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*^{Nt}, removing the nuclear localization signal of the Piwi protein. *piwi*^{Nt} 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*^{Nt} mutants are sterile most likely because of the disturbance of piRNA-mediated transposon silencing. Analysis of chromatin modifications in the *piwi*^{Nt} ovaries indicated that Piwi causes chromatin silencing only of certain types of transposons, whereas others are repressed in the nuclei 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.

oogenesis | short RNA

Drosophila Piwi (P-element induced wimpy testes) protein was first described as a factor required for germ-line stem cell (GSC) maintenance and normal development of the testes and ovaries (1–3). The same function was also demonstrated for Piwi orthologues in the germ lines of other organisms (2, 4). In *Drosophila*, the ovaries are composed of ovarioles consisting of a germarium and a string of egg chambers at consecutive developmental stages. Oogenesis starts in the anterior germarium containing two or three GSCs in close contact with somatic cap cells, the components of their niche. After stem cell division, the daughter cell adjacent to the cap cell remains within the niche as a stem cell, whereas the other daughter cell initiates differentiation into a cystoblast and eventually an egg (5). The Piwi protein is localized in the nuclei of somatic and germinal ovarian cells, but the production of signals to maintain GSC renewal requires Piwi expression only in the somatic niche (2, 3, 6). As a result of immediate GSC differentiation into cystoblasts, *piwi* mutant females usually contain germ line-less germaria and no more than two or three egg chambers (1, 2). Although several suppressors of *piwi* mutations restoring GSC maintenance were identified (7–10), the key niche signal regulated by *piwi* remains unknown (reviewed in refs. 11, 12). It was also shown that the intrinsic expression of Piwi in GSCs promotes their mitotic divisions (3, 6). Another role of Piwi in germ-line development is related to the formation of maternally inherited pole plasm (13). Finally, *piwi* mutations lead to transposable element overexpression and cause a transposition burst as a result of the loss of Piwi-interacting RNA (piRNA) silencing (14–18). Piwi is the founding member of the evolutionarily conserved piRNA-binding Piwi protein subfamily, which also includes Aub and Ago3 proteins in *Drosophila* (18). piRNAs are produced by the primary processing of single-stranded transcripts of heterochromatic master loci or by ping-pong amplification (19–21). Whereas germ cell-specific Aub and Ago-3 proteins are actively involved in the ping-pong cycle, the Piwi protein is mainly loaded with primarily processed piRNAs and represses transposons in germinal and somatic ovarian cells (18, 19, 22). Piwi is a predominantly nuclear protein, whereas most other piRNA machinery proteins are localized in the cytoplasm, particularly in the electron-dense perinuclear nuage organelle of germinal cells (23) and Yb bodies of ovarian somatic cells (24–26).

It has remained unknown whether Piwi functions in GSC self-renewal and piRNA-mediated silencing of transposable elements are interrelated. It has been suggested that a cessation of piRNA function can affect stem cell maintenance (8). Here we show that a mutant cytoplasmic Piwi is capable of supporting GSC self-renewal but loses the ability to repress transposable elements, leading to female sterility. We also show that Piwi-mediated silencing takes place within the nuclei of germinal cells and involves chromatin modification.

Results

Identification of *piwi*^{Nt} Mutation. While characterizing a female sterile mutation, hereafter *piwi*^{Nt} (i.e., *N*-truncated), on chromosome 2, we detected sterile transheterozygotes carrying the *piwi*^{Nt} 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*^{Nt} with *piwi*^{2} or *piwi*^{3} 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. S1*A*). 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. S1*A*). 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. S1*A*). The rest of the *piwi* gene encoding the PAZ and Piwi domains responsible for short RNA binding and target RNA slicing remained unchanged (Fig. 1*A*). Antibodies against the extreme N-terminal part of Piwi did not recognize the mutant

