Key role of piRNAs in telomeric chromatin maintenance and telomere nuclear positioning in *Drosophila* germline

Running title: Telomeric piRNA clusters

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Abstract

Background: Telomeric small RNAs related to PIWI-interacting RNAs (piRNAs) were discovered in different eukaryotes, however, their role in germline-specific telomere function remains poorly understood. Using a *Drosophila* model, we performed an in-depth study of the biogenesis of telomeric piRNAs and their function in telomere homeostasis in the germline.

Results: Telomeric transgenes were used to monitor the piRNA production and chromatin structure of particular telomeric loci. Small RNA-seq data has shown that telomeric arrays belong to a class of dual-strand piRNA clusters, but the ability to produce piRNAs varies along telomeric transposon arrays. Rhino, a germline-specific homolog of heterochromatic protein 1 (HP1), associates with all telomeric transgenes and its level correlates with the abundance of transgenic piRNAs. In contrast to conventional dual-strand piRNA clusters, piRNA loss causes sharp decrease of HP1, Rhino and trimethylated histone H3 lysine 9 levels at telomeric regions, Thus, the presence of piRNAs are required for telomere chromatin maintenance in the germline. Moreover, piRNA loss causes telomere translocation from the nuclear periphery towards the nuclear interior but does not affect telomere end capping or clustering. Chromatin analysis of telomere-associated sequences (TAS) revealed strong tissue specificity of silencing mechanisms exerted by subtelomeric regions.

Conclusions: piRNAs provide a germline-specific mechanism of telomeric chromatin assembly. Telomeric arrays belong to a unique type of self-targeting dual-strand piRNA clusters whose transcripts serve as piRNA precursors and their only targets at the same time. Telomeric chromatin is highly sensitive to piRNA loss suggesting a primary role of *Drosophila* telomeres in the response to the disruption of genome integrity.

Key words: telomere, germline, retrotransposon, transgene, piRNA cluster, HP1, Rhino, chromatin, transcription, Drosophila

Background

Telomere transcription is an evolutionary conserved feature of eukaryotic telomeres [1]. The biogenesis of telomeric transcripts has been shown to be tightly connected with telomere length control and telomeric chromatin formation. Telomeric transcripts serve as precursors of small RNAs (tel-sRNAs) involved in the epigenetic regulation of telomeric heterochromatin.

Tel-sRNAs were discovered in mammalian embryonic stem cells, in the ciliate *Tetrahymena thermophila*, in plants and *Diptera* indicating that their generation is a widely conserved feature [2-5]. Small RNAs generated by subtelomeric regions in fission yeast as well as some tel-sRNAs have been implicated in the assembly of telomeric heterochromatin [2, 4, 6, 7]. Plant and mammalian tel-sRNAs are related to the class of Piwi-interacting RNAs (piRNAs) generated in germline and in stem cells [8, 9]. However, the role of tel-sRNAs in germline-specific telomere function is not understood. Telomeric piRNAs and their role in telomere length control was first described in *Drosophila melanogaster* [3]. Using a *Drosophila* model, we performed in-depth study of the biogenesis and function of telomeric piRNAs in the germline.

The piRNA-mediated pathway provides silencing of transposable elements (TE) in the germline [9, 10]. In contrast to small interfering RNAs (siRNA), which are processed by the Dicer endonuclease from double strand RNA, piRNAs are generated from long single strand precursor transcripts. piRNA precursors are encoded by distinct genomic regions enriched by damaged TE copies, termed piRNA clusters [11]. Dual-strand piRNA clusters found in the *Drosophila* germline produce piRNAs from precursors transcribed by both genomic strands. Dual-strand piRNA clusters can be classified into several variants such as extended pericentromeric TE-enriched regions [11], individual TE-associated euchromatic clusters [12] and transgene-associated clusters [13, 14]. In all these instances, piRNAs and their targets are expressed by distinct genomic regions.

Pericentromeric piRNA clusters produce piRNAs that target nascent transcripts of active TEs resulting in posttranscriptional silencing as well as heterochromatin protein 1 (HP1)-mediated transcriptional silencing [15-18]. In the perinuclear compartment, piRNA-mediated cleavage of TE transcripts initiates further processing of cleavage products to increase piRNA abundance and diversity [11, 19-21]. Distinct chromatin components of the piRNA clusters that couple transcription and RNA transport appear to direct cluster-derived transcripts into piRNA processing machinery [22-24]. The germline-specific homolog of HP1, Rhino (Rhi), is essential for piRNA production from dual-strand piRNA clusters [25-27]. piRNAs are required at early embryonic stages for deposition of Rhi and Histone 3 lysine 9 trimethylation mark (H3K9me3) at dual-strand piRNA clusters but at later developmental stages the chromatin of piRNA clusters is maintained by an unknown Piwi-independent mechanism [28].

Drosophila telomeres appear to constitute a separate class of the piRNA clusters; with abundant piRNAs corresponding to telomeric and subtelomeric sequences being present in *Drosophila* ovarian small RNA libraries. The telomeres of *D. melanogaster* are maintained in the absence of telomerase by the transpositions of the specialized telomeric retrotransposons *HeT-A*, *TART* and *TAHRE* organized in tandem head-to-tail arrays; with *HeT-A* being the prevailing

telomeric retrotransposon [29-31]. Telomere associated sequences (TAS) consist of complex satellite-like repeats and are located proximally to retrotransposon arrays. Telomeric transcripts are processed into piRNAs that regulate telomeric TE expression and their transposition rate onto chromosome ends in the germline [3, 32].

Thus, telomeres need to produce both intact TE mRNA, required for the normal rate of telomere elongation, as well as piRNAs that target these TE mRNA, in order to regulate optimal telomere length. Indeed, violation of this balance can lead to disruption of telomere length control [3, 33]. Different factors including piRNA pathway components act cooperatively to regulate telomeric repeat expression and telomere protection in the germline, providing genome integrity during early development [34, 35]. It is clear, that involvement of telomeres in piRNA production should considerably affect telomere biology in the germline, however, telomeres are not yet characterized as piRNA clusters.

Analysis of ovarian small RNA-seq data revealed abundant piRNAs corresponding to both genomic strands of telomeric retrotransposons and TAS [11], thus telomeric piRNA clusters can be formally related to the dual-strand type. However, the main distinction of telomeric piRNA clusters is that their transcripts serve both as a source of piRNAs and as their only targets at the same time. While the genome-wide data argued against the existence of defined promoters at most dual-strand clusters [26], telomeric retroelements are characterized by the presence of bidirectional promoters that provide transcription of the piRNA precursors [36].

Experimental evidence indicates that *HeT-A* and related *TAHRE* elements are extremely sensitive to piRNA pathway disruption showing up to 1,000-fold hyper-expression in contrast to *TART*, which demonstrates modest upregulation [3, 32, 37, 38]. Therefore, *HeT-A* has been extensively used as an indicator of TE dysregulation, but not of piRNA cluster expression, upon piRNA pathway disruption. In contrast, expression of the germline piRNA clusters was diminished upon disruption of Rhi and Piwi depletion [16, 25, 26] indicating a fundamental difference between telomeric and conventional piRNA clusters. Accordingly, the organization of telomeric piRNA clusters remains unclear: Are they represented by discrete clusters or by continuous piRNA-producing region? How do specialized telomere components coexist with chromatin factors that associate with piRNA clusters in the germline?

Unique mapping of small RNA reads is the major source of information about the genomic origin of piRNAs. Endogenous telomeres consisting of repetitive sequences are subjected to recombination and healing by retro-transpositions that leads to high sequence variability in telomeres. Thus, the unique strain-specific mapping of small RNA reads to telomeres is technically challenging. Artificial sequences inserted into endogenous piRNA clusters serve as unique marks that allow for the exploration of highly repetitive genomic loci. Furthermore, it is

believed that TE insertions into native piRNA clusters provide adaptation to transposon invasion [39]. According to this principle, any heterologous sequences including transgenes being located within native piRNA clusters acquire its ability to produce piRNAs and silence complementary targets [40-42]. Such integration is possible because of enhanced read-through transcription in the piRNA clusters that is realized through the suppression of RNA polymerase II termination [22]. Long piRNA precursors comprising of alien sequences would be targeted by abundant endogenous piRNAs with subsequent 3'-directed endonuclease Zucchini-dependent piRNA production [20, 21] resulting in generation of novel piRNAs. Indeed, the construct P{lArB}when integrated into a subtelomeric piRNA cluster on the X-chromosome produces abundant piRNAs that suppress the expression of other *lacZ* transgenes in germ cells [40, 42].

Transgenic *Drosophila* strains carrying *P*-element copies in terminal retrotransposon array have been identified and characterized [43]. In contrast to TAS that exert polycomb group (PcG) protein-mediated silencing of transgenes inserted in these regions [44-46], telomeric retrotransposon arrays show euchromatic characteristics and do not silence transgene reporters in somatic tissues; which, in fact allowed for the selection of such transgenic strains [43]. JIL-1 kinase was shown to protect the telomeric retrotransposon *HeT-A* promoters from the spreading of repressive chromatin from the TAS providing positive regulation of telomeric retrotransposon expression [46, 47]. Therefore, based on their different ability to silence integrated transgenes, two telomeric subdomains were defined within the *Drosophila* telomere in somatic tissues; transcriptionally active retrotransposon arrays and heterochromatic TAS [43, 44].

