Separation of stem cell maintenance and transposon silencing functions of Piwi protein

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Piwi-interacting RNAs (piRNAs) and Piwi proteins have the evolutionarily conserved function of silencing of repetitive genetic elements in germ lines. The founder of the Piwi subfamily, Drosophila nuclear Piwi protein, was also shown to be required for the maintenance of germ-line stem cells (GSCs). Hence, null mutant piwi females exhibit two types of abnormalities, overexpression of transposons and severely underdeveloped ovaries. It remained unknown whether the failure of GSC maintenance is related to transposon derepression or if GSC self-renewal and piRNA silencing are two distinct functions of the Piwi protein. We have revealed a mutation, piwi<sup>fl</sup>, removing the nuclear localization signal of the Piwi protein. piwi<sup>fl</sup> females retain the ability of GSC self-renewal and a near-normal number of egg chambers in the ovarioles but display a drastic transposable element derepression and nuclear accumulation of their transcripts in the germ line. piwi<sup>fl</sup> mutants are sterile most likely because of the disturbance of piRNA-mediated transposon silencing. Analysis of chromatin modifications in the piwi<sup>fl</sup> ovaries indicated that Piwi causes chromatin silencing only of certain types of transposons, whereas others are repressed in the nucleus without their chromatin modification. Thus, Piwi nuclear localization that is required for its silencing function is not essential for the maintenance of GSCs. We suggest that the Piwi function in GSC self-renewal is independent of transposon repression and is normally realized in the cytoplasm of GSC niche cells.

Results

Identification of piwi<sup>fl</sup> Mutation. While characterizing a female sterile mutation, hereafter piwi<sup>21</sup> (i.e., N-truncated), on chromosome 2, we detected sterile transfertility carriers of the piwi<sup>21</sup> chromosome and an opposite chromosome with deletions uncovering the region containing piwi and aub genes. Sterility was also observed in flies carrying transheterozygous combinations of piwi<sup>21</sup> with piwi<sup>2</sup> or piwi<sup>3</sup> but not with aub mutations. We revealed a 5' truncation of the piwi gene as a result of P element vector insertion in the coding region of the first exon (Fig. S1A). RT-PCR analysis demonstrated the presence of the piwi transcript in the mutant ovaries, but 5'-RACE defined its start site at the first intron of piwi (Fig. S1A). This start site position was confirmed by RT-PCR. Analysis of this region using McPromoter software (27) predicted a cryptic promoter at this site. The presence of the ATG codon near the intron 1/exon 2 boundary enables initiation of translation of a shortened Piwi protein lacking 26 N-terminal amino acids including the nuclear localization signal (NLS), but with two additional amino acids (M and Q) encoded by the intron sequence (Fig. S1A). The rest of the piwi gene encoding the PAZ and Piwi domains responsible for short RNA binding and target RNA slicing remained unchanged (Fig. 1A). Antibodies against the extreme N-terminal part of Piwi did not recognize the mutant

The authors declare no conflict of interest.

The Direct Submission article had a prearranged editor. M.S.K. and O.A.S. contributed equally to this work.

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Author contributions: M.S.K., O.A.S., and V.A.G. designed research; M.S.K., O.A.S., E.Y.Y., A.D.S., E.A.M., and S.A.L. performed research; M.S.K., O.A.S., E.Y.Y., A.D.S., and V.A.G. analyzed data; and M.S.K. and V.A.G. wrote the paper.