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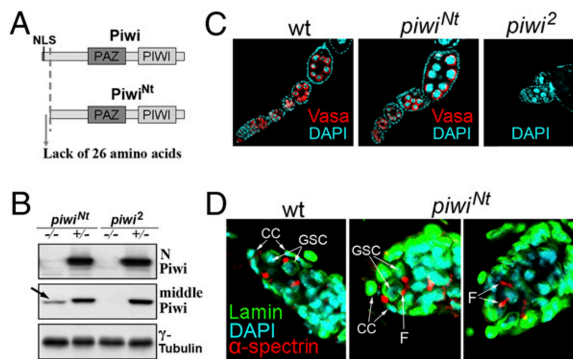

Fig. 1. Flies carrying the *piwi*^{Nt} mutation that leads to the formation of *N*-truncated Piwi protein have a normal number of egg chambers and germinal stem cells. (*A*) Truncated Piwi^{Nt} protein lacks 26 N-terminal amino acids including the NLS. (*B*) Antibodies against the middle Piwi region, but not against the N-terminal part (*Upper*), detect the shortened Piwi (arrow). (*C*) WT, *piwi*^{Nt}, and *piwi*^{2} (null mutant) ovarioles stained for Vasa (red), a marker of germinal cells, and DAPI (blue). (*D*) Staining of GSCs in the WT (*Left*) and *piwi*^{Nt} (*Middle* and *Right*) germaria by lamin (green) and α-spectrin (red) antibodies marking spectrosomes at the site of their contact with somatic cap cells (CCs); germ cell undergoing division can be observed by an elongated fusome material ("F").

protein, whereas those against Piwi C-terminal or middle regions showed the presence of the shortened Piwi protein (Fig. 1*B* and Fig. S1*B*). Both shortened polypeptide and transcript were not detected in the WT ovaries by Western analysis and 5′-RACE, respectively, and the corresponding sequences were not found in the available databases, indicating that Piwi^{Nt} represents a mutant protein but not an isoform encoded by the *piwi* gene.

Loss of Nuclear Piwi Localization Does Not Affect Stem Cell Maintenance. The null *piwi* mutants 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 ovaries of homozygous *piwi*^{Nt}, transheterozygous *piwi*^{Nt}/*piwi*^{2}, or *piwi*^{Nt}/*Df(2L)BSC145* females had a near-normal number of egg chambers in the ovarioles (Fig. 1*C*), thus indicating the *piwi*^{Nt} ability to maintain GSC self-renewal. *piwi*^{Nt} homozygous females aged 1 to 5 d contained an average of 4.3 egg chambers per ovariole ($n = 120$), and *piwi*^{Nt}/+ heterozygotes had 6.2 chambers ($n = 150$). The observed slight decrease of egg chamber number is characteristic of piRNA system mutants, which can be explained by a delay in GSC/cystoblast mitotic divisions (28) but not by GSC direct differentiation into cystoblasts. Oogenesis proceeds completely in *piwi*^{Nt} mutants and oocytes are correctly positioned in most *piwi*^{Nt} egg chambers, although some ovarioles (2%) have an abnormal phenotype reflected by characteristics such as fused egg chambers (Fig. S2*A*). We also observed oocyte axis specification defects typical for piRNA mutants (23), such as abnormal dorsal appendages and mislocalization of the posterior morphogen Oskar (Fig. S2 *B* and *C*). Only approximately 30% of *piwi*^{Nt} oocytes (21 of 65) had correctly positioned Piwi and Osk in the oocyte pole plasm.

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

Next we tested the expression pattern and localization of the mutant Piwi^{Nt} 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*^{Nt} 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^{Nt} protein was detected mainly in the cytoplasm of nurse cells and around their nuclei, similarly to nuage components, e.g., Aub (Fig. 2*B*). Mutant protein was found in the cytoplasm of follicle somatic cells (Fig. 2*C*) 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 germarium (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 germarium, Piwi is known to be strongly stained in the nuclei of niche cells and GSCs, weakly stained in cystoblasts and early mitotic cysts, and strongly stained in late mitotic and differentiating 16-cell cysts (3, 6). We observed Piwi^{Nt} in cytoplasmic inclusions in niche cells (Fig. 2*E*), although its amount in the anterior part of germarium was drastically lowered compared with germ-line cysts (Fig. 2*D*).