Taking the advantage of telomere transgene model in combination with experiments on endogenous telomeric elements, we investigated piRNA production and chromatin structure of the different telomeric loci in the ovaries of transgenic flies. We have shown that production of telomere-specific piRNAs contributes significantly to the chromatin structure and expression of studied telomeric regions in the germline. In contrast to somatic tissues, TAS and *HeT-A—TART—TAHRE* arrays show similar chromatin structure and transcriptional status in the germline and can be related to one piRNA-producing domain. At the same time, we found that piRNA production is not similar between transgenes integrated in different telomeric retrotransposons. Chromatin and cytological studies provide strong evidence that telomeric piRNA clusters are highly sensitive to piRNA loss in contrast to conventional dual-strand piRNA clusters. These data, in combination with previously observed discrepancy of telomeric and other dual-strand piRNA cluster response to the piRNA pathway disruption [16, 28, 48], suggest that a distinct type of piRNA clusters protects telomere integrity in *Drosophila* germline.

Results

Transgenes located at different positions in telomeres produce small RNAs in *Drosophila* ovaries

It is well known that transgenes inserted within TAS produce abundant piRNAs and exert piRNA-mediated silencing of complementary targets [40, 42, 49, 50]. However, the piRNA production ability of transgenes located within telomeric retrotransposon arrays has not been explored. In this study, we used four available transgenic EY strains on a y^1w^{67c23} strain background carrying the P{EPgy2} construct in telomeric retrotransposon arrays [43]. P{EPgy2} is a *P*-element-based vector containing *mini-white* and *yellow* genes. The transgene EY08176 was inserted into the GAG ORF of *HeT-A*-related *TAHRE* at 2R chromosome. The transgenes EY00453 and EY00802 were integrated into the 3' UTR of *TART-B1* at 3L while EY09966 was inserted into *TART-C* at 4th chromosome. Insertion locations in telomeric retrotransposons are shown schematically in Fig. 1a. DNA FISH on polytene chromosomes isolated from salivary glands confirmed the telomeric localization of transgenes (Additional file 1: Figure S1). All *TART* insertions were in promoter region located between sense and antisense transcription start sites [51] reflecting the known specificity of *P*-element insertions that preferentially target the gene promoters and replication origins associated with them [52]. All transgenes were mapped at 12-23 kb distance from TAS [43]. The orange eye colour of EY08176, EY00453 and EY00802 transgenic flies corresponds to the previously reported phenotype and indicates a high level of *mini-white* reporter gene expression [43, 53]. The EY03383 strain carries P{EPgy2} in 2R TAS [43]. The insertions in TAS (EY03383) and in the telomere of the 4th chromosome (EY09966) are silenced and demonstrate a white or variegated eye colour phenotype (Table 1).

We asked whether the same transgenes inserted in different positions of telomeric retrotransposon arrays produce similar amount of piRNAs. To address this question, we sequenced small RNAs from the ovaries of four EY transgenic strains. Abundant endogenous *HeT-A*, *TAHRE* and *TART*-specific small RNAs are found in y^1w^{67c23} and transgenic strains (Fig. 1a; Additional file 1: Figure S2) indicating that transcripts of all telomeric retroelements serve as a source of piRNAs, however, it is unclear, what the contribution of each particular telomeric element copy to the production of the piRNAs is. Mapping of small RNAs to the telomeric transgenes located at different positions revealed differences in the production of small RNAs (Fig. 1b) which may be attributed to piRNA production variations between integration sites. Small RNAs are mapped to both genomic strands of the entire transgene EY08176 located within *TAHRE* element. Most of small RNAs mapping to the transgene are 24-29 nt long and demonstrate 5' terminal uridine bias (1U bias), which is characteristic of piRNAs (Fig. 1c). We

found sense/antisense piRNA pairs (relative to transgene) overlapped by 10 nt, which is a signature of the ping-pong piRNA amplification cycle [11, 19] (Fig. 1d). This small RNA profile strongly suggests that this transgene is integrated within pre-existing piRNA cluster.

The transgenes EY00453, EY00802 and EY09966 inserted in *TART* elements within different chromosome arms demonstrate a lower efficiency of small RNA production. A significant fraction of the small RNAs produced by EY00453, EY00802 and EY09966 transgenes are 21-nt siRNAs; no ping-pong signal was detected for transgenic piRNAs showing 1U-bias. Interestingly, small RNAs mapping to canonical *TART* elements do not reveal gaps in the regions corresponding to the transgene insertions (Fig. 1a). At the same time, it is believed that transgene-mapped small RNAs should reflect small RNA production in the integration site. Therefore, one may suggest that particular *TART* copies are not involved in piRNA production. Conversely, transgene integration *per se* in *TART* promoter region could impair production of piRNA precursors. Unique mapping of small RNAs to all telomeric transgenes revealed piRNAs derived from P-element fragments and linkers, confirming that observed effects are transgene-specific (Additional file 1: Figure S3).

Northern blotting of *white*-specific small RNAs from transgenic strains confirmed the presence of abundant small RNAs in the ovaries of strain EY08176 and a reduced amount of small RNAs in the ovaries of strains EY00453, EY00802 and EY09966 (Fig. 1e). As expected, we detected small RNAs in EY03383 carrying an insertion in 2R TAS. In contrast, no *white* small RNAs were detected in EY03241 carrying P{EPgy2} in euchromatin indicating that piRNA production in other studied strains is telomere-specific. Mapping of small RNAs from the y^{1} w^{67c23} strain to P{EPgy2} revealed a negligible amount of small RNAs complementary to transgenic sequences (Fig. 1b).

Thus, telomeric retrotransposon arrays can be considered as a continuous piRNA cluster; however, piRNA-producing ability varies significantly between transgenes integrated in different positions of telomeric retrotransposon arrays (Table 1).

HP1, Rhino and H3K9me3 associate with different telomeric transgenes

Silencing of the main telomeric retrotransposon *HeT-A* in the germline is exerted by several mechanisms including the piRNA pathway [3, 32, 34, 36, 38]. piRNA-guided transcriptional silencing is mediated by deposition of HP1 and H3K9me3 [15-18]; whereas the germline-specific HP1 homolog Rhi serves as a chromatin marker of the dual-strand piRNA clusters and provides delivery of the piRNA precursor transcripts to the piRNA processing machinery [24-27, 54]. We asked whether repressive chromatin components and Rhino

associate with different telomeric transgenes in ovaries. To investigate this we performed chromatin immunoprecipitation (ChIP) and using transgene-specific primers compared HP1, H3K9me3 and Rhi accumulation at transgenes and an endogenous dual-strand piRNA cluster located at the genomic site *42AB*. Transcriptionally active *rp49* and *metRS-m* genes and the intergenic *60D* region were included in the analysis as negative controls. HP1 and H3K9me3 were considerably enriched in all studied transgenes (Fig. 2). As a positive control, we detected Rhi enrichment at two regions of the *42AB* piRNA cluster (Fig. 2). Strong enrichment by Rhi was observed in the EY08176 transgene, whereas a lower but statistically significant level of Rhi binding was detected at the transgenes EY00453, EY00802 and EY09966 (Fig. 2; Additional file 1: Figure S4). Rhi level also varies at different positions of *42AB* which likely reflects the intrinsic heterogeneity of the chromatin structure in natural piRNA clusters.

Judging by Rhi binding, which correlates with the ability to produce piRNAs, telomeric transgenes belong to the dual-strand piRNA clusters. Besides, our data show that all telomeric transgenes, regardless of piRNA production rate and Rhi binding, associate with HP1 and H3K9me3 in ovaries. These observations raise a question about the role of the piRNA pathway in the deposition of HP1 and H3K9me3, crucial for telomere functioning.

piRNAs are required for deposition and maintenance of HP1, Rhi and H3K9me3 chromatin components at telomeric retrotransposon arrays in ovaries

HP1 and H3K9me3 are important components of telomeric chromatin involved in telomere length control in mammals [55]. HP1 and H3K9me3 are also present in *Drosophila* telomeres in somatic cells [44, 56, 57], however, the mechanisms underlying their deposition at the telomere are not clear and likely differ between somatic and germline tissues. HP1 can directly bind to telomeric DNA in somatic cells providing telomere capping, while telomeric transposon silencing is mediated by its interaction with H3K9me3 [57]. In the germline, the piRNA pathway exerts HP1-mediated transcriptional silencing of telomeric retroelements [16, 32, 36, 38]. Using the transgene EY08176 which generates abundant piRNAs and binds Rhi, we studied the role of piRNA pathway in the deposition of HP1, H3K9me3 and Rhi in *Drosophilla* ovaries. Firstly, we performed ChIP on ovaries of the EY08176 strain carrying a mutation of the RNA helicase Spindle-E (*spnE*) that induces a strong reduction in the germline piRNA content including telomeric small RNAs [3, 58]. We show that *spnE* mutation causes a considerable decrease in the association of HP1, Rhi and H3K9me3 at the EY08176 transgene whereas these chromatin components remained intact at *42AB* and other endogenous dual-strand clusters (Fig. 3).

We next compared enrichment by HP1, Rhi and H3K9me3 at endogenous *HeT-A* and *TART* copies and dual-strand piRNA cluster in *spnE* mutants (Fig. 3). We show that *spnE* mutation causes a reduction of HP1, Rhi and H3K9me3 at endogenous *HeT-A* and *TART-A* elements (Fig. 3) which is in accordance with the previously observed loss of H3K9me3 and HP1 from telomeric transposons upon the piRNA pathway disruption [16, 38]. Of note and in contrast to telomeric transgene and *HeT-A* and *TART*, Rhi, HP1 and H3K9me3 are not displaced from the major piRNA cluster at *42AB* locus and other dual-strand piRNA clusters in *spnE* mutants (Fig.3). Our data demonstrate a strong piRNA-dependence of chromatin in telomeric piRNA clusters from ovaries that sharply contrasts with Piwi-independent maintenance of chromatin in conventional piRNA clusters. This result is in accordance with the previously observed maintenance of H3K9me3 chromatin marks at dual-strand piRNA clusters upon germline Piwi KD [16, 28].

