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PII: S0022-2836(20)30391-0

DOI: https://doi.org/10.1016/j.jmb.2020.06.004

Reference: YJMBI 66565

To appear in: *Journal of Molecular Biology*

Received date: 19 February 2020

Revised date: 25 May 2020

Accepted date: 2 June 2020

Please cite this article as: S. Cacchione, G. Cenci and G.D. Raffa, Silence at the End: How Drosophila Regulates Expression and Transposition of Telomeric Retroelements, *Journal of Molecular Biology* (2020), https://doi.org/10.1016/j.jmb.2020.06.004

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Silence at the end: how *Drosophila* regulates expression and transposition of telomeric retroelements.

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Abstract

The maintenance of chromosome ends in *Drosophila* is an exceptional phenomenon because it relies on the transposition of specialized retrotransposons rather than on the activity of the enzyme telomerase that maintains telomeres in almost every other eukaryotic species. Sequential transpositions of *Het-A*, *TART*, and *TAHRE* (HTT) onto chromosome ends produce long head-to-tail arrays that are reminiscent to the long arrays of short repeats produced by telomerase in other organisms. Coordinating the activation and silencing of the HTT array with the recruitment of telomere capping proteins favors proper telomere function. However, how this coordination is achieved is not well understood. Like other *Drosophila* retrotransposons, telomeric elements are regulated by the piRNA pathway. Remarkably, HTT arrays are both source of piRNA and targets of gene silencing thus making the regulation of *Drosophila* telomeric transposons a unique event among eukaryotes. Herein we will review the genetic and molecular mechanisms underlying the regulation of HTT transcription and transposition and will discuss the possibility of a crosstalk between piRNA mediated regulation, telomeric chromatin establishment and telomere protection.

Keywords: telomere capping; retrotransposons; piRNAs; terminin; Het-A

Introduction

The transition from circular to linear chromosomes about 1 Gy ago is a key event in eukaryotic evolution [1, 2]. The emergence of linear chromosomes posed two major problems to the first eukaryotic cell. First, chromosome ends would have been sensed as sites of DNA damage wreaking havoc to genome integrity [3]; second, the removal of the terminal primer used by DNA polymerases during replication determines a progressive erosion of chromosome ends leading to the loss of essential genes, thereby compromising cell viability [4, 5]. To cope with these two problems, cells evolved telomeres by the implementation of efficient strategies to specifically address the protection and the replication issues [6]. The accumulation of repeated sequences at chromosome ends turned out to efficiently counteract the loss of relevant parts of the genome due

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to incomplete end replication. The majority of modern telomeres consist of short G-rich repeats elongated by telomerase [7], which is thought to derive from an ancient retroelement [8, 9]. Telomeres are protected from unwanted DNA damage repair by several proteins that constitute specific capping complexes, namely shelterin in vertebrates and in fission yeast [10, 11] and CST in budding yeast [12]. The components of the capping complexes perform a number of conserved functions: 1) recognize and bind the ssDNA overhang that is present at chromosome ends in most organisms and provides the 3' OH for the primed synthesis of new DNA sequences; 2) recognize and bind the DNA duplex containing telomeric repeats; 3) inhibit the activation of the DNA damage repair pathways; 4) favor the recruitment of the telomere lengthening machineries.

Notably, in *Drosophila* different mechanisms evolved to ensure telomere homeostasis. Fruit flies have lost telomerase and (with the exception of the Ver protein) their genome does not encode obvious orthologs of shelterin or CST components that co-evolved with the telomerase-based system of telomere maintenance [13, 14]. The *Drosophila* capping complex, named terminin, shares the structural organization and performs all the functions of shelterin and CST. However, terminin proteins are not conserved in terms of sequence and bind telomeric DNA with no sequence specificity [15]. Indeed, telomeric DNA in *Drosophila* does not contain short repeats; instead, it consists of head-to-tail arrays of three different non-LTR retrotransposons, *Heterochromatic telomeric repeats, type A/Healing Transposon (HeT-A), Telomere Associated Retrotransposon (TART),* and *Telomere Associated and HeT-A Related (TAHRE)* (Figure 1), known as the HTT arrays, which are specifically targeted at chromosome ends through efficiently regulated transposition events at each fly generation (see below). The evolution of capping complexes, able to recognize chromosome ends independently of the sequence, implies that HTT arrays are not strictly essential for telomere capping and it is assumed that *Drosophila* telomeres are epigenetically determined structures [15, 16]. This is further supported by the observation that after telomerase loss in *Diptera* (estimated about 260 million years ago) [13], different telomerase-independent telomere elongation variants flourished in the *Drosophila* genus, with gains and losses of specific lineages of retroelements in different *Drosophila* species [17] and the concurrent rapid evolution of telomere capping proteins [17, 18].

Despite an extensive literature on how capping and length are maintained at *Drosophila* telomeres, the question of whether there might exist a functional interaction between HTT targeting and telomere capping remains quite elusive. Here we will describe the mechanisms exploited by fruit flies to regulate the telomeric retrotransposition, how this can be reconciled with the recruitment of telomere capping complexes and the relevance of these results for the telomere biology field.

How *Drosophila* caps its ends: a short overview

Drosophila telomeres are protected by a specialized telomeric complex (terminin) as well as by a large number of terminin-associated proteins [14, 15, 19]. Mutations in genes encoding these proteins result in frequent telomere fusions, a very peculiar phenotype that allowed their identification. Terminin consists of the proteins HP1/Orc-associated Protein (HOAP) [20] and HP1-HOAP-interacting Protein (HipHop) [21] (and its male germline specific counterpart, K81 [22, 23]), which bind double-stranded telomeric regions; Modigliani (Moi) [24], Verrocchio (Ver) [25, 26], and Telomere Ends Associated (Tea) [27] that interact with single-stranded DNA. Terminin components are fast evolving proteins [15, 19, 28]. As these proteins localize only at telomeres and serve exclusively telomeric functions, terminin is considered functionally analogous to shelterin. In contrast, several other non-terminin proteins important for telomere function are evolutionarily conserved and their roles are not restricted to telomeres. This class of proteins includes Heterochromatin Protein 1a (HP1a) [29], Without children (Woc) [30], the MRN complex [31, 32], ATM [33], Eff/UbcD1 [34], Peo/AKTIP [35] and Separase [36].

Genetic and molecular data have shed some light on the inter-relationships between terminin proteins and between terminin and terminin-associated factors. It has been extensively reported that the recruitment of all these proteins at somatic chromosome ends is independent of the DNA sequence including telomeric transposon elements. However, in the female germline, regulators of telomeric transposons are also required for chromosome stability (see below), indicating that telomere length maintenance and protection might not be as unlinked as generally thought.