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 germaria of *zuc* [*HM27*]/*zuc*[*Del*] mutant flies and observed a complete loss of Piwi from the nuclei of GSC niche cells (Fig. 2*F*, *Middle* and *Right*), whereas *zuc* mutants showed no GSC deficiency phenotype (Fig. 2*F*, *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*^{Nt} ovaries (Fig. 3*A*). To analyze the specificity of this effect on piRNA silencing, we compared it with that of the *piwi*^{2}-null mutation and with *aub*, *mael*, *spn-E*, and *armi* mutations affecting the cytoplasmic components of the piRNA machinery, some of which have been shown to be responsible for the activation of a number of transposons tested in our study (16, 32–38) (Fig. 3*A*). Transcript level of the *Gypsy* and *mdg1* retrotransposons increased 10- to 20-fold in the ovaries of homozygous *piwi*^{Nt} and *piwi*^{2} or transheterozygous *piwi*^{2}/*piwi*^{Nt} flies relative to the corresponding heterozygotes (Fig. 3*A*). 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. S4*A*) showed no or minor up-regulation caused by *aub*, *mael*, and *spn-E* mutations affecting the proteins of the germ-line piRNA machinery (except the effect of *spn-E* on *mdg1* expression; Fig. 3*A*). We found *mdg1* transcripts localized largely in the nuclei of somatic cells including GSC niche cells in the germaria of the *piwi*^{Nt} ovaries (Fig. 4 *A*–*C*). The quantitative effects on the germ line expressed transposons observed in *piwi*^{Nt} homozygotes and *piwi*^{2}/*piwi*^{Nt} transheterozygotes were more similar to the effects of *piwi*^{2} than to those of the *aub*, *mael*, *spn-E*, and *armi* mutations (Fig. 3*A*). Hence, *piwi*^{Nt} seems to abolish the silencing function of the Piwi protein completely and to be peculiar to the *piwi* loss of function mutation. Indeed, *I* and *G* elements, whose complementary piRNAs were preferentially found in the Aub and Ago3 complexes than in Piwi ones (18), showed a weak up-regulation both in *piwi*^{2} and *piwi*^{Nt} mutants and a stronger up-regulation in *aub*, *mael*, *spn-E*, and *armi* mutants (Fig. 3*A*). At the same time, the *aub*, *mael*, and *spn-E* mutations exhibited similar to *piwi*^{Nt} and *piwi*^{2} quantitative effects on the derepression of *HMS-Beagle* and