Loss of chromatin repressive marks leading to transcriptional activation was observed during aging in yeast and flies [59, 60]. Fly aging leads to enhanced expression of heterochromatic transposon copies and piRNA production [61, 62]. To test whether fly age affects telomeric chromatin in ovaries and efficiency of the telomeric piRNA production we utilized the EY08176 transgenic strain. We compared the level of HP1, Rhi and H3K9me3 at transgenes in the ovaries of 3- and 30-days old females. We did not detect statistically significant differences in the deposition of Rhi and H3K9me3, while decreased binding of HP1 at transgene was observed in 30-days old flies (Additional File 1: Figure S5). Northern blotting of short RNA did not reveal changes in the abundance of transgenic *white*-specific piRNAs in the ovaries of 3- and 30-days old EY08176 transgenic flies (Additional File 1: Figure S5). The steady-state level of EY08176 transgenic transcripts in ovaries of 3-days and 1-month flies was also not significantly changed (Additional File 1: Figure S5). These results indicate that HP1 binding at telomeres in ovaries is decreased with aging that however does not affect the piRNA production.

Thus, the piRNA pathway provides a germline–specific mechanism of HP1, Rhi and H3K9me3 deposition at telomeric retrotransposon arrays and is strongly required for maintenance of this chromatin state during gametogenesis unlike other dual-strand piRNA clusters.

piRNAs are required for telomere localization at nuclear periphery but are dispensable for telomere capping and clustering in the germline

We suggested that differences in chromatin structure and ability to produce piRNAs between telomeric transgenes may be determined by specific features of telomeric retroelements

themselves. Indeed, the transgene EY08176 is located within *HeT-A*-related *TAHRE*, whereas other studied transgenes are integrated in *TART*. Using DNA FISH combined with Rhi immunostaining we addressed the question about mutual positioning of endogenous *HeT-A* or *TART* copies and Rhi in the nuclei of highly polyploid nurse cells. In contrast to the giant polytene chromosomes of salivary glands, the chromatids of highly polyploid nurse cells are only partially conjugated allowing for the detection of numerous DNA FISH signals. *HeT-A* and *TART* fragments corresponding to their ORFs were used for DNA FISH probe preparation. Rhi and *HeT-A* DNA FISH signals are almost always superposed (Additional file 2: Table S1). Most *HeT-A* signals are clustered and overlapped with the largest Rhi foci forming rosette-like structures in different *D. melanogaster* strains (Fig. 4a; Additional file 1: Figure S6). Many but not all *TART* signals also colocalize with Rhi (Additional file 2: Table S1), however, in contrast to the numerous clustered *HeT-A* signals, single *TART* signals colocalize with small Rhi foci (Fig. 4b). This differential pattern of telomeric retrotransposon staining may be explained by the fact that *TART* copy number is lower than that of *HeT-A* and *TART* is not present in all telomeres.

ChIP shows that the piRNA pathway mutation *spnE* causes loss of Rhi from *HeT-A* and *TART*, but not from other dual-strand piRNA clusters (Fig. 3a,b). To confirm this observation, DNA FISH combined with Rhi immunostaining was performed on ovaries of *spnE* mutants. Colocalization of Rhi with *HeT-A/TART* signals was not observed in *spnE* mutants in contrast to *42AB* signals which remained colocalized with Rhi (Fig. 4c, Additional file 2: Table S1). Thus, the loss of piRNA pathway components in adult fly ovaries strongly affects the chromatin structure of the telomeric regions but not conventional piRNA clusters suggesting fundamental differences between these two types of piRNA clusters.

Next, we addressed a question about the role of the piRNAs in the deposition of the protective capping complex at telomere ends in the germline. Previously, reduction of *HeT-A* enrichment by HOAP, the main component of the *Drosophila* telomere capping complex [63], was shown by ChIP analysis in *aubergine* and *armitage* but not in *ago3* and *rhi* piRNA gene mutants [35]. We performed *HeT-A* DNA FISH combined with HOAP immunostaining on ovaries of control flies and *spnE* mutants. HOAP extensively colocalizes with clustered and individual *HeT-A* signals both in control and mutant nurse cell nuclei (Fig. 4d). Those HOAP signals that do not colocalize with *HeT-A* most likely correspond to telomeres lacking full-length *HeT-A* copies at the chromosome end. Thus, HOAP loading at telomere ends appears to be mediated by specific piRNA pathway components [35] but not by piRNAs.

We observed that clustered *HeT-A* signals are located toward the nuclear interior in *spnE* mutants in contrast to wild type nurse cell nuclei where *HeT-A* signals are located closer to the

nuclear periphery (Fig. 4a,d). We estimated the positioning of clustered *HeT-A* signals relative to the nuclear surface by 3D quantitative confocal image analysis of *HeT-A* DNA FISH samples on ovaries of wild type and *spnE* mutant flies. We found that the distance from the center of the *HeT-A* FISH signal to the nuclear periphery of nurse cells is significantly increased in *spnE* mutants comparative to control flies (from 1.7 to 4.4 μM in average, P=3.1x10-8, two-tailed t-test, Fig. 5).

Thus, piRNAs contribute significantly to the deposition of HP1, Rhi and H3K9me3 at the telomeric retrotransposon arrays and nuclear position of telomeres in the germline. However, they play a minor role in the formation of telomere capping complex and telomere clustering.

Comparison of subtelomeric chromatin in somatic and ovarian tissues.

Drosophila TAS regions consist of complex satellite-like repeats of 400-1800 bp in length and form heterochromatin domains that are able to induce silencing of transgenic constructs in somatic cells, a phenomenon known as telomeric position effect [64, 65]. TAS regions are enriched by H3K27me3 marks and bind Polycomb group proteins [44-46]. In the germline, we observed HP1, Rhi and H3K9me3 enrichment at EY03383 subtelomeric transgene (Fig. 6a). ChIP using an anti-H3K27me3 antibody also revealed a high level of this chromatin mark at the EY03383 transgene not only in imago but also in ovaries (Fig. 6b). This fact raises a question of how different chromatin complexes coexist within TAS regions.

An egg chamber comprises of an oocyte and fifteen nurse cells surrounded by somatic follicular cells. We suggested that TAS could recruit PcG proteins only in somatic follicular cells. To visualize relative position of the TAS and proteins, we conducted DNA FISH combined with immunostaining on ovaries. A DNA probe corresponding mostly to 2R-3R TAS was used for FISH combined with a/Rhi and a/H3K27me3 immunostaining on ovaries of the y^{1} w^{67c23} strain. We observed colocalization of TAS with H3K27me3 mark, associated with Polycomb silencing, in nuclei of follicular cells (91% co-localization, n=58, n is a number of follicular cell nuclei) (Fig. 6c). On the contrary, TAS signals are not associated with H3K27me3 staining but colocalize with Rhi foci in the nuclei of nurse cells (Fig. 6d, Additional file 2: Table S1). In *spnE* mutants and upon *piwi* germline knockdown, we observe a loss of colocalization between Rhi foci and TAS signals; however, no overlap between H3K27me3 staining and TAS was detected in the nurse cell nuclei (Fig. 6d, Additional file 1: Figure S7a). Similar results were obtained when a DNA probe corresponding to 2L-3L TAS was used (Additional file 1: Figure S7b). Thus, PcG-dependent silencing of TAS is established in ovarian somatic cells but not in the germline.

Next, we compared the expression level of telomeric transgenes in ovaries and revealed that steady-state RNA level of *mini-white* and 5' P-element regions is similar in all studied telomeric transgenic strains (Fig. 6e). Simultaneously, active expression of *white* reporter was observed in the eyes of EY08176, EY00802 and EY00453 transgenic strains but not in EY03383 and EY09966 strains where transgenes are located in heterochromatic TAS and the 4th chromosome telomere, respectively. Thus, transcription activity of transgenes located in different positions of telomere is similar in the germline but differs considerably in somatic tissues and appears to depend on tissue-specific chromatin structure.

Discussion

piRNA production and Rhi binding differ along telomeric region

piRNAs complementary to both genomic strands of telomeric retroelements are produced in the *Drosophila* germline [3, 11, 35, 66], but little is known about telomeres as piRNA clusters. We used P{EPgy2} constructs inserted into the telomeres as unique markers of piRNA production within the highly repetitive telomeric region. Our data show that the telomeric retrotransposon array can be considered as a single telomeric piRNA cluster as all studied transgenes produce piRNAs and bind Rhi. However, we observed heterogeneity in the properties of the dual-strand piRNA cluster along retrotransposon arrays; a transgene located within the *TAHRE-HeT-A* array produces considerably more piRNAs from both genomic strands and shows stronger enrichment by Rhi unlike transgenes inserted in full-length *TART* retrotransposons at 3L and 4th chromosomes. Irregular distribution of Rhi at different regions of the *42AB* piRNA cluster was also observed.

Differential effects of piRNA pathway disruption on the transcription of different regions in cluster *42AB* has been previously described [22, 67] suggesting that heterogeneity is an inherent characteristic of the endogenous piRNA clusters, while the nature of such variation is unclear. We observed that piRNA production by telomeric transgenes depends on the type of telomeric retrotransposon in which the transgene was inserted. Transgenes located in *TART* elements behave more like piRNA targets than piRNA clusters. Of note, in all cases transgene insertions into *TART* elements were located in the regulatory region between sites of sense and antisense transcriptions [51]. Taking into account that *TART*-specific piRNAs are evenly distributed along *TART* sequences and many *TART* DNA FISH signals colocalize with Rhi foci, transgenes inserted in *TART*s should be involved in the piRNA processing. However, it is likely that transgene insertions interfere with *TART* promoter activity and cause reduction in piRNA

precursor read-through transcription. Conversely, strong differences between *HeT-A* and *TART* telomeric retrotransposons were observed in many aspects such as genomic copy number, structure, patterns of transcription and response to piRNA pathway disruption [3, 29, 68, 69]. We also observed a very different pattern of *HeT-A* and *TART* localization in the nuclei of nurse cells; most but not all *TART* copies associated with faint single Rhi signals in contrast to clustered *HeT-A* signals associated with rosette-like Rhi aggregates. Most likely studied transgenes were inserted into those *TART*s which are not involved in piRNA processing. *TART* transcripts are more stable [68] which may be explained by its role in the providing reverse transcriptase (RT) for the transpositions of the main structural telomeric element *HeT-A* that lacks RT. Therefore, one may suggest that transcripts of full-length *TART* copies may be protected from piRNA processing to ensure encoding of the crucial enzyme for telomere elongation, *TART* RT.