Author contributions: M.S.K., O.A.S., and V.A.G. designed research; M.S.K., O.A.S., E.Y.Y., A.D.S., E.A.M., and S.A.L. performed research; M.S.K., O.A.S., E.Y.Y., A.D.S., and V.A.G. analyzed data; and M.S.K. and V.A.G. wrote the paper.
Next we tested the expression pattern and localization of the mutant Piwi<sup>Nt</sup> protein. The requirement of the NLS in the Piwi N-terminal region has been shown previously for Piwi import into the nuclei of cultured ovarian somatic cells (25, 29). In the piwi<sup>Nt</sup> ovaries, the nuclear localization of the mutant Piwi protein was lost completely in all cells in which Piwi is expressed, including germinal nurse cells, developing oocytes, and somatic follicle cells (Fig. 2 A–D and Fig. S3). The Piwi<sup>Nt</sup> protein was detected mainly in the cytoplasm of nurse cells and around their nuclei, similarly to nuage components, e.g., Aub (Fig. 2B). Mutant protein was found in the cytoplasm of follicle somatic cells (Fig. 2C) but at a significantly lower level than in germ cells.

Thus, our data provide an unexpected conclusion that cytoplasmic Piwi is capable of supporting GSC maintenance. This process is known to depend on Piwi expression only in somatic cells forming the GSC niche at the anterior-most end of the gerarium (2, 6). The GSC niche consists of the terminal filament, escort cells, and cap cells, which directly associate with GSCs via adherens junctions (11, 30). In the WT gerarium, Piwi is known to be strongly stained in the nuclei of niche cells and GSCs, weakly stained in cytoplasmic and early mitotic cysts, and strongly stained in late mitotic and differentiating 16-cell cysts (3, 6). We observed Piwi<sup>Nt</sup> in cytoplasmic inclusions in niche cells (Fig. 2E), although its amount in the anterior part of gerarium was drastically lowered compared with germ-line cysts (Fig. 2D).

It has been reported that the depletion of the Zuc protein, a piRNA system component, caused Piwi disappearance from follicle cell nuclei and its accumulation in cytoplasmic Yb bodies (24, 25). We analyzed Piwi localization in the gerarium of zuc [HM27]/zuc[Del] mutant flies and observed a complete loss of Piwi from the nuclei of GSC niche cells (Fig. 2F, Middle and Right), whereas zuc mutants showed no GSC deficiency phenotype (Fig. 2F, Left) (31). This observation also argues for the ability of cytoplasmic Piwi to provide a signal for GSC maintenance.

**Piwi Nuclear Localization Is Indispensable for Transposon Silencing in Germ Cells.** We revealed drastic transposon derepression in the piwi<sup>Nt</sup> ovaries (Fig. 3A). To analyze the specificity of this effect on piRNA silencing, we compared it with that of the piwi<sup>2</sup>-null mutation and with aub, mael, and armi. To this end, we compared the expression pattern and localization of the proteins of the germ-line piRNA machinery (except the effect of spin-E on mael expression; Fig. 3A). Transcripts of the Gypsy and mdg1 retrotransposons increased 10- to 20-fold in the ovaries of homozygous piwi<sup>Nt</sup> and piwi<sup>2</sup> or transheterozygous piwi<sup>Nt</sup>/piwi<sup>2</sup> flies relative to the corresponding heterozygotes (Fig. 3A). In line with the current view (19, 24), these elements known to be expressed mainly in the somatic ovarian cells (18, 19, 37, 38) (Fig. S4A) showed no or minor up-regulation caused by aub, mael, and spin-E mutations affecting the proteins of the germ-line piRNA machinery. The effect of spin-E on mael expression (Fig. 3A) was not observed in the ovaries of piwi<sup>Nt</sup> and piwi<sup>2</sup> or transheterozygous piwi<sup>Nt</sup>/piwi<sup>2</sup> flies. This observation also argues for the ability of cytoplasmic Piwi to provide a signal for GSC maintenance.