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The *Het-A, TART* and *TAHRE* retroelements maintain telomere length in *Drosophila melanogaster*

The three elements *Het-A, TART* and *TAHRE* (Figure 1A-C) are inserted exclusively at chromosome ends and share many features with mammalian LINEs: they are arranged in unidirectional arrays (HTT) containing 5' truncated elements, with their poly-A regions directed towards the centromere (Figure 1D). This arrangement reflects the mode in which they retrotranspose onto the chromosome end. In order to incorporate a new element that elongates the chromosome end, a poly-A full length sense transcript is docked to the telomere and the free 3' OH DNA end is used for target-primed reverse transcription of the antisense strand, followed by second strand synthesis. The 5' region of the element thus represents the exposed telomere extremity and remains subjected to terminal erosion. *HeT-A* [37] is about 6 kbp in length, has an open reading frame encoding a GAG protein, but does not encode for a reverse transcriptase (Figure 1A). Hence, for its transposition *Het-A* must rely on a reverse transcriptase provided *in trans* by a different element (probably *TAHRE*) [38, 39]. *Het-A* has a long 3'UTR (2,6 kbp) and the last 600 bp contain the sense promoter, which drives the transcription of a downstream element. Therefore, the production of a transposition-competent *Het-A* transcript requires cooperation between two adjacent elements. Two transcriptional start sites from which sense transcripts originate have been located at -31 and -62 bp upstream of the poly-A region [40]. Consequently, *Het-A* has a 5' UTR, which contains a tag region of variable length, derived from the 3' UTR of the upstream element. This tag includes the oligo-A tract and may act as a buffer sequence that shortens due to terminal erosion. Interestingly, the 3' UTR of *Het-A* contains G-rich tracts able to form G-quadruplex structures *in vitro* [41], a structural motif implicated in regulating transcription and replication [42] found also at the G-rich telomeric 3' DNA strand of telomerase-based telomeres. Due to this peculiarity, it has been therefore suggested that having *Het-A* at the chromosome termini might have conferred an evolutionary advantage.

The *TART* element (Figure 1B) is about 11-13 kbp long and has two ORFS (ORF1 and ORF2) separated by a short spacer, indicating that translation of the second ORF requires an

internal ribosomal entry site [43]. ORF1 and ORF2 encode for a Gag protein and a reverse transcriptase (Pol), respectively. *TART* has a pair of Perfect Non-Terminal Repeats (PNTRs, arrows in Figure 1B) within its 5' and 3' UTRs [43-45]. The promoter for sense transcripts is located in the 5' UTR and the transcriptional start site lies 75 bp upstream of the ORF1 ATG, so that the first codons of ORF1 overlap with the last 84 bp of the 5' PNTR. Hence, as it occurs also for *Het-A,* most of the 5' UTR is lost during transcription and thus the RNA template for retrotransposition does not contain the promoter sequence. In this respect, both *Het-A* and *TART* differ from other non LTR transposons, such as *jockey* or the *I element* [46], which have an internal promoter that drives transcription upstream of the promoter so that this sequence can be copied onto the new transcript [47]. Therefore, *Het-A* and *TART* face two challenges, which might jeopardize their success to produce full-length copies for retrotransposition: (a) terminal erosion that causes loss of 5' sequences, and (b) an inability to perpetuate the promoter. *Het-A* and *TART* adopted different strategies to solve these issues. *TART* 5' end is regenerated at each transposition event by reverse transcription, using the 3' PNTR as a template to copy the missing region of the 5' end that has not been incorporated into the transcript (dotted line, Figure 1B). This mechanism involves a template jump, a feature observed in some reverse transcriptases from non LTR elements [48]. As a consequence, the two 3' and 5' PNTRs in *TART* coevolved and changes observed in one PNTR are almost invariably also present in the other one on the same element [44]. In contrast to *TART,* which "plays solo" and behaves as an independent unit, to compensate for the loss of 5' end, *Het-A* operates in teamwork by exploiting a promoter sequence within the 3' UTR of an adjacent element that is closer to the chromosome end (Figure 1A). This is not possible for *TART*, because its 3' end, despite being capable of initiating transcription, is unable to allow the readthrough into a downstream element, due to the presence of a unique polyadenylation site that promotes RNA polymerase II termination [44].

The less abundant element, *TAHRE*, is about 10 kb in length and shows similarity to *HeT-A*, especially in the UTR regions (Figure 1C); like *Het-A*, *TAHRE* has a 3' sense promoter which drives the transcription of a downstream element. *TAHRE* also encodes a reverse transcriptase that shows similarities with the Pol protein encoded by the ORF2 of *TART* [38, 39]. It has been

suggested that *TART* and *TAHRE* originated from a common ancestral element and that *HeT-A* may have evolved from *TAHRE* by loss of the ORF2 coding sequence or by mobilization of a truncated copy that contained only the ORF1 [38].

The three elements have also promoters in their 3' ends, which drive the expression of antisense transcripts [45, 49-51].

Regulation of telomeric transposition as revealed by somatic tissues studies.

The RNA transcripts of *HeT-A, TART* and *TAHRE* play at least two crucial roles in the biology of transposition: they are reverse transcribed and they encode proteins essential for transpositions. Yet, the regulation of transposition at *Drosophila* telomere ends presents two fundamental questions on the mechanisms of transposition: 1) how the specific and exclusive targeting to chromosome ends is achieved; 2) how the transposon expression is controlled during development.

Studies on mitotic cells (either S2 cultured cells or larval brain cells) that express tagged telomeric transposons helped get insights into the regulation of transposon expression. The genetic and molecular characterization of lines with artificially induced Terminal Deletions (TDs) maintained over several generations, has also provided a tremendous source of information on telomeric transposon regulation especially during the *de novo* telomere formation. Although Muller's early studies unveiled the concept of telomeres based on the failure of recovering viable terminal deletions upon X-ray irradiation [52], successive studies showed that terminal deletions can indeed be retrieved in flies in different ways. *Drosophila* in fact endures terminally deleted chromosomes, which are efficiently healed and recruit the same proteins that normally cap natural telomeres. Interestingly, some of these new telomeres do not contain the HTT array indicating that i) telomeric retrotransposons are neither necessary nor sufficient for establishing the protective cap at telomeres and ii) new telomeres can be assembled on any DNA sequence. TDs have been induced either by X-rays, dicentric chromosome breakage or by cleavage into specific target sequences, within transgenes inserted at a very distal location on chromosome ends, in flies expressing non-*Drosophila* endonucleases (i.e. I-SceI) [37, 53-59]. The occurrence of TDs induces

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the inactivation of appropriate genetic markers and can be monitored through visible phenotypes in the adult. More importantly, the expression of these visible phenotypes can be modulated by events taking place in close proximity to the TDs such as telomeric erosion or transposition, thus making TDs a valuable tool to study the dynamics of telomere retrotransposon activity during *de novo* telomere formation. Interestingly, the findings that terminal deletions likely occur at high frequency on all chromosomes of natural populations strengthen the view that information obtained from TD studies can be used to understand the origin and the maintenance of naturally deleted chromosome ends [60, 61].

Studies carried out in flies bearing TDs as well as in somatic cells revealed that transposition events are regulated at different levels and involve distinct classes of regulators: a) cis-acting factors b) telomere capping proteins c) chromatin factors d) DNA repair proteins and e) still-uncharacterized genetic factors. The evidence that the majority of regulators are factors that are not encoded by telomeric retroelements supports the view of a host-transposon mutualistic interaction that reduces the cost to the host of transposon activity [17, 61, 62]

Cis acting factors. Transient transfection assays and studies on TDs showed that only 400 bp of the *Het-A* 3'UTR is required for the activity of this promoter and highlighted the most crucial feature of this element in promoting transcription (for a review see [63]). However, the same 400 bp of 3'UTR is also apparently active in driving the expression of reporter genes when moved into euchromatic sites. Yet, increasing the length of flanking sequence with the addition of 5'UTR repressed this activity to levels comparable to those of endogenous *Het-A* [64]. This suggests that in addition to the 400 bp sequence stretch, other sequences of the 3'UTR serve as regulatory elements to delimit the promoter activity to its native telomeric heterochromatin. This promoter is bidirectional and activates transcription in opposite directions [51].