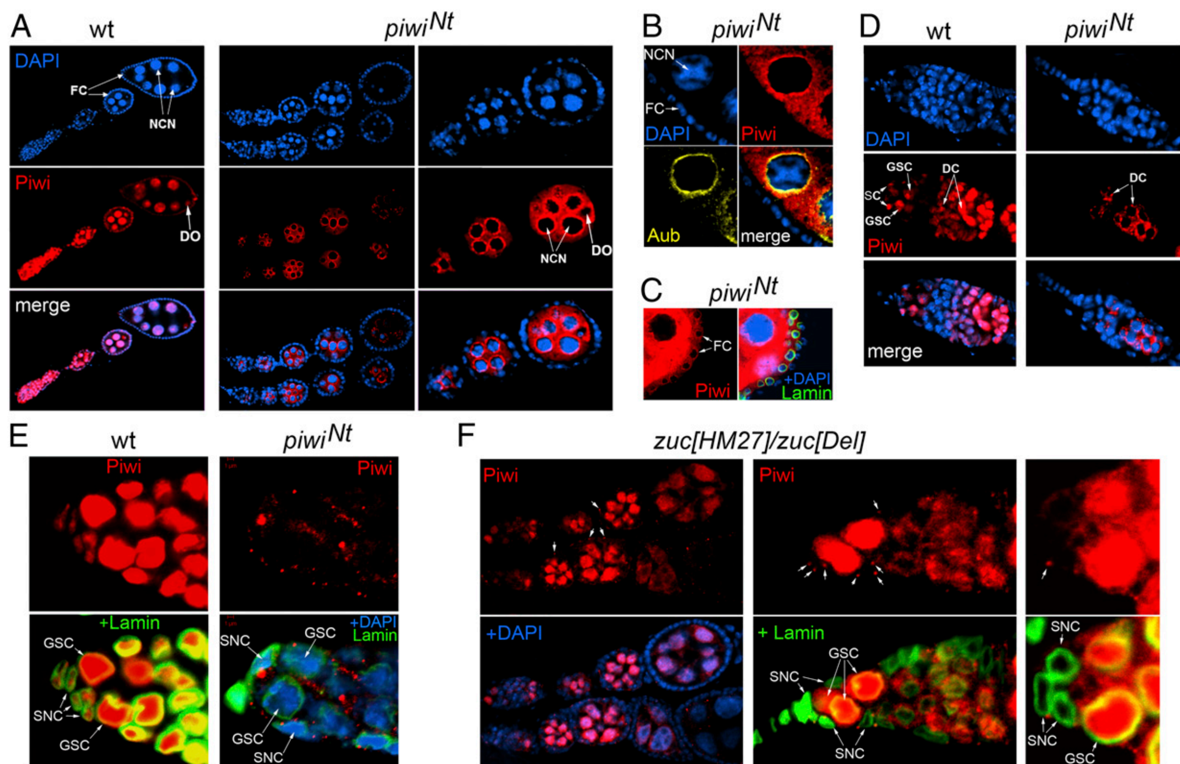

Fig. 2. Localization of the Piwi protein in the ovaries of *piwi*^{Nt} and *zuc* mutants. (*A*) Piwi (red) and DAPI (blue) staining of the WT (*Left*) and *piwi*^{Nt} (*Middle* and *Right*) ovarioles. Piwi protein is known to be localized in the nuclei of nurse cells (NCN), developing oocytes (DO), and somatic follicle cells (FC) enveloping egg chambers. In the *piwi*^{Nt} mutant, Piwi nuclear localization is lost completely. The mutant Piwi protein is mainly localized in germinal cells and is undetectable in somatic cells by the microscope setup. (*B*) Fragment of *piwi*^{Nt} egg chamber demonstrating partial colocalization of cytoplasmic Piwi (red) and Aub protein (yellow) around the nurse cell nucleus. (*C*) Piwi^{Nt} protein can be observed in the cytoplasm of follicle somatic cells by using the microscope setup for enhanced detection. (*D*) Piwi staining of the WT (*Left*) and *piwi*^{Nt} (*Right*) germaria. SC, somatic cells; DC, differentiating cysts. Piwi mutant protein is detected basically in DC. (*E*) Piwi^{Nt} mutant protein localization in the anterior part of the germarium with the GSC niche. In the somatic niche cells (SNC), the Piwi^{Nt} protein accumulates in cytoplasmic granules. (*F*) *Left*: Normal number of egg chambers in *zuc*[*HM27*]/*zuc*[*Del*] ovarioles. Piwi accumulates in follicle cell cytoplasm (arrows). *Middle* and *Right*: Piwi is lost from the nuclei of *zuc* mutant niche cells (SNC) and accumulates in cytoplasmic granules (arrows).

Gate retrotransposons (Fig. 3*A*) and caused a distribution of their transcripts in germ-line cells similar to that of *piwi*^{Nt} (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. S4*B*), where it amassed in the form of cytoplasmic 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. S4*C*). 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 process 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 *piwi*^{2} mutant (Fig. 3*B*), 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. 3*B*), which can be attributed to a lower total amount of Piwi^{Nt} protein than in the WT (Fig. 1*B* and Fig. S1*B*).