**Loss of Nuclear Piwi Localization Does Not Affect Stem Cell Maintenance.** The null mutants piwi<sup>2</sup> have severely degenerate ovarioles with an extremely small amount of egg chambers because of the complete differentiation of GSCs with no renewal divisions (1, 2). By contrast, the ovarioles of homozygous piwi<sup>Nt</sup>, transheterozygous piwi<sup>Nt</sup>/piwi<sup>2</sup>, or piwi<sup>Nt</sup>/Df(2L)BSC145 females had a near-normal number of egg chambers (Fig. 1C), thus indicating the piwi<sup>Nt</sup> protein ability to maintain GSC self-renewal. piwi<sup>Nt</sup> homozygous females aged 1 to 5 d contained an average of 4.3 egg chambers per ovariole (n = 120), and piwi<sup>Nt</sup>/+ heterozygotes had 6.2 chambers (n = 150). The observed slight decrease of egg chamber number is characteristic of piRNA mutants (19, 24), these elements known to be expressed mainly in the somatic ovarian appendages and mislocalization of the posterior morphogen Oskar (Fig. S2 B and C). Only approximately 30% of piwi<sup>Nt</sup> oocytes (21 of 65) had correctly positioned Piwi and Osk in the oocyte pole plasm.

Whereas the adult ovarioles in the piwi<sup>Nt</sup>-null mutants contain the germaria depleted of germ-line cells (1, 2), the piwi<sup>Nt</sup> germaria carries developing germ-line cysts and a normal amount of GSCs (two or three per gerarium) as visualized by α-spectrin staining of spectrosomes, specific germ cell organelles at the sites of GSC contacts with cap cells (Fig. 1D). Undergoing divisions of germ cells were indicated by the fusome material at the cytoplasmic bridges connecting mother and daughter cells (Fig. 1D, Right).

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In the current view (19, 24), these elements known to be expressed mainly in the somatic ovarian cells (18, 19, 37, 38) (Fig. S4A) showed no or minor up-regulation caused by aub, mael, and spin-E mutations affecting the proteins of the germ-line piRNA machinery. The effect of spin-E on mael expression (Fig. 3A) was not observed in the ovaries of piwi<sup>Nt</sup> and piwi<sup>2</sup> or transheterozygous piwi<sup>Nt</sup>/piwi<sup>2</sup> flies. This observation also argues for the ability of cytoplasmic Piwi to provide a signal for GSC maintenance.
Gate retrotransposons (Fig. 3A) and caused a distribution of their transcripts in germ-line cells similar to that of piwiNt (Fig. 4 D–J). As a result of the derepression, the HMS-Beagle RNA accumulated mainly in the developing oocytes and nurse cells (Fig. 4 D–G and Fig. S4B), where it amassed in the form of cytoplasmatic clouds around the nuclei and as separate small dots within the nuclei (Fig. 4 E–G). The Gate transcripts predominantly accumulated in the nuclei of nurse cells (Fig. 4 H–J and Fig. S4C). These data indicate Piwi intranuclear activity contributing to transposon repression and also suggest that cytoplasmic piRNA pathway proteins (Aub, Mael, and Spn-E) may influence the Piwi-mediated silencing in the germ-line nuclei. What underlies different ability of transposon RNAs for nuclear export remains unclear. Most likely, it is determined by the degeneration of transposon RNA sequences involved in the interaction with the nuclear export machinery.

By Northern analysis, we detected germ line-specific piRNAs species which were absent in the piwiNt mutant (Fig. 3B), suggesting that piRNA biogenesis and its loading into Piwi complexes occur in the cytoplasm of germ-line cells. piRNA amount decreased compared with the heterozygotes (Fig. 3B), which can be attributed to a lower total amount of PiwiNt protein than in the WT (Fig. 1B and Fig. S1B).