Telomeric chromatin plays a pivotal role in telomere protection and maintenance. HP1 and H3K9me3 regulate capping, telomeric repeat silencing and control of their transpositions onto chromosome ends [56, 57, 70]. In the germline, tissue-specific characteristics of telomeric chromatin have been previously revealed [34]. Here, we show that the germline-specific HP1 homolog Rhi binds telomeric retrotransposon arrays and TAS. Surprisingly, strong enrichment of the EY08176 transgene by Rhi, which recognizes the same H3K9me3 marks as HP1, does not abolish or reduce HP1 binding compared with insertions in *TART* elements indicating that Rhi and HP1 do not compete for binding sites at telomeric chromatin. Thus, the piRNA pathway to a large extent determines telomeric chromatin shaping in the germline.

Telomeric region represents distinct type of self-targeting dual-strand piRNA cluster

In the *Drosophila* germline, piRNA sources and piRNA targets are mainly represented by different genomic sequences; piRNA clusters enriched by damaged TE fragments provide piRNA precursor transcripts processed into piRNAs that target active TEs at transcriptional and post-transcriptional levels [11, 16]. It is well known that piRNA targets are silenced at the transcriptional level through the assembly of repressive chromatin; loss of piRNAs cause strong reduction of HP1 and H3K9me3 marks at complementary targets leading to their overexpression [15-17, 32, 38]. However, piRNA clusters themselves show other response to piRNA pathway disruption in ovaries of imago; loss of piRNA production in the adult germline fails to activate piRNA cluster transcription and switching from a repressive to an active chromatin state [16, 26, 28]. No discrete well-defined promoters were revealed within conventional piRNA clusters suggesting that non-canonical transcription generates piRNA precursors [26]. It was also

reported that subtelomeric piRNA clusters differ from conventional germline piRNA clusters. Production of piRNAs from the *42AB* dual-strand piRNA cluster was far less sensitive to the germline depletion of Rhi or HP1a than the 3R TAS and transgenes located in this region [48].

We performed an in-depth analysis of the nature of telomeric piRNA clusters and revealed strong differences in the chromatin dynamics between telomeric piRNA clusters and conventional dual-strand clusters. Strong sensitivity of *HeT-A* chromatin to the piRNA pathway disruption has been experimentally observed [16, 18, 38]. We have shown using different approaches that piRNA pathway mutations induce the loss of HP1, H3K9me3 and Rhi from transgenes located in *HeT-A—TAHRE* arrays as well as from endogenous telomeric retrotransposons in contrast to other dual-strand piRNA clusters. It should be mentioned that maternally inherited piRNAs are deposited in the germ cells of the piRNA mutants because females carrying mutations in the heterozygous state are used for genetic crossings. It was shown that maternal and/or zygotic piRNAs are sufficient to induce formation of repressive chromatin at robust piRNA clusters in early embryogenesis and that this state is maintained during germ cell development, even upon loss of piRNAs at later developmental stages [28].

For telomeric retrotransposon arrays piRNAs are required at all stages of germline development to maintain their repressive state. Thus, the chromatin dynamics of telomeric retrotransposons more resembles those of piRNA targets than of piRNA clusters. At the same time, telomeric regions bind Rhi and produce piRNA precursors, hence being related to the dual-strand piRNA clusters.

The fundamental property of telomeric piRNA clusters is the presence of distinct strong retrotransposon promoters, the activation of which, upon loss of piRNAs may cause a switch from a repressive to an active chromatin state [32, 36]. It is believed that the chromatin state of the genomic locus determines its expression level; however, it is also well known that the transcriptional status of the PcG target genes plays a crucial role in the switching from a repressive to an active state, which provides the transcription-sensitive mechanism of epigenetic memory [71]. In fission yeast, high transcriptional activity at an siRNA target locus prevents heterochromatin assembly apparently through the displacement of the silencing complex [72]. Jil-1 kinase serves as a positive regulator of *HeT-A* transcription likely by protecting an open chromatin state at the *HeT-A* promoter [47, 73]. Jil-1 could provide displacement of the repressive marks and activation of *HeT-A* promoter upon loss of piRNAs.

We found that clustered *HeT-A* copies, normally located at the nuclear periphery were located more towards the nuclear center following the loss of piRNAs. We suggest that this process is induced by massive *HeT-A* overexpression and is related to the expression-dependent nuclear positioning phenomenon described by several groups (for review [74].

Telomere clustering near the nuclear periphery was observed in yeast [75]. In *Drosophila*, telomere clustering nearby nuclear envelope was shown in somatic cells [76, 77]. In *Drosophila* oocytes at the pachytene stage of meiosis, telomeres are not clustered but do associate with the nuclear envelope [78]. We observed clustering of *HeT-A* DNA FISH signals near the nuclear periphery in the nuclei of polyploid nurse cells; however, which particular telomeres are involved in clustering is unclear. piRNA loss leading to transcriptional activation and chromatin changes within telomeres affects the peripheral localization of telomeres in the germline; however, it does not affect telomere clustering because the majority of *HeT-A* signals remains associated. Thus, piRNA pathway is required for the proper nuclear localization of telomeres.

Besides telomeric regions, some individual transcriptionally active TE copies inserted in euchromatin are related to the piRNA targets that also produce piRNAs [12]. Strong reduction in H3K9me3 and Rhi association upon *piwi* depletion is observed for such TE copies [26]. The main difference between telomeric arrays and individual TE copies is that the latter are targeted by piRNAs mainly produced by other piRNA clusters or TE copies. We conclude that telomeric piRNA clusters demonstrate a specific type of self-targeting and are highly sensitive to the presence of piRNAs dual-strand piRNA clusters; the transcripts of which serve as both the piRNA precursors and the only targets of these piRNAs simultaneously (Fig. 7). Specific accumulation of ribonucleorprotein particles consisting of *HeT-A* RNA and HeT-A-GAG protein near the centrosomes at the early syncytial stage of embryogenesis was observed upon germline depletion of several different factors including piRNA pathway components [34, 79]. Given the putative cytotoxic effect of *HeT-A* RNA in mitosis we speculate that burst of *HeT-A* overexpression upon piRNA pathway disruption makes telomeres a highly sensitive sensor guarding genome stability.

Germline-specific chromatin structure of *Drosophila* telomeres

Comparison of the expression and chromatin structure of telomeric transgenes in ovaries and somatic tissues shows fundamental differences. Based on their ability to silence transgenes in somatic tissues, TAS regions were defined as a heterochromatic domain, while telomeric retrotransposon arrays were considered as a transcriptionally active subdomain [43, 44]. Remarkably, subtelomeric regions of diverse organisms consist of highly variable sequences that exert a silencing effect on transgenes integrated within these regions [80]. Thus, the conserved silencing capacity of TAS is apparently important in telomere functioning. In the *Drosophila* germline, we observe similar chromatin properties of TAS and terminal *HeT-A—TART—TAHRE* arrays. Both telomeric regions produce piRNAs, bind Rhi and are expressed at a similar level

(Table 1). Our data raise an intriguing question about competition or developmentally regulated replacement of different chromatin complexes at TAS. PcG protein binding sites were revealed in TAS repeats [45]. Indeed, immunostaining and genetic analysis of PcG protein mutants clearly demonstrated that the TAS zone serves as a platform for PcG protein-mediated chromatin assembly in somatic tissues [44, 45] and in ovarian somatic cells (Fig. 6). We suggest that initiation of piRNA precursor transcription in TAS displaces PcG complexes or prevents their deposition in the germline. Tissue-specificity of silencing mechanisms has been observed by other groups; for example, polycomb repressive complexes were shown to silence transgenes carrying retrotransposon *Idefix* in somatic tissues but not in ovarian follicular cells [81]. Interestingly, in ovarian somatic cells, retrotransposon *mdg1* copies marked by H3K27me3 were not susceptible to piRNA-mediated transcriptional silencing [17]. Our results in combination with previous studies indicate that complicated and competitive relationships between various chromatin complexes define the chromatin structure of the genomic loci including telomeres, particularly in the developmental context.

Conclusions

Our results show that the piRNA pathway provides a strong germline-specific mechanism of telomere homeostasis. piRNAs play a pivotal role in the establishment and maintenance of telomeric chromatin in the germline providing loading of HP1 and H3K9me3, highly conservative telomere components, at different telomeric regions. piRNA pathway disruption causes a loss of heterochromatic components and translocation of telomeres from the periphery to the nuclear interior. However, piRNAs are not required for telomere capping and telomere clustering. In contrast to somatic tissues, where TAS represents a repressed domain, *HeT-A—TART—TAHRE* arrays and TAS show similar chromatin structure and transcriptional status in the germline and can be related to one piRNA-producing domain binding Rhi, a germline-specific HP1 homologue. However, strong heterogeneity in the ability to produce piRNAs and bind Rhi is observed along telomeric transposon arrays. Thus, telomeric piRNA clusters are related to a specific type of self-targeting and are highly sensitive to the presence of piRNAs dual-strand piRNA clusters; the transcripts of which serve as both the precursors of piRNAs and their only targets at the same time.