Promoter studies and 5' RACE experiments on total RNAs from *D. melanogaster* S2 cells, larvae and adults revealed a single sense start for the *TART* transposition intermediate RNA, located in the 5' PNTR [44, 45]. Other putative sense 5' ends were also shown to map within the PNTRs suggesting that these repeats could play an important role in *TART* transcription [45].

Furthermore, reporter mapping identified sense promoter activity at the 3' end, but this 3' sense strand promoter was responsible mainly for short 3'UTR transcripts. Interestingly, 3'UTR contains also a *TART* antisense promoter, which produces a nearly full-length antisense RNA that is significantly more abundant than sense strand RNA [49]. The antisense transcript contains introns but does not encode proteins indicating that it is not providing sequence information needed for transposition [45].

Telomere specific transposition is also dependent on Gag proteins encoded by *Het-A* and *TART*. These proteins, which share amino acid sequence motifs with retroviral Gags, interact cooperatively at chromosome ends in interphase nuclei, likely promoting the specific end-targeting of telomeric transposons. Transient transfection studies on *Drosophila* cultured cells carried out by the Pardue group revealed that *Het-A* Gag protein forms discrete Nuclear foci (*Het-A* dots) that colocalize with the terminin protein HOAP [65, 66]. *TART* Gag also is targeted to the nucleus, but it localizes at telomeres only if co-expressed with *Het-A* Gag [65].

Other independent studies by the Rong group using an anti-*Het-A* Gag antibody revealed that in wild-type larval brains endogenous *Het-A* Gag proteins can also form nuclear telomere foci, which appear as hollow spheres (*Het-A* spheres, [56]). These spheres are filled with *Het-A* RNAs and colocalized with replicating telomeres suggesting that telomere elongation and end-replication are coupled events also in *Drosophila* as in other organisms [67, 68]. Interestingly, mutations in *Su(var)2-5* and *verrocchio (ver)* genes, which encode the capping factors HP1a and Ver, respectively, affected the organization of *Het-A* spheres [56] indicating that a relationship between telomere capping and elongation could exist also in flies (see below). It has been speculated that *Het-A* transcription at telomeres could therefore initiate *Het-A* Gag multimerization, which in turn attracts the transposition machinery. However, it is still unknown whether *Het-A* dots or spheres also contain RT activity, suggesting that some of the targeting would not result into effective transposition.

Het-A/Gag spheres (see the scheme in Figure 2, left) have also been detected in the nuclei of the female germline cells upon piRNA loss and consequent overexpression of *Het-A* (see next

section). Presumably these structures mediate increased telomeric transpositions observed in the piRNA pathway gene mutants [69, 70].

Telomere capping factors. The analysis of TDs, which are devoid of the HTT array but do not fuse and are stably transmitted, led to the hypothesis that, unlike other organisms, *Drosophila* telomere protection and elongation are uncoupled events [15, 16]. However, the evidence that capping proteins regulate the expression of telomeric transposons suggests that elongation and capping are not intrinsically separated as previously suggested. The most evident example is represented by HP1a (encoded by *Su(var)2-5*), an essential terminin associated protein that is involved in both telomere capping and telomeric DNA transcription, as well as in telomere elongation [29, 71, 72]. Indeed, different *Su(var)2-5* mutations have a strong dominant effect on the frequency of attachment of *HeT-A* and *TART* to TDs, activate *HeT-A* transcription, and show cytologically visible elongated polytene chromosome tips. This effect is independent of the HP1a capping activity as increased levels of *HeT-A* RNAs are also found in *Su(var)2-5* alleles that do not elicit TFs and encode a mutant HP1a, which normally localizes at telomeres [29]. Finally, *Su(var)2-5* mutant larval brain cells displayed an increase in the size of *Het-A* spheres [56], thereby confirming that HP1a appears to repress *HeT-A* transcription at telomeres. This function, however, is not limited to somatic cells as HP1a knockdown in the female germline also led to derepression of telomeric transposons, very likely as a consequence of a reduction of Piwi-dependent transcriptional repression [73]. The activation of *HeT-A* transcription may partly contribute to the high rate of transposition-mediated *HeT-A* additions in the *Su(var)2-5* mutant background.

Very interestingly, *HeT-A* transcription and targeting can be also regulated by Ver, a terminin factor with an OB-fold domain, which shares significant structural similarities with the Stn1 protein [25, 26], a component of the conserved CST complex (Cdc13-Stn1-Ten1 in budding yeast, CTC1-STN1-TEN1 in higher eukaryotes) required for telomere replication and elongation [74-76]. It is worth noting that, in yeasts, plants and human, Stn1 negatively regulates telomerase activity [77-80]. FISH analysis and qPCR revealed that in *ver* mutants, clustered *HeT-A* transcripts disappeared and the steady-state level of *HeT-A* transcripts was also significantly reduced leading

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to a complete absence of *HeT-A* spheres [56], indicating that Ver might positively regulate transposon targeting. The finding that also in Drosophila an OB-fold protein plays a role in telomere elongation mechanisms, reinforces the view that the recruitment of telomere retrotransposons in flies shares mechanistic similarities with the recruitment of telomerase and telomere replication.

Chromatin Factors. HTT transposition is also regulated by protein complexes that, although not directly involved in capping chromosome ends, strongly associate with telomeric chromatin. Deletion of the *Drosophila* hnRNPA1 homolog, Hrb87F/Hrp36, increases the expression of *HeT-A* transcripts and elongated telomeres [81]. As Hrb87F plays several roles in different processes such as gene expression, organization of nuclear matrix and heterochromatin formation, it remains elusive whether its effect on HTT regulation is indirect or due to a specific function at chromosome ends. Interestingly, hnRNPA1 has a role in telomere regulation in higher eukaryotes [82, 83], suggesting that its involvement in telomere regulation is evolutionarily conserved.

HeT-A transcripts also increase considerably upon depletion of Proliferation Disrupter (Prod) protein, a chromatin factor that has been localized at the promoter of *HeT-A* element [84], where it potentially recruits additional factors to maintain a proper telomere chromatin structure [85]. However, since *prod* mutant alleles only show a slight, although significant, increase of *HeT-A* copy number, Prod can be considered a negative regulator of *HeT-A* transcription, which is unlikely to be involved in regulating *HeT-A* targeting at chromosome ends.