Piwi Is Involved in Chromatin Silencing of Transposons in Ovaries. The role of Piwi in heterochromatin formation in somatic cells as well as the direct interaction of Piwi with chromatin and the HP1 protein have been reported (8, 39–44). However, it remained unknown whether Piwi-mediated intranuclear silencing of transposons in the germ line is based on chromatin regulation. Previously, we have shown that the snp-E mutation leads to the opening of retrotransposon chromatin structure in the ovaries (36). As Piwi is a single Drosophila piRNA-interacting protein with nuclear localization, it is the most probable effector of the putative piRNA-mediated chromatin silencing pathway. The close to normal phenotype of the piwiNt/piwiNt ovaries allowed us to carry out an adequate analysis of HP1 and histone mark occupancies in transposon sequences by ChIP in Piwi-depleted nuclei, which would be impossible in severely underdeveloped ovarian tissues of the piwi-null mutants. ChIP analysis of whole ovaries mainly reflects the chromatin state in the nurse cells, whereas follicle cell chromatin can also have its contribution, because a mature egg chamber contains 15 polyploid nurse cells with a DNA content of as much as 2,048C (45) and approximately 1,000 follicle cells, which undergo polyploidization up to 4C to 16C (46, 47). We found that the piwiNt mutation increased the active H3K4me2 mark but decreased repressive H3K9me3/me2 modifications and HP1 enrichment in the chromatin of telomeric HeT-A element (Fig. 5) compared with the heterozygotes. In the chromatin of the HMS-Beagle, mdg1, and copia nontelomeric retroelements, we observed a mutation-induced increase of the H3K4me2 mark, but in contrast to the telomeric elements, no significant changes in the abundance of H3K9me3/me3 or HP1 were detected (Fig. 5). The mutation had no effect on both analyzed histone marks and HP1 content in the chromatin related to two different regions of the Gate retrotransposon (Fig. 5), despite
the accumulation of its transcripts mainly in the nucleus (Fig. 4H). Overall, these data suggest that Piwi is involved in transposon repression by altering their chromatin structure, whereas some other Piwi-mediated nuclear mechanisms of silencing may occur related specifically to a transposon type.

**Discussion**

*Drosophila* Piwi protein is known to be implicated in GSC self-renewal (2, 6), as well as in piRNA-mediated repression of transposable elements (19–23). Here we describe the phenotype of a unique mutation in the *piwi* gene that leads to the formation of cytoplasmic PiwiNt (i.e., N-truncated Piwi protein) lacking the NLS. The properties of this mutant made the direct influence of the piRNA pathway on GSC maintenance unlikely, as the *piwi*Nt mutant displayed normal GSC self-renewal (Fig. 1C and D) but lost Piwi-mediated transposon repression completely in ovarian cells (Fig. 3A) including niche cells responsible for GSC self-renewal signaling (Fig. 4B). Thus, the Piwi regulatory function in GSC maintenance is distinct from the nuclear piRNA silencing mechanism.

The details of Piwi-mediated transposon silencing process as well as the interactions between Piwi and other piRNA machinery proteins remain poorly understood. We found that the *aub*, *spn-E*, and *mael* mutations, affecting cytoplasmic and nuage components of the piRNA system in germinal ovarian cells (19, 35, 48–50), lead to the nuclear accumulation of transcripts of *HMS-Beagle* and *Gate* transposons similarly to the effect of *piwi*Nt mutation (Fig. 4D–J and Fig. S4 B and C). Probably, nuage proteins, which are dispensable for Piwi nuclear import (19) (Fig. S5), may nevertheless ensure proper Piwi protein function in nuclear silencing. The reasons why Piwi nuclear localization is required for silencing of a large number of transposons in the germinal cells are not entirely clear. Possibly, particular Piwi-regulated transposons avoid the degradation by Aub/Ago-3 cytoplasmic piRNA machinery as a result of their masking by cytoplasmic proteins. Several reports have implicated Piwi in chromatin status maintenance (8, 39–43). We revealed that the loss of the nuclear Piwi in the *piwi*Nt ovaries enriches the chromatin context of telomeric transposons with H3K4me2 euchromatic marks and decreases the occupancy of heterochromatic marks such as H3K9me2/3 histones and HP1 protein (Fig. 5). These effects resemble those caused by the *spn-E* mutation (36). The HP1 level in *HeT-A* repeats was also shown to be decreased in the ovaries of *aub* and *armi* piRNA system mutants (51). The *piwi*Nt mutation also leads to enrichment of the *HMS-Beagle* and *mdg1* transposons in the *H3K4me2* modification (Fig. 5). These elements are highly up-regulated in the mutant ovaries (Fig. 3A), and a portion of their transcripts amassed as discrete dots within nuclei (Fig. 4C and E), suggesting their elevated transcription. However, no significant changes in the repressive H3K9me2/me3 marks and HP1 abundance were observed in the *HMS-Beagle* and *mdg1* chromatin (Fig. 5). The absence of a negative correlation between the H3K9me2/me3 and HP1 abundance were observed in the chromatin context of these elements due to the *piwi*Nt mutation might result from a preferential derepression of euchromatic transposon copies lacking the H3K9me2/me3 modifications. In the chromatin context of the *Gate* element, we detected no changes in active and repressive marks in the *piwi*Nt mutant (Fig. 5), although *Gate* transcription was also drastically up-regulated according to RT-PCR (Fig. 3A), and was shown to actively accumulate in the nuclei (Fig. 4H–J and Fig. S4C). These data suggest that Piwi may induce nuclear transposon silencing not only by chromatin-based repression but also by other mechanisms such as nuclear posttranscriptional RNA degradation. This suggestion is in concert with the observation that the nucleoplasm is enriched in the Piwi protein compared with DNA-containing areas of ovarian nuclei (3). Although the presence of RNA degradation complexes including decapping and exoribonuclease activities associated with the piRNA system in the cytoplasm of *Drosophila* germ-line cells has been established (50), the partners of Piwi in chromatin repression and intranuclear transposon transcript elimination remain to be elucidated.