Methods

Drosophila transgenic strains

Transgenic strains EY08176, EY00453, EY00802, EY09966 and EY03383 carrying the EPgy2 element and inserted within different telomeric regions were described previously [43] and were kindly provided by J. Mason. *Misy* natural strain was obtained from the collection of Institut de Genetique Humaine (CNRS), Montpellier, France. y^1w^{67c23}; *P{EPgy2}Upf3*^{EY03241} (stock #16558) was obtained from Bloomington Drosophila Stock Centre. Strains bearing *spindle-E* (*spn-E*) mutations were ru^1 st^1 *spn-E*^{1} e^1 ca^1/*TM3*, Sb^1 e^s and ru^1 st^1 *spn-E*^{hls3987} e^1 ca^1/*TM3*, Sb^1 e^s. KD (from "knockdown") flies were F1 of the cross of two strains bearing construct with short hairpin (sh) RNA (spnE_sh, #103913, VDRC; piwi_sh, #101658, VDRC) and strain #25751 (*P{UAS-Dcr-2.D}1*, w^{1118}, *P{GAL4-nos.NGT}40*, Bloomington Stock Center) providing GAL4 expression under the control of the germline-specific promoter of the *nanos (nos)* gene.

Fluorescence *in situ* hybridization (FISH) with polytene chromosomes was performed as previously described [82]. A PCR fragment amplified using *white*-specific primers 5'-catgatcaagacatctaaaggc-3' and 5'- gcaccgagcccgagttcaag-3' was labeled with a DIG DNA labeling kit (Roche).

RT-PCR analysis

RNA was isolated from the ovaries of 3-day-old females. cDNA was synthesized using random hexamers and SuperScriptII reverse transcriptase (Life Technologies). cDNA samples were analyzed by real-time quantitative PCR using SYTO-13 dye on a LightCycler96 (Roche). Values were averaged and normalized to the expression level of the ribosomal protein gene *rp49*. Standard error of mean (SEM) for two independent RNA samples was calculated. The primers used are listed in Additional file 2: Table S2.

Small RNA library preparation and analysis

Small RNAs 19-29 nt in size from total ovarian RNA extracts were cloned as previously described in [40]. Libraries were barcoded according to Illumina TrueSeq Small RNA sample prep kit instructions and submitted for sequencing using the Illumina HiSeq-2000 sequencing system. After clipping the Illumina 3'-adapter sequence, small RNA reads that passed quality control and minimal length filter (>18nt) were mapped (allowing 0 mismatches) to the Drosophila melanogaster genome (Apr. 2006, BDGP assembly R5/dm3) or transgenes by bowtie [83]. Small RNA libraries were normalized to 1 Mio sequenced reads. The plotting of size distributions, read coverage, and nucleotide biases were performed as described previously [14].

Ovarian small RNA-seq data for y^1w^{67c23} and transgenic strains EY08176, EY00453, EY00802, EY09966 were deposited at Gene Expression Omnibus (GEO), accession number GSE98981.

Chromatin immunoprecipitation

~200 pairs of ovaries were dissected for every IP experiment. ChIP was performed according to the published procedure [84] . Chromatin was immunoprecipitated with the following antibodies; anti-HP1a (Covance or C1A9 Developmental Studies Hybridoma Bank), anti-trimethyl-histone H3 Lys9 (Millipore), Rhi antiserum [36]. Primers used in the study are listed in Additional file 2: Table S2. Quantitative PCR was conducted with a Light cycler 96 (Roche). Obtained values were normalized to input and compared with values at *rp49* gene as a control genomic region. Standard error of mean (SEM) of triplicate PCR measurements for three-six biological replicates was calculated. Normalization of ChIP data on the *HeT-A* and *TART* copy number was done using PCR on genomic DNA for each genotype. No substantial differences in the telomeric retrotransposon copy number were observed between *spn-E/+* and *spn-E/spn-E* flies.

FISH and immunostaining

The combination of protein and DNA localization was done according to the previously described procedure [82]. Rabbit anti-H3K27me3 (Abcam) and rat anti-Rhi antibodies [36] were used. The probes used for DNA FISH and labeled using a DIG DNA labeling kit (Roche) were; *TART*, cloned fragment of *TART-A* ORF2, corresponding to 434-2683 nucleotides in GenBank sequence DMU02279; *HeT-A*, cloned fragment of *HeT-A* ORF, corresponding to 1746 to 4421 nucleotides in GenBank sequence DMU 06920. Probes corresponding to 2R-3R TAS, 2L-3L TAS and *42AB* region were PCR fragments obtained using primers listed in Additional File 2: Table S2 and labeled with PCR DNA labeling mix (Roche). To stain DNA, ovaries were incubated in PBS containing 0.5 μg/ml DAPI. Three biological replicas were obtained for each experiment. The confocal microscope Zeiss LSM 510 Meta was used for visualization. Confocal image z-stacks were generated with slice step of 1.05 μM.

Calculations of distance from clustered *HeT-A* DNA FISH spots to the nuclear periphery

The calculations are performed in Imaris 7.4.2 software using manual segmentation of nuclei basing on DAPI staining, automatic segmentation of in situ signal spots and automatic calculation of a center of homogeneous mass corresponding to the main *HeT-A* cluster of in situ hybridization signals. FISH spot size is the diameter of sphere encompassing all the spots in XY plane and Z position corresponding to center of mass. The distance between the center of image masses and the nearest point on nuclear surface was measured by increasing the radius of the sphere originated from the center of image masses until it intersects with nuclear surface, and recording the radius as a distance.

Additional files

Additional file 1: Supplementary figures S1-S7. Figure S1. Localization of telomeric transgenes. **Figure S2.** The profiles of telomeric small RNAs (related to Fig. 1a). **Figure S3.** Generation of small RNAs by telomeric transgenes (related to Fig. 1b). **Figure S4.** Rhi occupancy at telomeric transgenes (related to Fig. 2). **Figure S5.** The role of aging in the deposition of HP1, Rhi and H3K9me3 at telomeric transgene in ovaries. **Figure S6.** Endogenous *HeT-A* telomeric retrotransposons associate with largest Rhi domains**. Figure S7. Subtelomeric chromatin in the germline (related to Fig. 6d)** (PDF 1494 kb)
Additional file 2: Supplementary Tables. Table S1 Colocalization of *HeT-A, TART* and TAS with Rhino. Table S2 Primers used in the study (5'-to-3'). (XLSX 369kb)

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Competing interests

The authors declare that they have no competing interests

Availability of data and materials

Ovarian small RNA-seq data for y^1w^{67c23} and transgenic strains EY08176, EY00453, EY00802, EY09966 were deposited at Gene Expression Omnibus (GEO), accession number GSE98981.

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Figure legends

Figure 1 Generation of small RNAs by telomeric transgenes. (A) The schematic structure of telomeric elements is shown above. Insertion sites of transgenes are indicated as triangles situated above and below schemes which corresponds to their genomic orientation. The profiles of small RNAs in ovaries of y^1 w^{67c23} strain along the canonical sequences of *HeT-A, TAHRE, TART-B* and *TART-C* telomeric retrotransposons are shown. Normalized numbers of small RNAs (RPM, reads per million, 0-3 mismatches) in a 30 bp window were calculated. Length distribution of the telomeric element small RNAs is shown below. Percentages of reads having 1U are indicated for each strand (only 24-29 nt reads were considered). (B) Normalized numbers of small RNAs mapped to transgenic constructs (blue – sense; brown – antisense; no mismatches allowed). Scheme of the P{EPgy2} transgene is shown above. (C) Length distribution of transgenic small RNAs is shown. Percentages of reads having 1U are indicated for each strand (only 24-29 nt reads were considered). (D) The relative frequencies (Z-score) of 5' overlap for sense and antisense 24-29-nt piRNAs (ping-pong signature) is shown. (E) Northern analysis of the RNA isolated from the ovaries of EY08176, EY00453, EY00802, EY09966 and EY03383 telomeric transgenic strains. In EY03241 strain, transgene is inserted in euchromatin and does not produce piRNAs. Hybridization was done with *white* riboprobe to detect antisense piRNAs. Lower panel represents hybridization to *mir-13b1* microRNA. P^{32}-labeled RNA oligonucleotides were used as size markers.

Figure 2. Chromatin components of the telomeric transgenes. HP1, H3K9me3 and Rhi occupancy at P{EPgy2} transgenes was estimated by ChIP-qPCR using primers corresponding to 5'-P-element transgenic sequence. Two regions of *42AB* piRNA cluster are enriched by all studied chromatin components. *rp49, metRS-m* and *60D* regions are used as negative controls. Asterisks indicate statistically significant differences in Rhi enrichment relative to EY03241 (* $P < 0.05$ to 0.01, ** $P < 0.01$ to 0.001, *** $P < 0.001$, unpaired t-test).

Figure 3. The role of piRNA pathway in the deposition of HP1, Rhi and H3K9me3 at telomeric transgenes in ovaries. ChIP-qPCR analysis of HP1, Rhi and H3K9me3 enrichment at EY08176 telomeric transgene, endogenous *HeT-A, TART* and several dual-strand piRNA clusters in ovaries of hetero- and trans-heterozygous ($spn\text{-}E^1/spn\text{-}E^{hls3987}$) *spindle-E* mutants. Asterisks indicate statistically significant differences in chromatin protein levels at indicated regions between spnE/+ and *spnE/spnE* (* $P < 0.05$ to 0.01, ** $P < 0.01$ to 0.001, *** $P < 0.001$, unpaired t-test).

Figure 4. piRNA pathway disruption causes loss of Rhi from *HeT-A* and *TART* but not from the *42AB* piRNA cluster. DNA FISH with *HeT-A* (A), *TART* (B) and *42AB* (C) probes (green) combined with Rhi staining (red) was done on ovaries of y^1 w^{67c23} strain and of *spn-E*^{1}/*spn-E*^{hls3987} mutants. (D) piRNAs are dispensable for telomere capping and telomere clustering. DNA FISH with *HeT-A* probe combined with HOAP staining was done on ovaries of y^1 w^{67c23} strain and of *spn-E*^{1}/*spn-E*^{hls3987} mutants. DNA was stained with DAPI (blue). Nuclei of nurse cells from VIII-X stages of oogenesis are shown.