DNA repair proteins. Studies on TDs have also revealed that some DNA repair proteins can act as negative regulators of *HeT-A* and *TART* transposition at telomeres. It has been shown that Ku70 and Ku80 proteins that promote DNA repair through NHEJ limit transposition events by rendering chromosome ends less accessible to retrotransposon transcripts. Indeed, flies deficient for Ku70 and Ku80 exhibit a dramatic increase in the frequency of *HeT-A* and *TART* attachments to a broken chromosome end, as well as an increase in elongation of terminal DNA by gene conversion [86]. However, this increase is not associated with a boost of transcription of *HeT-A*

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and *TART*. Beaucher et al [55], developed an assay to monitor the rate of *de novo* telomere formation events in *Drosophila*, which normally occur at a very low frequency. With this assay, they showed that functional NHEJ inhibits the formation of a novel telomere at a broken end. Indeed, the recovery of healed double strand breaks is enhanced by loss of the NHEJ factor Ligase 4. Whereas loss of Ku70 does not affect the healing rate, it increases the attrition at a broken end, before it is healed by formation of a new telomere [55]. Interestingly, the same assay revealed that depletion of ATR Interacting Partner (ATRIP) resulted in a striking increase of transposition events and consequently of new telomere formation. Although this assay could not provide an absolute measurement of healing, the resulting observations indicate that in *Drosophila*, like in yeast cells, mechanisms promoting the recruitment of proteins involved in DNA repair at telomeres generally inhibit *de novo* telomere formation [87].

Uncharacterized factors. Two dominant viable mutations, *Telomere Elongator (Tel)* and *Enhancer of telomere gene conversion (E(tc))* result in a marked increase in *HeT-A* and *TART* copy number at telomeres and to extensively elongated telomeres [16]. The *Tel* mutation, which originated from the *Gaiano* stock, leads to a ten-fold increase of *HeT-A* transcription with respect to wild-type, more likely due to the high number of template copies than to an increase of transcription rate [88]. However, *Tel* mutation did not appear to affect transposon attachment to broken chromosome ends [55]. *Tel* mutants have also been reported to exhibit a high content of spliced antisense *HeT-A* transcripts for the 3'UTR (but not for the *gag* fragment) in somatic tissues [89]. This deregulation of *Het-A* expression may involve a mechanism similar to that observed in ovaries defective for the piRNA pathway ([50], see below), even if it remains still unaddressed whether the accumulation of these antisense transcripts can account for the extended telomere phenotype seen in *Tel* mutants.

In the *E(tc)* mutant, telomere DNA is elongated by gene conversion using the homologous sequences as a template and contain *HeT-A* and *TART* sequences that increase with the number of generations [90]. However, like *Tel, E(tc)* mutation does not enhance the frequency of telomeric element transposition indicating that *E(tc)* affects the function of factors regulating gene

conversion.

Tel and *E(tc)* have been mapped at the same chromosome 3 genomic region suggesting that both mutations are likely affecting the same gene. The molecular identification of this factor(s) will unveil new functions in the regulation of *Drosophila* telomere length.

Regulation of telomeric transposition in the germline: Piwi pathway and chromatin remodeling come into play

As mentioned above, *Het-A*, *TART* and *TAHRE* produce both sense and antisense transcripts [45, 50, 51]. Although *Het-A* and *TART* share common genomic niche, these elements exhibit also specific genetic requirements for their silencing and regulation [44, 49, 91-94].

Telomere function and stability, particularly in the germline, require the establishment of a specific epigenetic pattern characterized by an enrichment in heterochromatic marks, such as trimethylated lysine 9 of H3 histone (H3K9me3) and HP1a binding [91]. In *Drosophila,* trimethylation of H3K9 is catalyzed by distinct histone methyltransferases, Su(var)3-9 and SetDB1/Eggless, active in the soma and the germline respectively [71, 95, 96]. Importantly, depletion of either SetDB1 or HP1a results in derepression of retrotransposons in ovaries, including *Het-A* [72, 95, 97] suggesting that a proper telomere chromatin set up is associated with regulation of the transcription of telomeric transposons. In *Drosophila* ovaries, transposon expression is silenced by the piRNA pathway, whose major players are a germline-specific class of small RNAs known as Piwi-interacting RNAs (piRNAs), and the Piwi clade of Argonaute proteins, Piwi, Aubergine (Aub), and Argonaute3 (Ago3) (reviewed in [98-101]). Figure 2 summarizes the complex activities in play at telomeres, based on recent data on the regulation of piRNA pathway and on the modulation of *Het-A* activity in the female germline. Indeed, downregulation of the piRNA pathway factors shown in Figure 2 and in Figure 3 results in the accumulation of retrotransposons transcripts, including those produced by *Het-A.* The piRNA pathway is thought to be triggered by maternally inherited factors [100, 102]: piRNAs bound by Piwi and Aubergine are deposited into the developing egg by the mothers [103-105]. piRNAs originate from genomic loci named ‘piRNA clusters’ [106]. HTT arrays represent specialized piRNA clusters, whose transcripts

are both the piRNA precursors and their unique targets [91]. piRNA producing loci are characterized by a peculiar chromatin organization, enriched in H3K9me3 bound by the RDC complex formed by Rhino (Rhi, a paralogue of the heterochromatic protein HP1), Deadlock (Del), and Cutoff (Cuff) [107]. The RDC complex recruits transcription initiation factors that set up a non-canonical transcription of piRNA precursors (refs. [108-110], see Figure 2). Therefore, at piRNA clusters the transcriptional machinery is not recruited by DNA sequence signals but by a specific epigenetic mark. Next, a subset of sense and antisense *Het-A* transcripts are channeled to become piRNA precursors (Figure 2, on the right). These precursors are exported in a perinuclear structure called nuage and processed in the cytoplasm to eventually produce mature 23–29-nt piRNAs.

In the cytoplasm piRNA precursors enter the ping-pong piRNA amplification pathway, in which Aub and Ago3 direct the cleavage of transposon targets, and the phased piRNA biogenesis pathway, in which Piwi-piRNA mature complexes are assembled and imported into the nucleus (Figure 3; see [100, 101, 111] for detailed reviews). Armitage (Armi) connects the ping-pong and the phased piRNA pathways by shuttling Aub-bound pre-piRNAs from nuage to mithochondrial outer membrane [112].

The Piwi-piRNA complexes emerging from the phased pathway enter the nucleus, where they can fuel non-canonical synthesis from piRNA clusters and induce the establishment of a repressive chromatin environment at transposon loci (see legend of Figure 2 and refs. [113-116] for details). The binding of HP1a reinforces the establishment of heterochromatin and directly contributes to piRNA production at telomeres [73]. It is important to note that *Het-A* sense and antisense RNAs undergo extensive degradation in the nucleus, promoted by the CCR4/NOT complex [92] (see below).

Het-A sense transcripts produced by canonical transcription, which escape degradation (Figure 2, left side) and behave like intronless mRNAs, are exported in the cytoplasm and are translated to produce Gag proteins. Gag proteins form spheres, which assemble with sense transcripts, to generate the ribonucleoproteins that likely serve as intermediates for retrotransposition. The Gag/*Het-A* spheres are reimported in the nucleus and are recruited at

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telomere ends for retrotransposition, probably by interacting with the capping protein Ver, as it occurs in the soma [56].

Specific features of HTT transcription regulation affect telomere organization and stability.