The principal result of this study is that the cytoplasmic, but not nuclear, Piwi protein is required for GSC maintenance. It is known that GSC self-renewal requires Piwi expression only in somatic niche cells (2, 6), and *piwi* overexpression in these cells...
Fig. 4. Localization of mdg1 (A–C), HMS-Beagle (D–G), and Gate (H–J) transposon transcripts (green) by RNA in situ hybridization in the piwi\textsuperscript{\textit{Nt}} ovaries. In WT and heterozygotes, no signal was observed with the same microscope settings. (A and B) mdg1 is expressed predominantly in follicle cells (FC) and nicherotic cells (SCN). (C) Dot-like mdg1 RNA accumulation in the nuclei of follicle cells (indicated by arrows) stained with DAPI (blue). (D–G) HMS-Beagle retrotransposon transcripts accumulate in the developing oocytes (DO) and in nurse cells in the ovaries of piwi\textsuperscript{\textit{Nt}} (D and E), spn-E (F), and aub (G) mutants. In nurse cells, the transcripts amassed as cytoplasmic clouds and distinct nuclear dots (d). (E) Fragment of the egg chamber of the piwi\textsuperscript{\textit{Nt}} mutant stained for HMS-Beagle RNA (Upper) and for Lamin and DAPI (Lower). (H–J) Gate retrotransposon transcripts are observed mainly in the nuclei of nurse cells.