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 *spn-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 *piwi*^{Nt}/*piwi*^{2} 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 *piwi*^{Nt} 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 H3K9me2/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

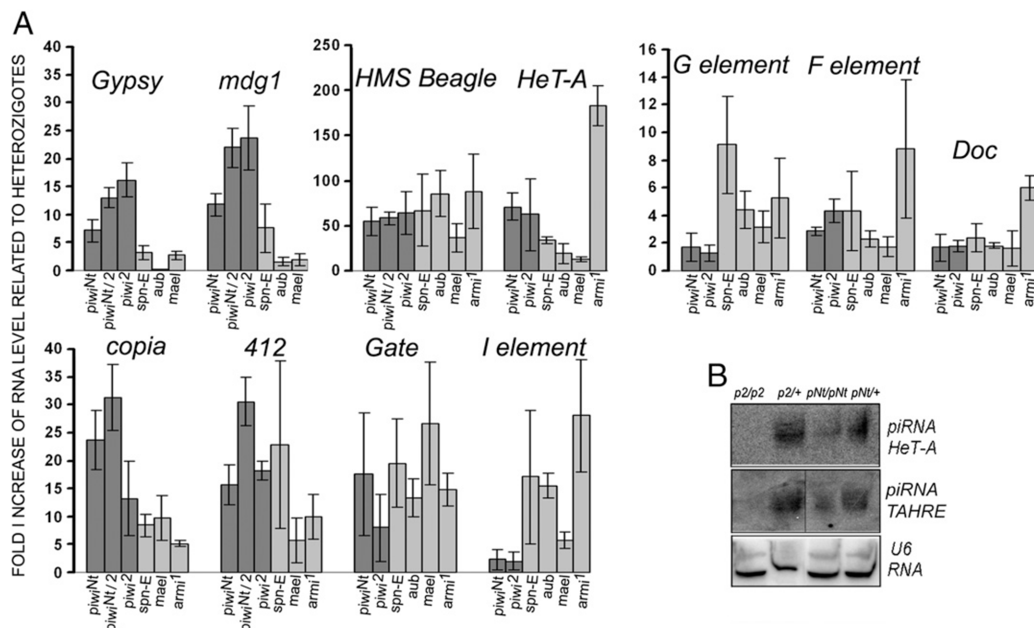

Fig. 3. The *piwi*^{Nt} mutation leads to transposon desilencing. (*A*) Quantitative RT-PCR evaluation of the fold accumulation of transposon transcripts in the homozygous *piwi*^{Nt} and *piwi*^{2} or transheterozygous *piwi*^{Nt}/*piwi*^{2} ovaries relative to heterozygotes (mean ± SD; $n = 3$). (*B*) Northern blot detection of piRNAs complementary to the germ line expressed *HeT-A* and *TAHRE* retrotransposons in ovarian RNA of *piwi*^{2} (p2) and *piwi*^{Nt} (pNt) mutants and the corresponding heterozygotes, normalized to *U6* RNA.

the accumulation of its transcripts mainly in the nucleus (Fig. 4*H*). 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 (19–21, 23). Here we describe the phenotype of a unique mutation in the *piwi* gene that leads to the formation of cytoplasmic Piwi^{Nt} (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. 1 *C* and *D*) but lost Piwi-mediated transposon repression completely in ovarian cells (Fig. 3*A*) including niche cells responsible for GSC self-renewal signaling (Fig. 4*B*). 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* mutations (Fig. 4 *D*–*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. 3*A*), and a portion of their transcripts amassed as discrete dots within nuclei (Fig. 4 *C* 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 H3K4me2 modifications 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. 3*A*), and was shown to actively accumulate in the nuclei (Fig. 4 *H*–*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