Some of the factors involved in the transcriptional regulation of telomeric retrotransposons also play direct roles in the control of telomere length or telomere stability in the female germline. Mutations in *aub* or in *spindle-E* (*spn-E*, a gene encoding a helicase essential for the ping-pong cycle), result in derepression of *Het-A* and *TART* and in telomere lengthening [70]. Moreover, *spn-E* mutations and *piwi* knockdown cause strong reduction of H3K9me3 marks, HP1a and Rhino at HTT arrays and increase of H3K4me2, a mark of active chromatin [91, 114, 117], indicating a key role of chromatin remodeling in telomeric transposons homeostasis. As noted above, a singular aspect of HTT arrays is that they are both source of piRNA and targets of gene silencing [91, 118, 119]. H3K9me3 residues recruit Rhino, which favors production of telomeric piRNAs, and concomitantly HP1a forms repressive chromatin domains [91]. Since *Drosophila* telomeres are 50 kbp long on average [61], the two chromatin patterns might occupy different parts of the HTT array. In addition, it remains to be established whether the two complexes form at the same time. Moreover, recent results show that HP1a also plays a role in telomeric piRNA biogenesis, possibly by stabilizing Rhino [73], adding complexity to the regulation of telomeric piRNAs. Importantly, piRNA production in the germline is necessary at all stages for establishing and maintaining a heterochromatic state at telomeric transposons [91], while at non-telomeric piRNA clusters maternal and/or zygotic piRNAs are sufficient to form a repressive chromatin state that is maintained by a piRNA-independent mechanism during oogenesis [120].

HTT expression in the germline is also regulated by a fine balance between stability and degradation of *HTT* RNAs. The proteins CCR4 and NOT form a complex involved in the deadenylation of specific germline mRNAs [121]. Knockdown of the *twin* gene (encoding CCR4) increases polyadenylation of *Het-A* (but not of *TART*) [122]. Moreover, depletion of CCR4 or NOT causes the accumulation of full-length *Het-A* (but not of *TART*) transcripts in germ cells [92, 122, 123]. CCR4 and Piwi associate in the nucleus at telomeres [92], suggesting that CCR4-NOT is

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recruited to mediate the degradation of nascent transcripts. Thus, *Drosophila* has evolved an additional regulatory system that restricts the abundance of *Het-A* transcripts by preventing their accumulation on chromatin. Similarly, the depletion of the RNA-binding protein Ars2 and of the transcription factors Trf2 and Woc result in *Het-A* transcript accumulation [122]. In addition, knockdown of *twin, Ars2, woc* and *Trf2* cause chromosome instability during mitosis [122]. Notably, Woc is also required to prevent telomeric fusions in somatic cells [30].

Telomeric Transposon Silencing and Telomere protection: Convergence of Functions?

The finding that mutations in *aub* and *armi* decrease the binding of the terminin protein, HOAP, to chromosome ends and result in telomere fusions in embryos [124] could suggest that an excessive accumulation of telomeric transposons may be detrimental for telomere stability and function. In contrast, mutations in *rhi* and *ago3* do not disrupt HOAP and HP1a binding [124]. Both Aub and Armi are involved in the phased piRNA pathway that produces most of the piRNAs bound by Piwi (see above) [112]. These results indicate that the Piwi-piRNA mediated transcriptional silencing of telomeric transposons could promote the establishment of a chromatin pattern that might also be important for the recruitment of the terminin capping complex. The transcriptional silencing of telomeric retrotransposons could represent an early step during the assembly of the capping complex at telomeres, possibly through the formation of specific heterochromatic domains enriched with HP1a bound to H3K9me3 containing nucleosomes.

It has been suggested that the formation of a proper telomeric chromatin might require DNA damage response (DDR) factors such as the MRE11–RAD50–NBS1 (MRN) complex and ATM [125, 126]. Indeed, in fruit flies mutations in the genes encoding the components of the MRN complex or the combined deficiency of ATM and ATR strongly reduce HOAP/Hiphop abundance at telomeres and cause extensive telomere fusions [31, 32, 127-130]. Furthermore, MRN and ATM are two of the very few factors essential for preventing fusigenic events in the female germline [33, 130]. As HOAP (and/or HipHop) fail to properly localize at telomeres upon perturbation of factors involved in the piRNA-mediated silencing, it is tempting to speculate that DDR and piRNA pathways could at some point converge into a common route to maintain telomeres. Recent

evidence showed that HP1a, which is also involved in the HTT transcriptional regulation, interacts physically with the MRN complex and that mutations in the MRN components reduce HP1a levels [131]. These results could give further support to our hypothesis that the piRNA-mediated transcriptional silencing and DDR factors might cooperate to recruit the terminin components.

As described earlier, both strands are transcribed at telomeres [51] and a small RNA response is mounted by the complementary piRNAs. At these actively transcribed loci, the nascent RNA is targeted for degradation by the piRNAs that simultaneously mediate the chromatin remodeling of the same loci from which they are generated. This situation implies that loss of piRNAs (i.e as consequence of depletion of either Aub or Armi) induces both the loss of the epigenetic marks at telomeres and a failure to silence the telomere transcripts, which in turn accumulate on chromatin, just like the telomeric repeat-containing RNA (TERRA) coats yeast and mammalian telomeres [91, 132, 133].

A correlation between small RNA responses at telomere and telomere maintenance is not unprecedented. Dysfunctional mammalian telomeres mount a small RNA response in which a damage-induced long non-coding RNA (dilncRNA) generated by Pol II transcription at a dysfunctional end, is processed in a dicer-dependent manner to generate DNA damage response RNAs (ddRNAs) [134]. The small RNAs form ribonucleoproteins that in turn, mediate recruitment of DNA repair factors (i.e. 53BP1) and favor DNA repair. Though the RNA biogenesis pathways of piRNA and ddRNAs are different, in this respect a damaged mammalian telomere is as transcriptionally active as a fly telomere and in both cases telomere transcripts are targeted by the small RNAs produced by themselves (both precursors and target).

The sense and antisense transcriptional activity at telomeres likely confers intrinsic instability to the chromosomes as it renders telomeres potentially exposed to transcriptional stress as well as to RNA:DNA hybrids that would normally be perceived as sites of damage. In this case, HTT transcription could thus favor the recruitment of DDR factors such as ATR, ATM, or the MRN proteins (Figure 2, refs [135-137]). We can speculate that DNA repair proteins at telomeres could resolve conflicts between the replication and transcription machineries and, in concert with the piRNA pathway, could attract terminin components to finally cap the telomeres and shield the

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chromosome end from unwanted DNA damage repair. However, when piRNA production is impaired, and the accumulation of transcripts at telomeric chromatin is unleashed, the presence of large amounts of HTT transcripts could become a threat to chromatin stability, due to their potential to form R-loops that would induce DNA damage response (this model is indicated by question marks in Figure 2). Such a strong transcriptional and replicative stress could impede severing of the DNA damage signaling at telomeres. This scenario would be further worsened if depletion of piRNAs was associated with the concomitant decrease of HOAP or HipHop and could ultimately result in telomere dysfunction and fusions. Further experiments are needed to address the functional relationships between the DNA damage response, the regulation of HTT transcription and telomere protection. With regard to this, careful attention should be paid in the definition of potential telomere fusion phenotypes. Assessing the presence of clear telomere-telomere fusions in the oocyte chromosomes is not as straightforward as in cultured cells or in larval neuroblasts. Therefore, sequencing of the DNA amplified from telomere junctions can provide the ultimate proof for the occurrence of covalent telomere fusion events [33, 130].