increases the number of GSC-like cells (3). Recent studies have revealed a possibility of Piwi transient localization within cytoplasmic Yb bodies in somatic ovarian cells (24, 25). Presumably, the Yb body mediates piRNA biogenesis and loading into Piwi complexes, which is a prerequisite for Piwi nuclear import (24, 25). Yb bodies are also present in niche cells (22), and their key component, the Yb protein, is indispensable for GSC maintenance (26, 52–54). Defects of GSC self-renewal observed in the Yb mutant ovaries lacking Yb bodies are very similar to those in piwi mutants (26, 52–54). Piwi protein is uniformly distributed throughout the cytoplasm of Yb-deficient somatic cells (24, 26). Thus, it is likely that a signal for GSC maintenance may be produced by the Piwi-Yb complex compartmentalized in Yb bodies of niche cells. This suggestion is in line with the observed granular localization of the Piwi\textsuperscript{\textit{Nt}} protein in the niche cytoplasm (Fig. 2E). Although the amount of the Piwi\textsuperscript{\textit{Nt}} protein in niche cells is drastically lower compared with the WT Piwi level (Fig. 2 D and E), it is sufficient to maintain GSCs. Similarly, only a tiny portion of the Piwi protein in WT ovarian cells seems to be located in cytoplasmic Yb bodies, where it was detected only by coimmunoprecipitation experiments (24, 25, 55). An additional argument for the role of cytoplasmic Piwi in GSC maintenance is provided by the phenotype of the zuc mutants, which show no evidence of stem cell renewal disturbance (31) (Fig. 2F), whereas Piwi is absent from the nuclei of niche cells and accumulates in Yb bodies (Fig. 2F). The loading of piRNAs into Piwi complexes was shown to be prevented in somatic cells lacking Zuc (24, 25, 55). In light of our results, this observation argues for the piRNA-independent mechanism of Piwi function in GSC maintenance, although we cannot be certain that short RNAs are completely absent from niche cells of zuc mutants. Genetic screens revealed that a mutation in the corto gene, which encodes a chromodomain protein, restored GSC division in both piwi and Yb mutants (10). It can be suggested that a putative GSC signal produced by niche cells may be regulated both in the nucleus by Corto and in the cytoplasm by Piwi-Yb.

Taking into account the evolutionarily conserved role of Piwi orthologs in GSC maintenance, further studies of the molecular mechanism of Piwi functions in the signaling pathways unrelated to piRNA silencing appear to be very intriguing.

Materials and Methods

Drosophila Strains. Flies were maintained at 25 °C on standard medium. The ovaries from 1- to 6-d-old flies were dissected. Oregon-R and y\textsuperscript{w}\textsuperscript{1}/y\textsuperscript{w}\textsuperscript{2} strains were also used to check piwi\textsuperscript{Nt} phenotype. Df(2L)BSC145 and Df(2L)BSC213 deletions in the 32C1;32C5 region containing the piwi gene

Fig. 5. ChIP analysis of transposon chromatin status. The bars indicate quantitative PCR-measured occupancies of HP1 and the modified histones in piwi\textsuperscript{\textit{Nt}}/piwi\textsuperscript{\textit{Nt}} transheterozygotes (dark) and piwi\textsuperscript{\textit{Nt}}/+ heterozygotes (light) normalized to the corresponding values for the intergenic 60D cluster (mean ± SD; n = 3). The ribosomal rp49 and heterochromatic light genes were used as examples of actively transcribed and repressed chromatin, respectively.
were obtained from the Drosophila stock center (Bloomington, IN). Other mutant fly stocks used in this work are indicated in SI Materials and Methods.

Molecular Characterization of piwi<sup>MM</sup> Mutation, RT-PCR Analysis, ChIP, Immunohistochemistry, and Western Blot. Methods for molecular characterization of piwi<sup>MM</sup> mutation, quantitative RT-PCR analysis, ChIP, immunohistochemistry, and Western blotting are provided in SI Materials and Methods.

RNA in Situ Hybridization. RNA in situ hybridization using DIG-labeled strand-specific riboprobes was performed basically as previously described (32). For the synthesis of the mdg1 riboprobe by T7 in vitro transcription, the plasmid containing the cloned PCR fragment of mdg1 retrotransposon (15) was used. PCR products carrying T7 promoters were used for T7 transcription of riboprobes to detect transcripts of HMS-Beagle and Gate transposons as well as piwi. Additional details can be found in SI Materials and Methods.

Short RNA Detection. Detection of short RNA was performed by Northern blot essentially as described previously (36). Additional details can be found in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Alexey Aravin, Gregory Hannon, Haruhiko Sioni, and Mikiko Sioni for providing antibodies; Alla Kalmykova for the mdg1-containing plasmid; and Trudi Schupbach for zebrafish flies. This work was supported by the Molecular and Cell Biology Program of the Russian Academy of Sciences, Grants MK-65160.2010.4 from the President of the Russian Federation, and Russian Foundation for Basic Research Grants 10-04-01812-a and 11-04-12027.