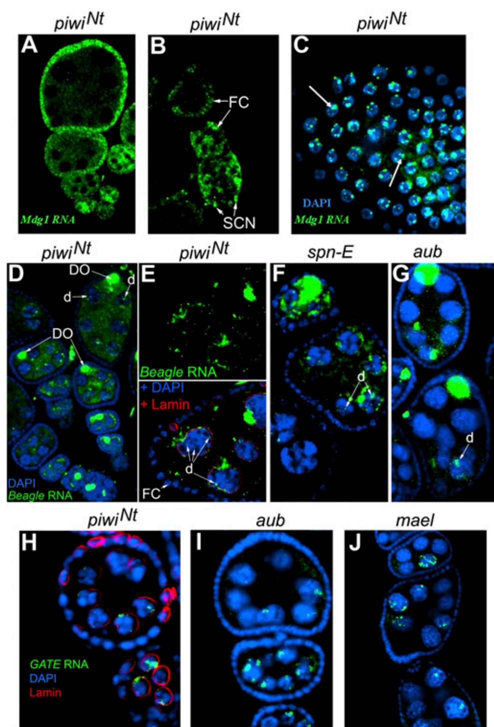

Fig. 4. Localization of *mdg1* (*A–C*), *HMS-Beagle* (*D–G*), and *Gate* (*H–J*) transposon transcripts (green) by RNA in situ hybridization in the $piwi^{Nt}$ ovaries. In WT and heterozygotes, no signal was observed with the same microscope settings. (*A* and *B*) *mdg1* is expressed predominantly in follicle cells (FC) and niche somatic 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 $piwi^{Nt}$ (*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 $piwi^{Nt}$ 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 (52), 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 $Piwi^{Nt}$ protein in the niche cell cytoplasm (Fig. 2*E*). Although the amount of the $Piwi^{Nt}$ 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. 2*F*), whereas Piwi is absent from the nuclei of niche cells and accumulates in Yb bodies (Fig. 2*F*). 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^1 w^{67c23} strains were used as a WT control. The $piwi^{Nt}$ cn^1 bw^1 chromosome was kept over the *CyO* balancer. Homozygous $piwi^{Nt}$ flies were rare and transheterozygous $piwi^{Nt}/piwi^2$ flies were also used to check $piwi^{Nt}$ phenotype. *Df(2L)BSC145* and *Df(2L)BSC213* deletions in the 32C1;32C5 region containing the *piwi* gene

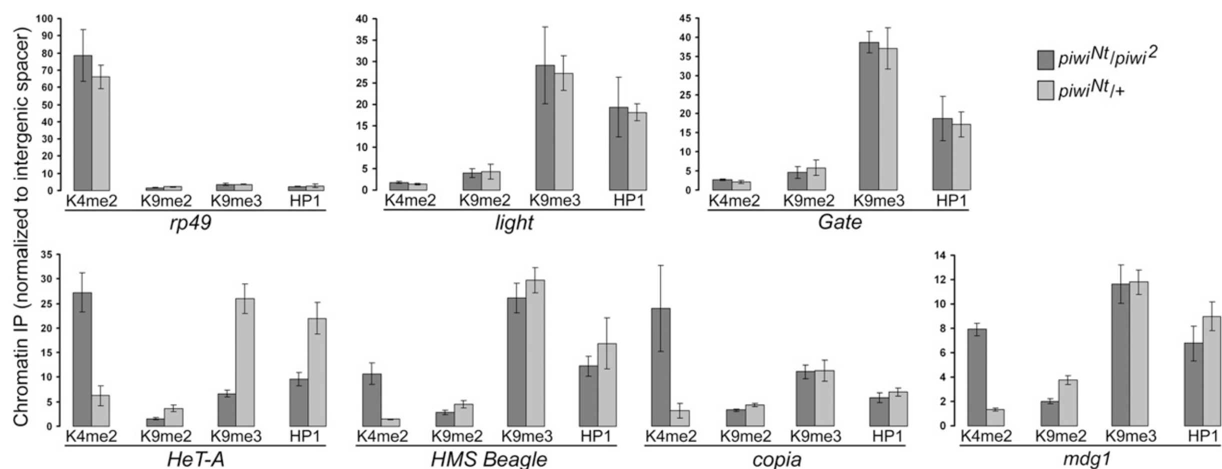

Fig. 5. ChIP analysis of transposon chromatin status. The bars indicate quantitative PCR-measured occupancies of HP1 and the modified histones in $piwi^{Nt}/piwi^2$ transheterozygotes (dark) and $piwi^{Nt}$/+ heterozygotes (light) normalized to the corresponding values for the intergenic 60D cluster (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*^{Nt} Mutation, RT-PCR Analysis, ChIP, Immunohistochemistry, and Western Blot. Methods for molecular characterization of *piwi*^{Nt} 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 (56). Additional details can be found in *SI Materials and Methods*.

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