced fertility.^{28,29} Abundant anti-sense transcription of the *crystal* locus is responsible for the production of antisense *Su(Ste)* piRNAs complementary to *Stellate* transcripts. Nuage proteins, such as Aub, AGO3, Spindle E, Armitage, Tejas, Krimper, Maelstrom, Vasa and Squash are found to be necessary for the piRNAs-mediated *Stellate* silencing. Mutations in these genes result in the loss of *Su(Ste)* piRNAs and *Stellate* derepression.^{5,6,18,19,24,25,30,31} It should be noted that the proteins, which deficiencies lead to *Stellate* derepression, are largely

concentrated to nuage. Piwi, the founder of PIWI clade, is known to be expressed mainly in somatic cells of the testis germinal proliferative center.²⁷ It is considered dispensable for *Stellate* silencing.⁶

Large-scale sequencing of testis Aub-associated short RNAs revealed that 70% of these piRNAs were *Su(Ste)*-derived, predominately in antisense orientation.¹⁸ About 90% of *Su(Ste)* piRNAs were presented by a piRNA of a single type, called *Su(Ste)-4*, or its variants. This observation strongly favors the assumption that *Su(Ste)* piRNAs are non randomly produced from precursor anti-sense *Su(Ste)* transcripts, but rather from a limited number of “hotspots.” However, among AGO3-associated piRNAs only 6% belongs to *Su(Ste)* piRNAs abundantly represented by *Su(Ste)-4* too. Only a trace amount of sense *Su(Ste)* piRNAs was found in Aub and AGO3 complexes.¹⁸ Also, a few complementary pairs of *Su(Ste)* piRNAs overlapping by 5'-ends (bearing signatures of the ping-pong cycle) were found. Thus, the bulk of testis piRNAs is generated by primary processing rather than the ping-pong mechanism. In spite of AGO3 expression level being significantly lower in the testes than that of Aub,¹⁸ the biogenesis of *Su(Ste)* piRNAs and *Stellate* silencing are dependent on both Aub and AGO3.^{5,18,25} A biological rationale for these requirements is still unclear now.

Only 54% of piRNAs from the AGO3-derived library and 7% from the Aub-derived one belong to transposon sequences.¹⁸ A significant part of transposon-associated piRNAs demonstrated ping-pong signatures and generated ping-pong pairs similarly to the situation in the ovaries. However, contrary to their effects in the ovaries, Aub and AGO3 deficiencies only slightly affect transposon expression in the testes.^{5,18} Apparently, mobilization of transposons in the testes is essentially under the control of an alternative mechanism.

In our recent paper¹⁹ we found at least two types of perinuclear nuage granules in germinal cells of the testes: a lot of small particles of about 0.6 μm that were concentrated around the nuclei, and significantly larger structures of about 2.4 μm , usually one per cell. The volume of larger granules is found to be more than 50 times that of the smaller ones. Large granules are enriched by the known nuage proteins: Vasa, Aub, AGO3, Tudor, Spindle E, Belle, Squash, Cutoff and also AGO1, the principal component of the microRNA pathway. Since most of the identified components are known as participants of the piRNA pathway, the new structure was named the piNG-body (piRNA Nuage Giant body). Vasa and Aub are shown to be completely colocalized at the periphery of the piNG-bodies, whereas AGO3 is located in the central lobe. The piNG-bodies appear in primary spermatocytes during active transcription period and are lost before meiotic divisions (Fig. 1). Neither piNG-bodies nor small nuage granules are detected in round spermatids. Mutational analysis demonstrated a strong association of piNG-bodies formation with the *Stellate* silencing.¹⁹ Symmetrical arginine methylation of PIWI clade proteins provided by arginine methyltransferase Capsuleen (Dart5)³² is found to be essential for piNG-body formation. *Capsuleen* mutations leading to Aub delocalization to random cytoplasmic foci induce strong *Stellate* derepression and loss of *Su(Ste)*