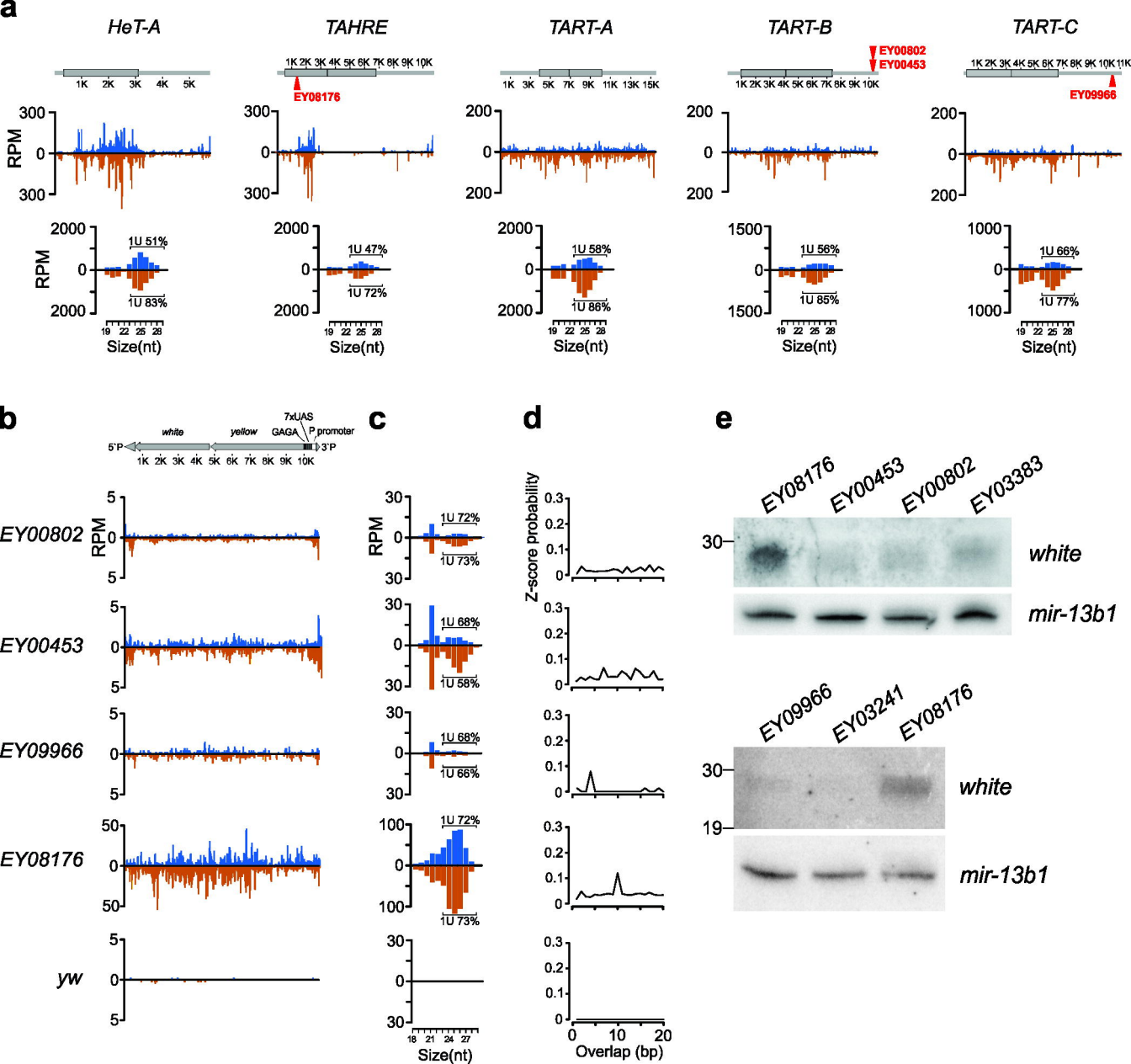
Figure 5. piRNAs are required for telomere localization at nuclear periphery Estimation of the positioning of clustered *HeT-A* signals relative to nuclear surface of nurse cells by 3D quantitative confocal image analysis of *HeT-A* DNA FISH on ovaries of y^1 w^{67c23} strain and of *spn-E*^{1}/*spn-E*^{hls3987} mutants.

Figure 6. Comparison of subtelomeric chromatin structure in somatic and ovarian tissues. (A) HP1, H3K9me3 and Rhi occupancy at EY03383 transgene located in 2R TAS was estimated by ChIP-qPCR. *rp49* and *metRS-m* regions are used as negative controls. (B) ChIP-qPCR analysis of H3K27me3 enrichment at subtelomeric transgene and *bxd* endogenous Polycomb-binding site on ovaries and adult males of EY03383 strain. Asterisks indicate statistically significant differences in chromatin protein levels at EY03383 relative to *rp49* (* $P < 0.05$ to 0.01, ** $P < 0.01$ to 0.001, unpaired t-test). (C) DNA FISH with TAS 2R-3R probe (green) combined with H3K27me3 staining (red) was done on ovaries of y^1 w^{67c23} strain. The nucleus of a follicular cell is shown. (D) DNA FISH with TAS 2R-3R probe (green) combined with Rhi (red) or H3K27me3 (red) staining was done on ovaries of y^1 w^{67c23} strain and of *spn-E*^{1}/*spn-E*^{hls3987} mutants. Nuclei of nurse cells (stage VIII-X) are shown. (E) RT-qPCR analysis of the expression levels of two regions (5' P-element fragment and *mini-white*) of the P{EPgy2} construct in ovaries of transgenic strains. *white*-specific primers detect only transgenic transcripts because endogenous *white* is partially deleted.

Figure 7. Telomeres represent distinct type of self-targeting dual-strand piRNA cluster. Schematic representation of three types of dual-strand piRNA clusters. Chromatin structure of canonical piRNA clusters is established by maternally inherited piRNAs but maintained by a piRNA-independent mechanism. On the contrary, piRNAs are strongly required for maintenance of chromatin state of telomeric and euchromatic TE-associated piRNA clusters during oogenesis. Only telomeric piRNA clusters produce piRNA precursors and piRNA targets at the same time. Assembly of telomere protection capping complex is not affected by piRNAs.

Tables

Table1 Comparison of telomeric transgene properties in somatic tissues and in the germline