With the exception of *Su(var)2-5 and woc,* it is noteworthy that mutations in all genes required for HTT transcription regulation in oocytes have no effect on somatic telomeres ([29]; our data) reinforcing the observations that the actions of these genes are restricted to female germline. However, this is quite unexpected, given that transcription of telomeric transposons and a specific telomere targeting of these elements are believed to occur in somatic tissues as well (see above). It is possible that, unlike germline cells, somatic cells do not require stringent piRNA-mediated regulation of transposition or that this regulation is redundant during mitosis. Finally, the acquisition of *Het-A*, *TART* and *TAHRE* at telomeres of mitotic chromosomes might rely more on recombination and/or gene conversion events rather than on transposition. Both events indeed account for telomere healing and *de novo* telomere formation that frequently occur in natural populations [60, 61], which underscore their importance in telomere maintenance. It is worth noting that recombination between chromosome end sequences is at the base of the Alternative Lengthening of Telomeres (ALT) pathway that a subset of cancers uses to elongate their telomeres in order to prevent the telomere shortening normally occurring in proliferating cells [138].

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Conclusions and Perspectives

Drosophila devised a peculiar solution to counteract terminal erosion and to shield chromosome ends from repair machineries. These problems have been solved by taming three retrotransposons to target chromosome ends and with a sequence-independent capping complex.

Several questions await an answer. Why have different proteins developed distinct roles at telomeres in the soma and in the germline? What makes a newly or freshly established germline telomere different from an already “seasoned” somatic telomere, which underwent many cycles of replication? And is it just a coincidence that telomere establishment occurs in a highly transcriptionally active chromatin environment, threatened by extensive transcriptional and replicative stresses that would require a high demand for repair factors such as ATM, ATR and MRN?

The signaling of DNA damage is necessary to maintain functional telomeres in *Drosophila* in both the soma and the germline; it is possible that the regulation of telomeric transposon transcription may contribute both to managing the stress inherent in an environment rich in nascent sense and antisense transcripts (initiation, elongation, termination, R-loops resolution) and to the recruitment of terminin. If DNA-RNA hybrid structures indeed occurred at fly telomeres, as at human telomeres [139-141], unresolved R-loops might be sensed as damaged foci and trigger the DNA damage response, thus contributing to telomere instability (Figure 2). Thus, recruitment of the protection complex occurs in a dynamic chromatin environment in which DNA damage response factors constitute a prerequisite for capping, but in the presence of unresolved chronic transcriptional stress, they might constitute a driver for telomere instability.

It should be noted that another whole level of complexity for telomere maintenance is found in the zygote, when paternal telomeres need to be completely reprogrammed [22, 23, 142] by switching from the sperm configuration enriched in the K81 protein to the canonical telomere. Whether the small RNA pathway is also involved in this process is currently unknown.

An additional question arising from studies in the female germline is whether zygotic genome instability is promoted only by accumulation of telomeric transposon transcription per se or

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also by the implicit follow up event, that of telomere elongation. Elongated telomeres per se in the female germline should not threaten genome integrity in the female germline. Long telomeres, such as those harbored by natural populations (i.e. Bejing populations) [61] or by *Tel* and/or *Et(c)* mutants, are indeed stably transmitted despite their remarkable increase in the *Het-A* and *TART* copy number. Very long telomeres are also efficiently inherited after introgression of either *Tel* or *Et(c)* mutant chromosomes in a different wild-type background [88, 90], indicating that their stability does not result from adaptive events. A plausible explanation for this tolerance is that in *Tel* and *Et(c)* mutants, despite the large number of telomeric transcripts produced by the high number of template copies, the HTT transposition rate does not increase and telomere elongation derives mainly from recombination and gene-conversion events. However, how *Drosophila* can measure transposition rate remains a fundamental issue to solve. The characterization of genes identified by *Tel* and/or *Et(c)* mutations will definitely provide new insights to clarify this important point. It is worth mentioning that break-induced replication (BIR) can restore the termini of broken dicentric chromosomes in flies by using sequences from the homolog. Interestingly, BIR-mediated restoration can lengthen chromosome termini by more than 1.3 Mb, compared with normal gene conversion, whose extension capacity is below 20 kb [143]. Further studies are needed to address the question of whether BIR-dependent chromosome healing influences the HTT attachments.

With the increasing number of species being studied, other telomerase-independent telomere maintenance mechanisms emerged in different taxa [13, 144-147]. Given the phylogenetic relationships between telomerase and retrotransposons [8, 148, 149] it is unsurprising that the ability of retrotransposons to target chromosome ends turned out to be a successful adaptation as a substitute for telomerase-based telomere elongation in eukaryotes [150]. The interactions between the telomere retroelements and the host genomes underwent extensive reshaping during evolution and the resolution of the host-transposon conflicts might have depended on a fine balance between the rate of piRNA production and the number of transposition events that maintained enough active copies of a certain transposon [151]. In some instances, as for *Drosophila biarmipes* and possibly also for *Drosophila takahashii,* telomere retroelements became inactive and recombination-based mechanisms emerged as the principal mean to regulate

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telomere length [17]. Furthermore, in *Drosophila melanogaster* retrotransposition of active telomeric elements coexists with gene conversion and very likely with BIR-mediated healing [90, 143, 152].

Noteworthy, alternative ways of telomere maintenance are present also in telomerase expressing species when the telomerase pathway is inactivated. *Schizosaccharomyces pombe* can survive telomerase loss by preserving telomeres via homologous recombination [153] or by replacing telomeric repeats with heterochromatic blocks (Heterochromatin Amplification dependent And Telomerase Independent (HAATI) cells, [154]). In humans, homologous recombination is thought to be at the basis of ALT [138], which allows maintaining telomeres in about 15% tumors [155]. Interestingly, both HAATI and ALT telomeres, similar to *Drosophila* telomeres, are characterized by an active transcriptional state [156-158].

Taken together, these observations suggest that the ability to implement recombination/gene conversion at telomeres and active transcription appear to be a common denominator shared by telomeres that are not maintained by a telomerase-dependent system [139, 149, 157, 159, 160].

Drosophila melanogaster telomeres combine an active transcriptional state (as they are a source of both transposon RNA and piRNA precursors) with their intrinsic proneness to recombination and/or gene conversion, as in this species retrotransposition and recombination both contribute to telomere maintenance [152]. It is also possible to postulate a potential competition between the two mechanisms, as suggested by the case of *Drosophilids* in which recombination prevailed as the main mode of telomere maintenance [17]. *Drosophila*, therefore, represents a unique model to understand the development of ALT mechanisms, which made possible the transitions between telomerase-dependent and telomerase-independent mechanisms of telomere maintenance, seen multiple times during evolution [13] as well as in experimentally-induced [161, 162] or pathogenic conditions [155].

We would like to propose a holistic view of the germline telomere, a chromatin domain that extends for tens of kilobases, in which different transcription regulation and chromatin remodeling activities take place concomitantly with the recruitment of the capping proteins that protect the very

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end of the chromosome. Future studies will clarify whether other proteins that are required to modulate the small RNA pathways at germline telomeres affect the loading of the terminin components, possibly enforcing sequence-independent recruitment of the capping complexes.

Acknowledgements

We are grateful to Alla Kalmykova for critical reading of this manuscript.

Figure legends

Figure 1. Schematic representation of the telomeric *Drosophila* retrotransposons.