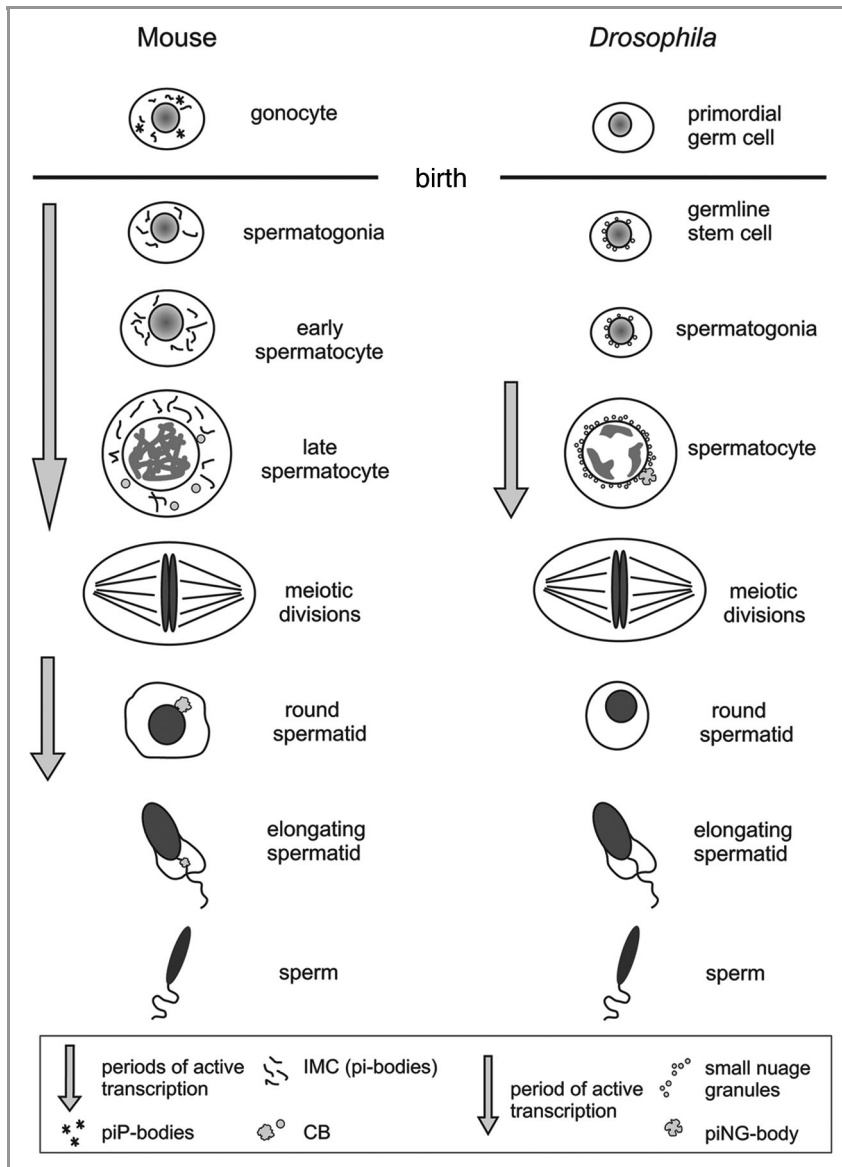


Figure 1. Scheme of spermatogenesis in mouse and *Drosophila*. Left: Germ granules in mouse gonocytes (IMC or pi-bodies, piP-bodies^{13,14}), spermatogonia, early spermatocytes (IMC), late spermatocytes (IMC, CB precursors) and haploid round spermatids (CB) are depicted. Adapted from ref. 2. CB formation coincides with periods of active transcription (arrows) and emerging of abundant pachytene piRNAs. In elongating spermatids the CBs gradually degenerates. Right: Two types of germ granules are found in the *Drosophila* male germline. There are a lot of small nuage granules, that appear early in germline stem cells, and significantly larger structures, the piNG-bodies. The piNG-bodies form in primary spermatocytes and disappear before meiotic divisions. Arrow indicates massive transcription during spermatocyte growth and maturation. No germ granules are found in round spermatids, where transcription program dramatically ceases.

piRNAs.¹⁹ Since AGO1 is found to be a piNG-body component, the functions of the large granules in the microRNA-mediated mRNA silencing can also be expected. In addition to that we detected DCP1, a decapping enzyme and marker of somatic P-bodies, as a piNG-body constituent (Kibanov, unpublished data).

mRNA degradation machinery.^{1,2,33,34} Biochemical isolation of mouse CBs reveals a high concentration of piRNAs, named late or pachytene piRNAs,³⁴ which do not demonstrate ping-pong signatures unlike pre-pachytene piRNAs that are expressed earlier in gonocytes.^{9,13} Pachytene piRNAs originate mainly from large non-repeated clusters located in non-annotated genome regions,

Whereas no piNG-body-like large nuage structures are observed in the ovaries, a question concerning their role in the testes is raised. Taking into account that the conventional nuage granules in the ovarian nurse cells appear to be enough for functioning of the “ping-pong” piRNA silencing mechanism, the piNG-bodies seem to be dispensable for the amplification cycle. We know that *Stellate* transcription occurs in primary spermatocytes³⁰ and temporarily coincides with the piNG-body formation.¹⁹ The analysis of *Su(Ste)* piRNAs derived from the testis libraries favors their generation by primary processing rather than by the “ping-pong” cycle.¹⁸ The arranged internal structure of the piNG-body and a high concentration of piRNA pathway components¹⁹ seem to contribute to effective kinetics of the *Stellate* silencing process. Abundant transcription of antisense *Su(Ste)* starts in spermatogonial cells, earlier than that of *Stellate* genes.³⁰ It is tempting to speculate that *Su(Ste)* piRNAs are produced before the start of *Stellate* transcription by a variant of primary processing. However, details of *Stellate* repression by the piRNA pathway and *Su(Ste)* piRNA biogenesis remain obscure to date.

Functional Relations Between piNG-body and CB

The mammalian CB is another large granular organelle involved in posttranscriptional mRNA processing in the testes. Although it appears in late pachytene spermatocytes and finally forms in round spermatids as a single structure per cell (Fig. 1), its persistence, similar to the piNG-body, coincides with the strong wave of transcription.^{1,2} Now more than 40 various proteins are identified as CB components.^{1,2} The CB contains MVH, the mammalian homolog of *Drosophila* Vasa protein; MIWI and MILI, PIWI clade proteins; Tudor domain-containing proteins; Dicer, the nuclease necessary for the microRNAs processing.^{33,34} MicroRNAs and microRNA pathway proteins were found in the CBs suggesting their role in microRNA-mediated expression regulation.³³

The CBs also contain proteins of the general

but not from transposon-related sequences.³⁵⁻³⁷ Targets of pachytene piRNAs are largely unknown. Although the exact roles of the CB and pachytene piRNAs in mouse spermatogenesis remain to be determined, we suggest that the piNG-body and CB have shared attributes and can be functionally related structures.

The recent studies of the piRNA pathway using *Drosophila* testes provide important insights into the biological role of the germ granules. Similar characteristics and protein content of the

CB and piNG-body allow using *Drosophila* as the model organism for further investigation of molecular principles of the germ granules organization and functioning.

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