a
HeT-A
TAHRE
TART-A
TART-B
TART-C
EY08176
EY00802
EY00453
EY09966
RPM
1U 51%
1U 83%
1U 47%
1U 72%
1U 58%
1U 86%
1U 56%
1U 85%
1U 66%
1U 77%
Size(nt)
b
7xUAS
GAGA
P promoter
white
yellow
5'P
3'P
EY00802
EY00453
EY09966
EY08176
yw
c
1U 72%
1U 73%
1U 68%
1U 58%
1U 68%
1U 66%
1U 72%
1U 73%
d
Z-score probability
Overlap (bp)
e
EY08176
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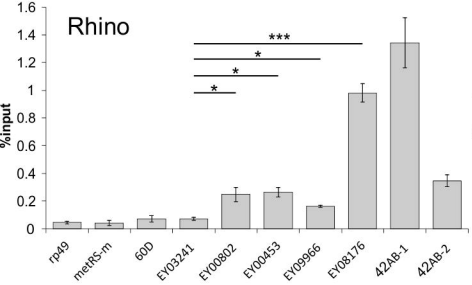
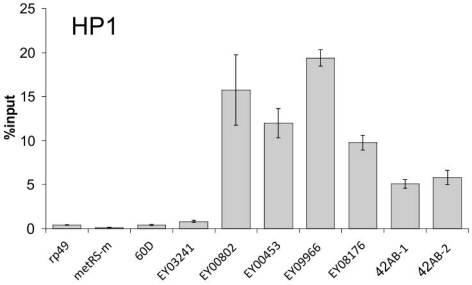
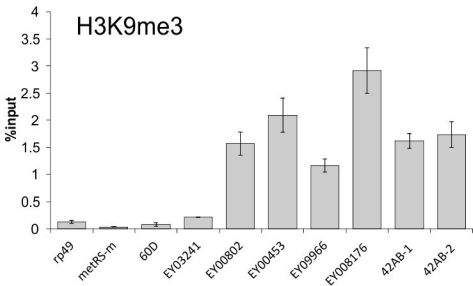
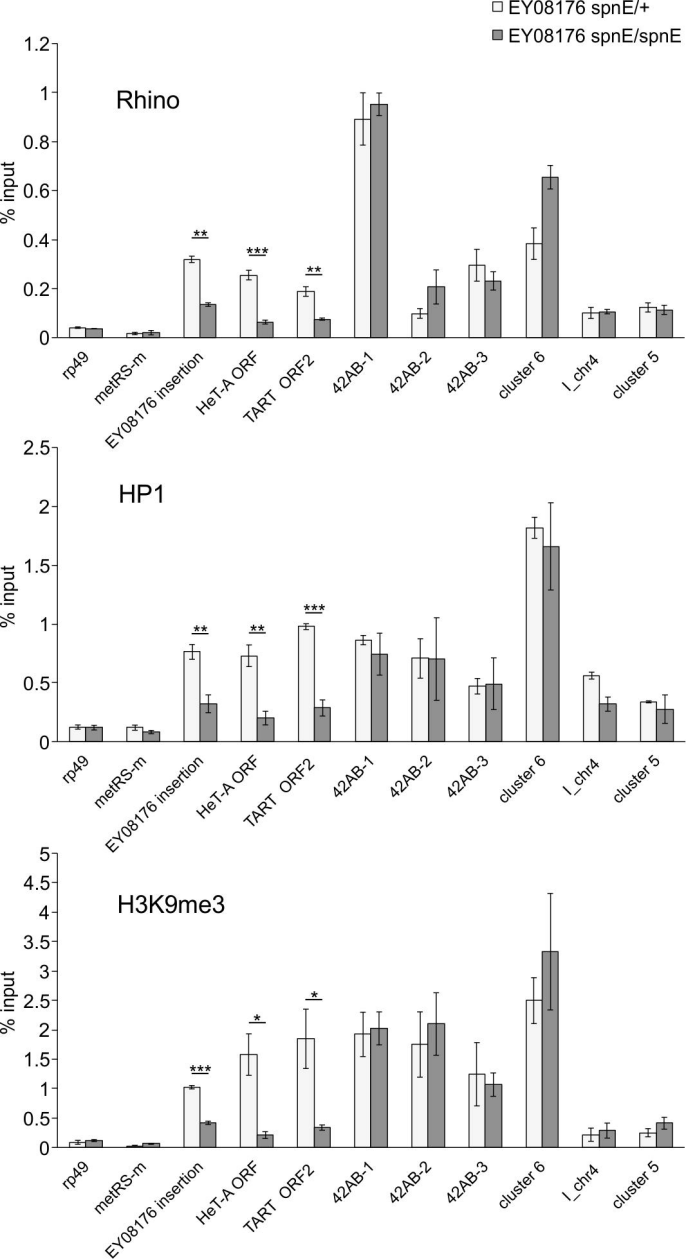
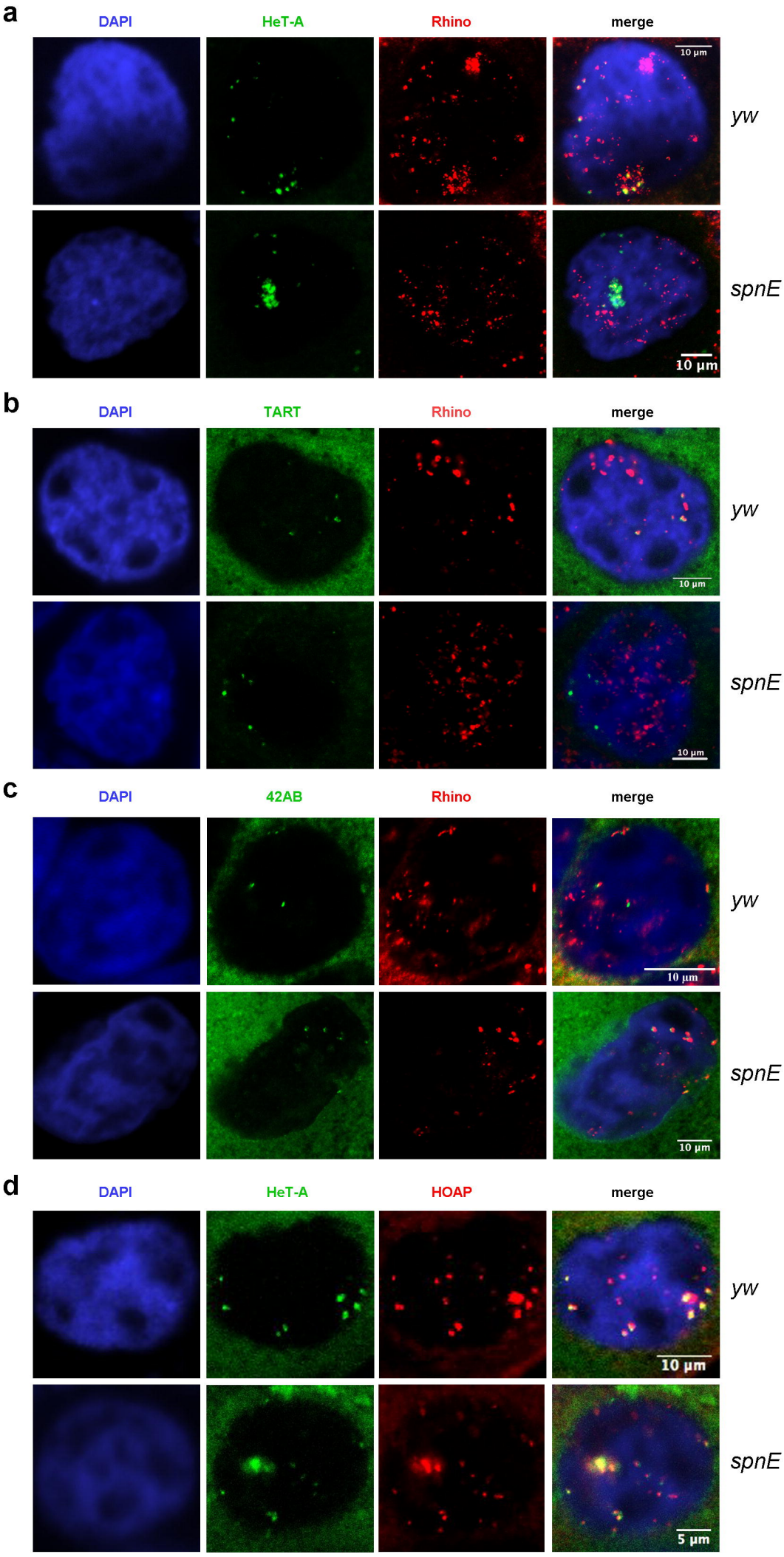
white
mir-13b1

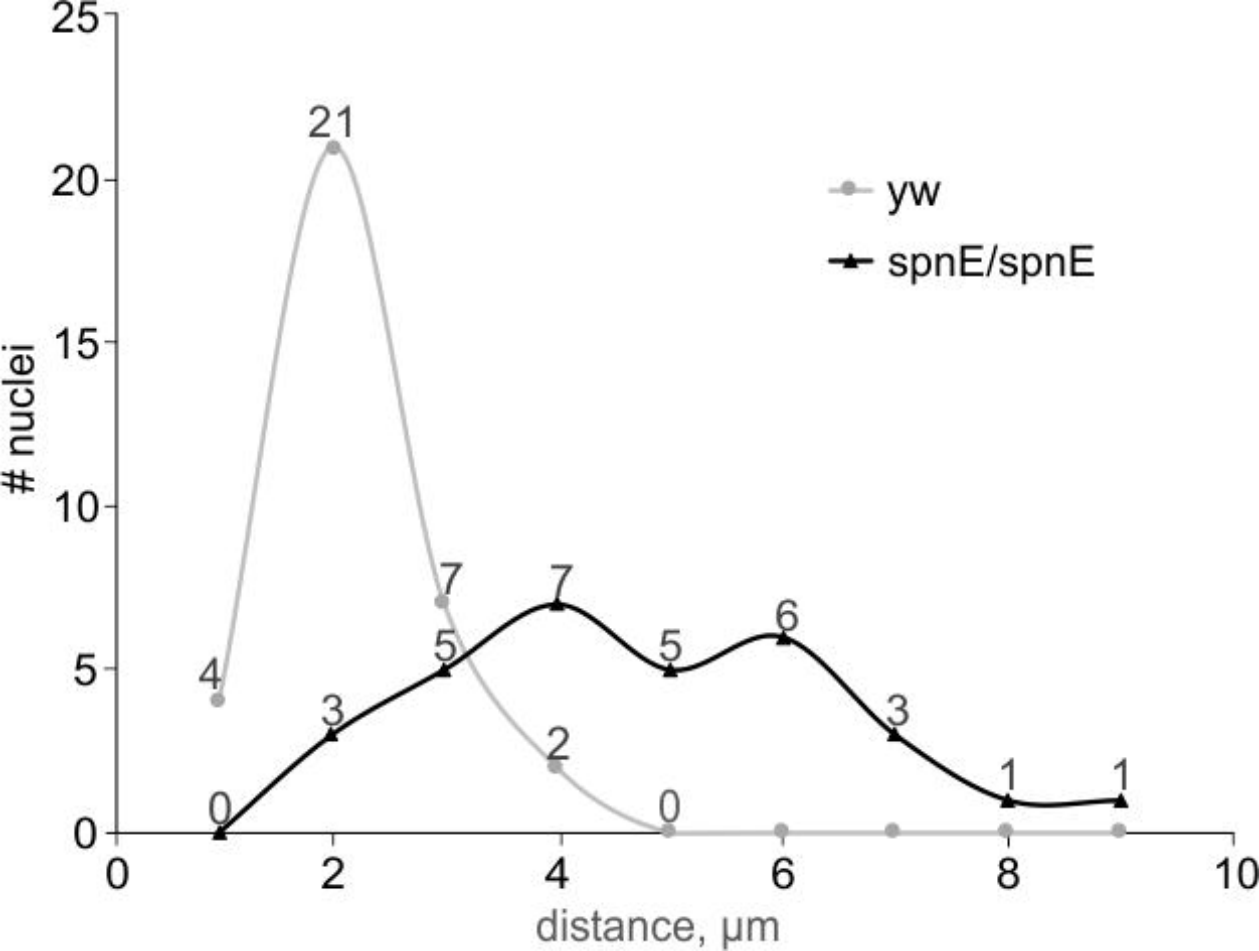

Rhino
%input
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
*
*
*
rp49
metRS-m
60D
EY03241
EY00802
EY00453
EY09966
EY08176
42AB-1
42AB-2