(A) Two Tandem copies of the *HeT-A* element. One complete *HeT-A* element (highlighted with a red bordered rectangle) is flanked by an upstream 5' truncated *Het-A* copy, which provides the promoter (P, red line) that drives transcription (black arrow) of a full-length sense transcript (green undulated line) from the element located downstream. This intronless, full-length transcript is the putative transposition intermediate. The arrows below the diagram indicate promoters for the antisense transcripts [51], which contain introns and undergo alternative splicing events [89, 163]. Blue boxes: 3'UTR; green boxes: oligo-A regions of variable length (An). Blue-striped boxes: 5'UTR; this region contains a “tag” that includes the most distal part of the 3'UTR and the oligo-A of an upstream *Het-A* element, which provided the promoter that has been used in the previous cycle of transcription [40, 164]. Pink box: open reading frame encoding the GAG protein. Grey box: another element located downstream in the array (derived from the GenBank sequence U06920.2).

(B) Schematic representation of a generic *TART* element. Three *TART* subfamilies exist (*TART-A, TART-B, TART-C*) which differ for the sequence of the UTRs (purple boxes) [44]. The UTR regions contain Perfect Non Terminal Repeats (PNTRs, white arrows). The dotted line within the 5' PNTR indicates the region that is copied by reverse-transcription, using the 3' PNTR as a template, during a de novo retrotransposition event. Pink boxes: ORF1 and ORF2, encoding the Gag and

Reverse transcriptase proteins, respectively. The promoter that drives transcription of the major sense transcript (green line) is contained within the PNTRs and the initiation site is marked by a black arrow; the end of the 3' PNTR contains termination sequences (term) [44]. The PNTRs comprise also promoters that drive the transcription of antisense transcripts (pink lines), which contain multiple introns [45].

(C) The *TAHRE* element has an organization similar to *Het-A* [38, 39], but in addition to the ORF1, encoding the Gag protein, it also contains a second ORF, encoding for a Reverse transcriptase (Pol; pink boxes). Orange-striped boxes: 5'UTR; orange boxes: 3' UTR (modified from ref [144]).

(D) Schematic representation of HTT arrays. In an average telomere, the HTT arrays contain 12 head-to-tail element insertions, spanning about 50 kbp. The 5' end of the most distal element is located at the extremity of the chromosome and may thus be subjected to terminal erosion until a new element is added onto the end. Only 20% of the *Het-A* or *TAHRE* insertions and 7% of the *TART* insertions are represented by full-length elements [38, 61].

Figure 2. Transcriptional fates of a *Het-A* element in the female germline.

The cartoon represents a series of epigenetic events occurring at telomeres in the female germline, which may regulate the activity of the *Het-A* telomeric retroelements. On the right, Piwi-piRNA complexes can either induce the formation of a repressive heterochromatin or promote non-canonical transcription. Transposon repression is initiated by targeting of the piRNA-Piwi complex at transposon loci, and requires the Panoramix (Panx), Nxf1 and Nxf2 (SFiNX) complex [165]. SFiNX recruits Lsd1, which demethylates the euchromatic mark H3K4me2, and the histone methyltransferase SetDB1 along with its co-factor Wde, which catalyzes H3K9me3 enrichment [166, 167]. SetDB1/Wde recruitment requires the small ubiquitin-like protein SUMO and the SUMO ligase Su(var)2-10 that links the piRNA-guided target recognition complex to the establishment of transcriptional silencing which requires the recruitment of HP1a [168]. H3K9me3 can be also bound by the RDC complex formed by Rhino (Rhi, a paralogue of the heterochromatic protein

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HP1), Deadlock (Del), and Cutoff (Cuff). The RDC complex binds H3K9me3 through the Rhino chromodomain and promotes transcription initiation of piRNA precursors through Rhi-dependent recruitment of Moonshiner, a paralogue of a basal transcription factor IIA (TFIIA) subunit, which in turn recruits other factors of the transcription initiation complex [108, 109]. This process is favored by the protein Maelstrom (Mael), which represses the canonical promoter-dependent Pol II transcription in a Piwi-dependent manner [169]. Next, a subset of sense and antisense Het-A transcripts are channeled to become piRNA precursors. These precursors are exported in the cytoplasm to be processed in the ping-pong and in the phased piRNA pathways (see Figure 3).
On the left there is a schematic representation of the telomere capping complex terminin. HOAP/HipHop bind the telomeric DNA duplex and load the Moi/Ver/Tea proteins that bind the single stranded overhang. In the female germline, HOAP/HipHop recruitment is thought to depend on the MRN complex and the ATM or ATR kinases. Depletion of these DNA damage response proteins results in loss of the protective cap and telomere fusion. HOAP recruitment might also be facilitated by piRNAs, as loss of Aub or Armi (key players in piRNA production pipelines in the cytoplasm), results in reduced HOAP loading and extensive telomere instability in embryos. Excessive accumulation of *Het-A* transcripts on chromatin, in CCR4-NOT mutants, results in chromosome instability. Accumulation of *Het-A* at *Drosophila* telomeres is reminiscent of the accumulation of the TERRA transcripts at both human and yeast telomeres [170]. If not properly managed, TERRA transcription is the source of conflicts at replicating telomeres, resulting in the formation of RNA/DNA hybrids, i.e. R-loops [141]. It is tempting to speculate that *Drosophila* telomeres, which produce transcripts in both directions, can also elicit R-loops.

Figure 3 – The ping-pong piRNA pathway and the phased piRNA pathway

In the cytoplasm piRNA precursors enter the ping-pong piRNA amplification pathway, in which Aub and Ago3 direct the cleavage of transposon targets, and the phased piRNA biogenesis pathway in which PIWI-piRNA mature complexes are assembled and exported to the nucleus [100, 101, 111]. In the ping-pong cycle an antisense piRNA with a U at its 5'-end (1U bias), possibly of maternal origin, is loaded with Aub [106, 171]. Aub directs the cleavage of

complementary sense transposon RNAs and generates two fragments. Ago3 binds at the 5' end of the fragment with an A at position 10 (10A bias), which is then trimmed by the 3'-5' exoribonuclease Nibbler [172]. Ago3-bound piRNAs then direct the cleavage of an antisense piRNA precursor, which, after cleavage, in turn is bound by Aub, leading to the amplification of piRNAs that will target transposons [173]. The process is favored by several Tudor proteins in the nuage, such as Krimp, which facilitates the formation of Aub/Ago3 complexes [174]. The phased piRNA pathway is localized on the mitochondrial outer membrane where the endonuclease Zucchini cleaves antisense pre-piRNA to 23-29 nt mature piRNAs that are bound by Piwi [172]. Armitage connects the two pathways by shuttling Aub-bound pre-piRNAs from nuage to mitochondrial outer membrane [112].