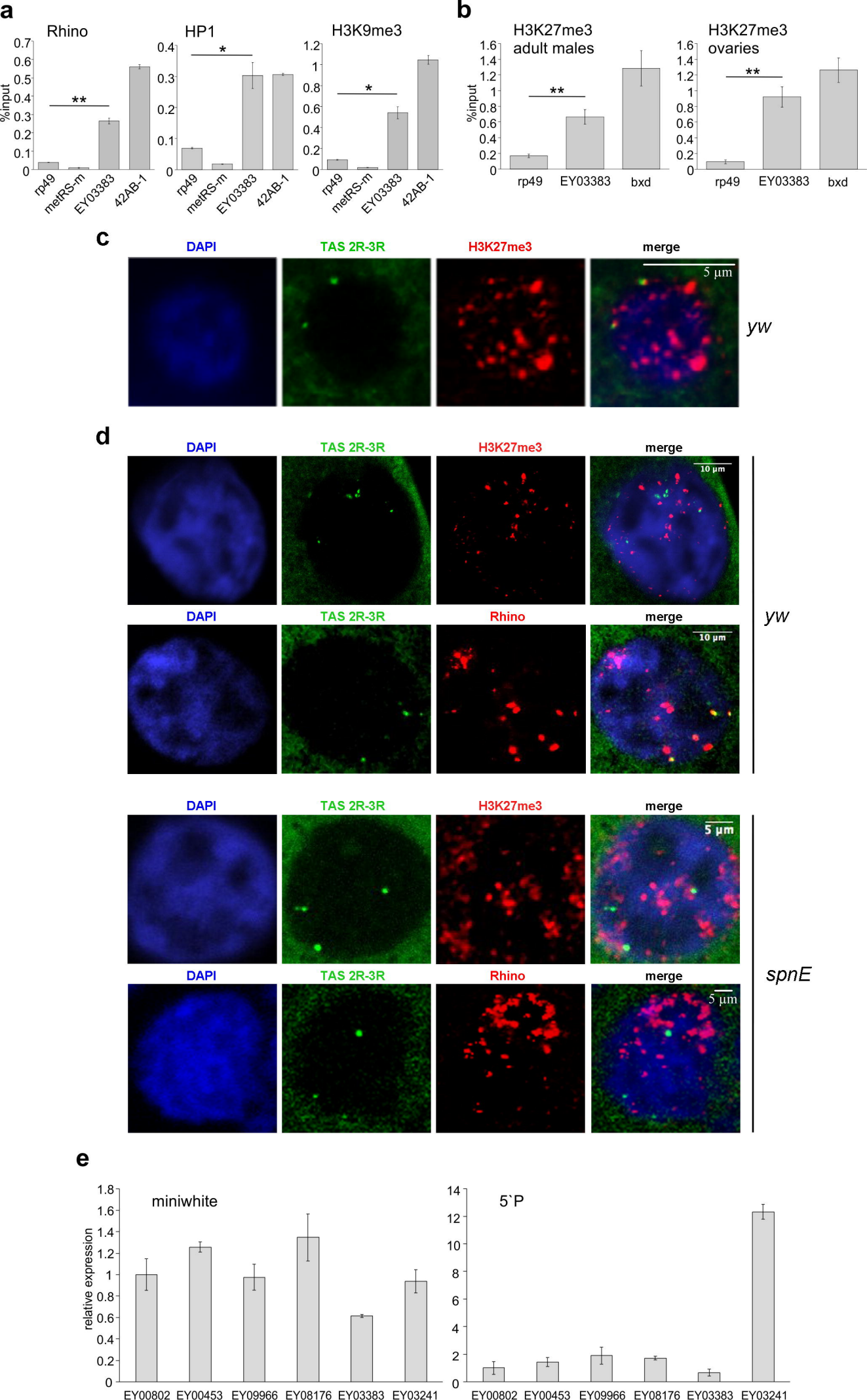

HP1
%input
25
20
15
10
5
0
rp49
metRS-m
60D
EY03241
EY00802
EY00453
EY09966
EY08176
42AB-1
42AB-2

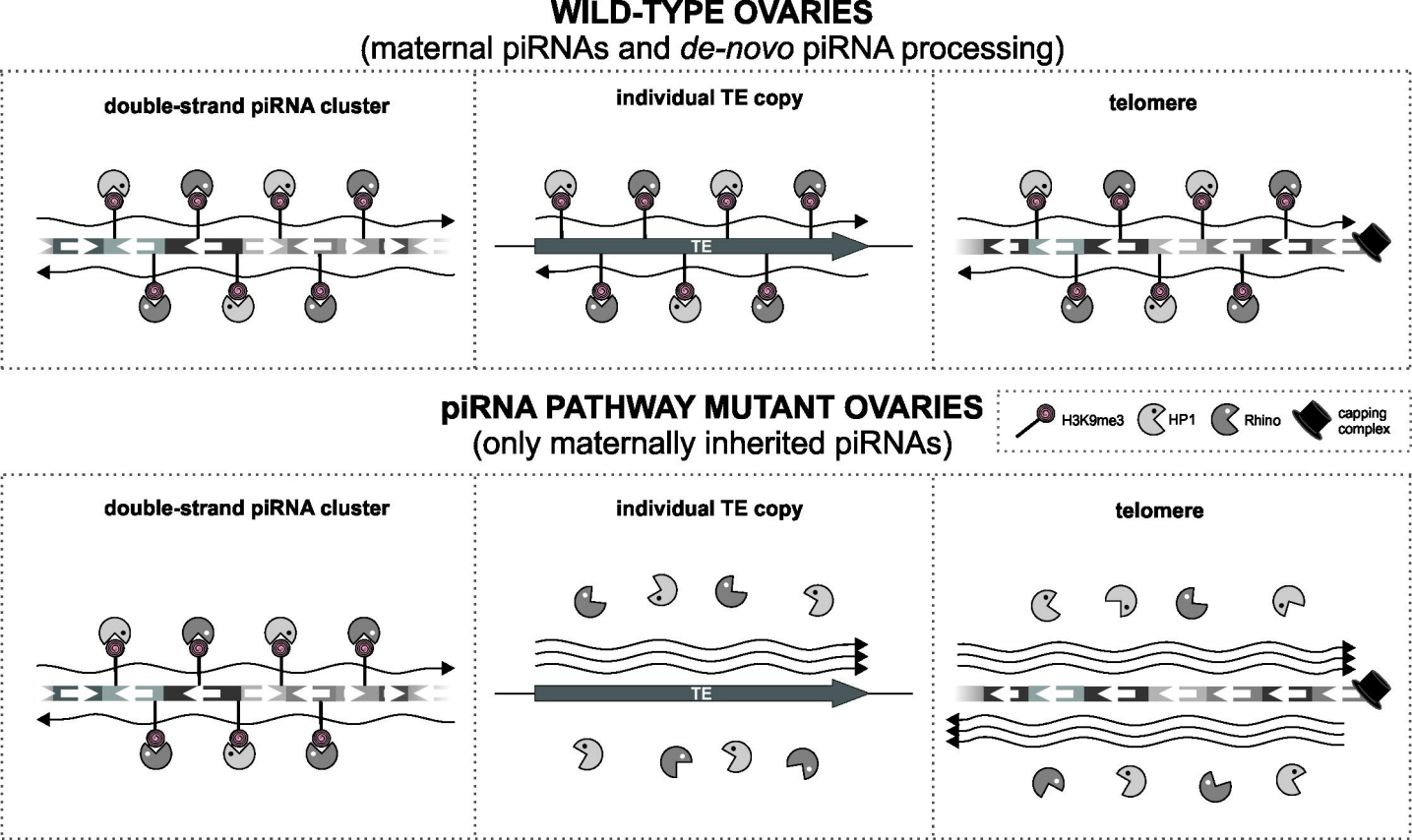

H3K9me3
%input
4
3.5
3
2.5
2
1.5
1
0.5
0
rp49
metRS-m
60D
EY03241
EY00802
EY00453
EY09966
EY08176
42AB-1
42AB-2


EY08176 spnE/+
EY08176 spnE/spnE
Rhino
% input
1.2
1
0.8
0.6
0.4
0.2
0
**
***
**
rp49
metRS-m
EY08176 insertion
HeT-A ORF
TART ORF2
42AB-1
42AB-2
42AB-3
cluster 6
I_chr4
cluster 5
HP1
% input
2.5
2
1.5
1
0.5
0
**
**
***
rp49
metRS-m
EY08176 insertion
HeT-A ORF
TART ORF2
42AB-1
42AB-2
42AB-3
cluster 6
I_chr4
cluster 5
H3K9me3
% input
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
*
*
rp49
metRS-m
EY08176 insertion
HeT-A ORF
TART ORF2
42AB-1
42AB-2
42AB-3
cluster 6
I_chr4
cluster 5


a
DAPI
HeT-A
Rhino
merge
10 µm
yw
spnE
10 µm
b
DAPI
TART
Rhino
merge
yw
10 µm
spnE
10 µm
c
DAPI
42AB
Rhino
merge
yw
10 µm
spnE
10 µm
d
DAPI
HeT-A
HOAP
merge
yw
10 µm
spnE
5 µm


25
20
15
10
5
0
nuclei
0
2
4
6
8
10
distance, µm
yw
spnE/spnE
21
7
4
2
0
0
3
5
7
5
6
3
1
1


a
Rhino
HP1
H3K9me3
%input
rp49
metRS-m
EY03383
42AB-1
b
H3K27me3
adult males
H3K27me3
ovaries
rp49
EY03383
bxd
c
DAPI
TAS 2R-3R
H3K27me3
merge
5 µm
yw
d
DAPI
TAS 2R-3R
H3K27me3
merge
10 µm
Rhino
yw
5 µm
spnE
e
miniwhite
5`P
relative expression
EY00802
EY00453
EY09966
EY08176
EY03383
EY03241


WILD-TYPE OVARIES
(maternal piRNAs and de-novo piRNA processing)
double-strand piRNA cluster
individual TE copy
TE
telomere
piRNA PATHWAY MUTANT OVARIES
(only maternally inherited piRNAs)
H3K9me3
HP1
Rhino
capping complex
double-strand piRNA cluster
individual TE copy
TE
telomere