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Drosophila melanogaster telomeres are sequence-independent epigenetic structures
Telomere DNA in *D. melanogaster* is made of three specialized retrotransposons targeting chromosome ends
The piRNA pathway finely tunes telomere retrotransposon activity in *Drosophila* ovaries
Drosophila telomere assembly requires the interplay between transcription and chromatin factor activities
Fly telomeres can be considered a good model for ALT telomeres

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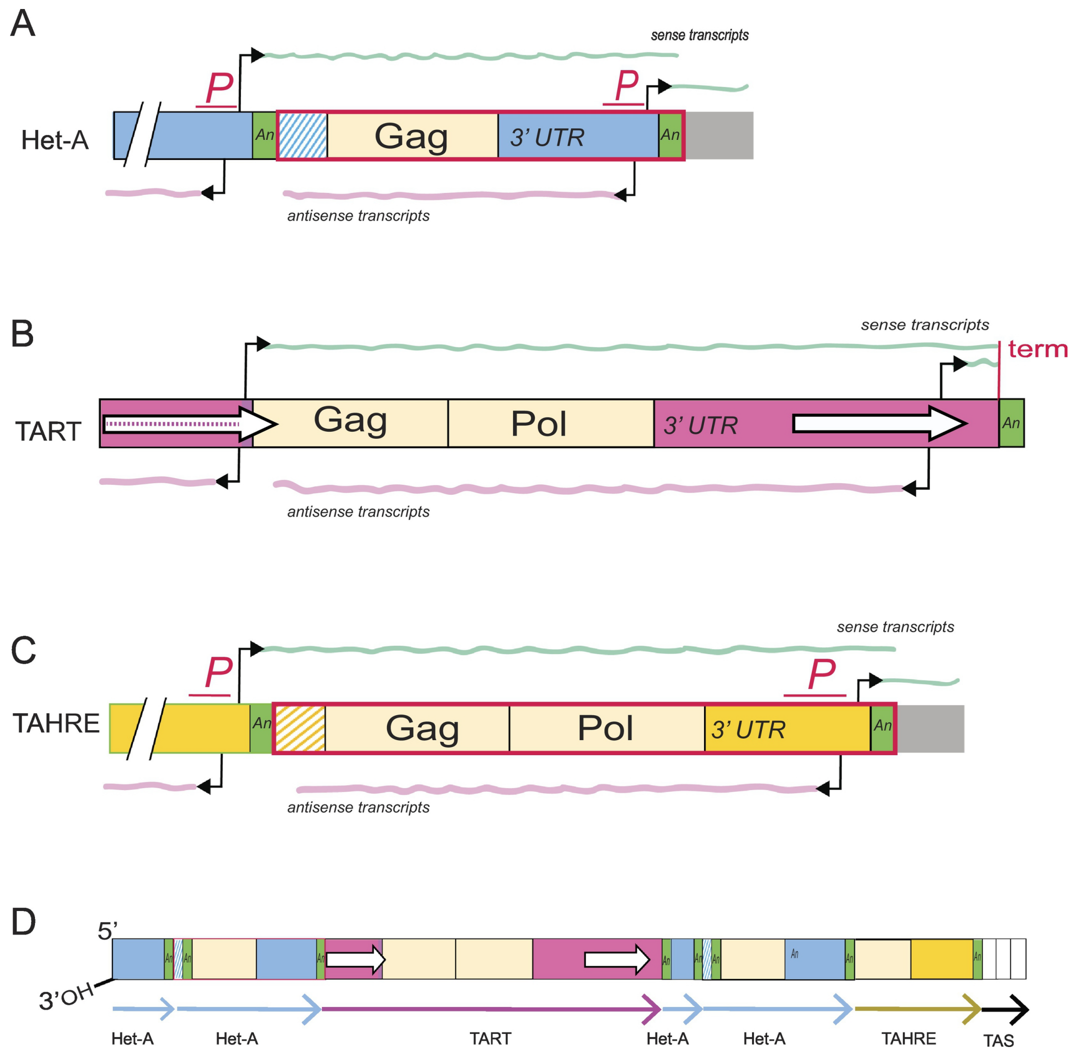

Figure 1

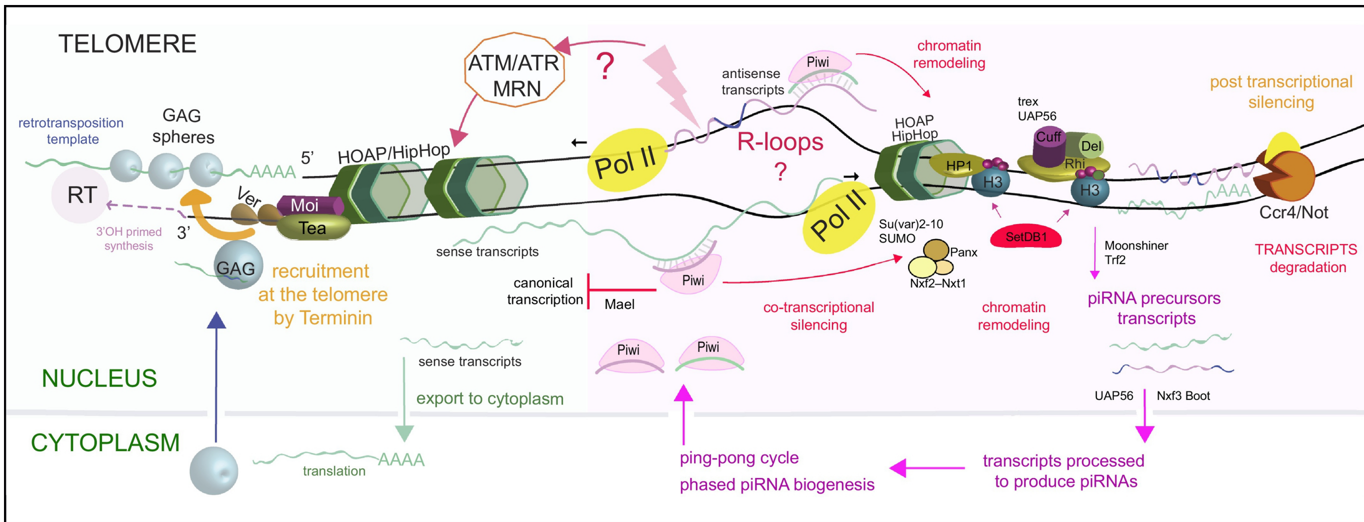

Figure 2

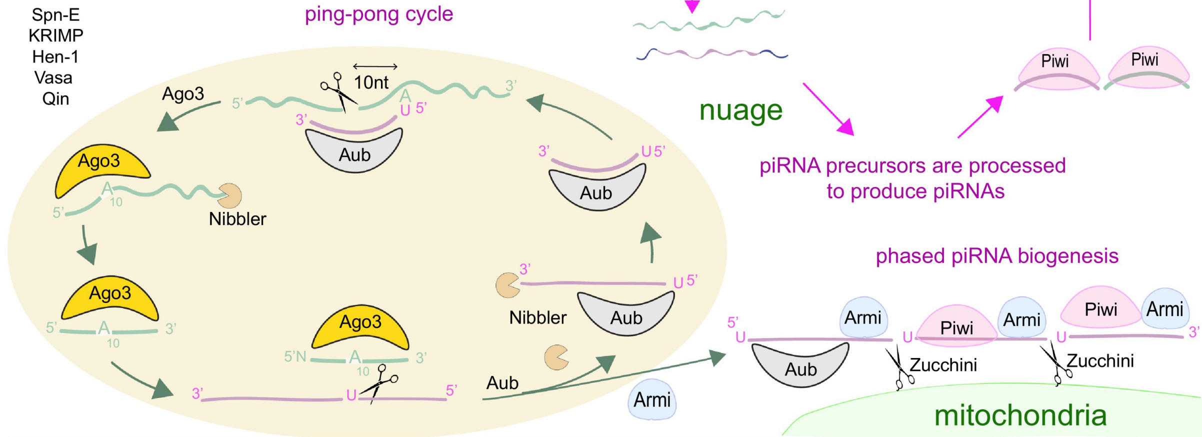

Figure 3