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Drosophila telomeres: an exception providing new insights

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Summary

Drosophila telomeres are comprised of DNA sequences that differ dramatically from those of other eukaryotes. Telomere functions, however, are similar to those found in telomerase-based telomeres, even though the underlying mechanisms may differ. *Drosophila* telomeres use arrays of retrotransposons to maintain chromosome length, while nearly all other eukaryotes rely on telomerase-generated short repeats. Regardless of the DNA sequence, several end-binding proteins are evolutionarily conserved. Away from the end, the *Drosophila* telomeric and subtelomeric DNA sequences are complexed with unique combinations of proteins that also modulate chromatin structure elsewhere in the genome. Maintaining and regulating the transcriptional activity of the telomeric retrotransposons in *Drosophila* requires specific chromatin structures, and while telomeric silencing spreads from the terminal repeats in yeast, the source of telomeric silencing in *Drosophila* is the subterminal arrays. However, the subterminal arrays in both species may be involved in telomere-telomere associations and/or communication.

Keywords

Drosophila; telomere; gene silencing; chromatin; capping

Introduction

At the dawn of the genetic age, William Bateson (1861-1926) produced what has become the mantra for formal genetics: “Treasure your exceptions.” By this he meant that a careful examination of a mutant's phenotype would shed light on the normal wild type process. The same can be said of understanding the nature of wild type structures in species that diverge from a consensus structure. Thus, while *Drosophila* telomeres have been described as unusual (1), they can provide a unique insight into structure function relationships. While it is becoming increasingly obvious that telomere functions are conserved in evolution, the structures that perform these functions are rather flexible (2). Studying *Drosophila* telomeres may help to understand how certain proteins have evolved to perform essential functions at telomeres with very different DNA organization and answer such questions as, “what is important about telomere structure in terms of specific telomere functions?”

To answer this, we must first ask what are the functions of a telomere. Telomeres were initially described as structures at the ends of linear chromosomes that are necessary for the stability of chromosomes, sometimes referred to as chromosome caps. After irradiation rearranged chromosomes, except ring chromosomes, which have no ends, virtually always carried a

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previously existing telomere at each end (3). In modern terms, we would say that telomeres are necessary to distinguish a natural chromosome end from a double strand DNA break (DSB). As a result, telomeres are not normally subject to resection necessary for recombination repair; nor are they subject to nonhomologous end joining (NHEJ) or any other mechanism that would tend to remove DSBs by ligation.

Second, as DNA polymerase cannot replicate a linear DNA molecule completely (4,5), and further manipulation at the chromosome end may be necessary to maintain the proper DNA structure at the tip, telomeres are also needed for length homeostasis (6). The so-called end replication problem applies to all linear DNA molecules, but at least three mechanisms to maintain chromosome length, despite the inevitable terminal erosion, have been described. First, a specialized reverse transcriptase, termed telomerase, may add short repeat units to the chromosome end using an RNA contained in the telomerase complex as a template. This mechanism is wide-spread but not exclusively used in eukaryotes. Even in species with telomerase alternative mechanisms may be used in some circumstances. Second, non-reciprocal recombination (gene conversion) between repeated regions on different telomeres may result in the elongation of one participant. Third, targeted transposition of telomere-specific retrotransposons to chromosome ends is used in *Drosophila* to increase chromosome length.

Other activities have been described for telomeres. In some cell types telomeres tend to associate with each other and with the nuclear envelope. They also inactivate genes in their vicinity, a process known as telomeric silencing or telomeric position effect (TPE). As genes near the nuclear periphery show reduced activity, telomere associations might conceivably play a role in TPE. Telomeres may also be important for homologous chromosome pairing during meiosis (7,8).

Considering function as the defining telomere characteristic, it is convenient to divide the telomere region into three components (Fig. 1). At the extreme end of the chromosome is the cap. This structure comprises several proteins that act in concert. Depending on the species, some of these proteins may recognize specific features of the terminal DNA sequence (9). A second region is defined by the presence of the terminal DNA sequence outside of the cap. As the length of this DNA array may be several kilobases, it may easily be larger than the cap itself. A third region is identified by an irregular array of repeated sequences often termed the subterminal repeat, or the telomere associated sequence (TAS). While TAS has been identified in a wide variety of species (10), their sequence and arrangement varies considerably from one species to another and even from one telomere to another within the same cell.

While *Drosophila* terminal repeats consist of arrays of telomere-specific non-long terminal repeat (LTR) retrotransposons, *HeT-A*, *TART* and *TAHRE* (collectively abbreviated here as HTT elements), instead of short telomerase-generated repeats (Fig. 1), *Drosophila* telomeres appear in other ways to be similar to telomeres in other organisms. The chromosome cap consists of a number of proteins necessary for chromosome stability and to regulate chromosome elongation. Many of these proteins are found at telomeres in other species (11). TPE is also observed at *Drosophila* telomeres, although it seems to originate in the TAS region rather than the telomeric repeats. Here, we relate the specific structural features of *Drosophila* telomeres to universal telomere functions.

The chromosome cap

After extensive genetic and cytological analysis of ionizing radiation-induced chromosome rearrangements in *Drosophila*, Muller (3,12) proposed that a specific structure is required on each chromosome end for viability, which he termed a telomere or cap. These studies were extended by McClintock (13,14), who found that a broken chromosome end in some maize

tissues may induce a cycle of chromosome fusion, anaphase bridges and new chromosome breaks. A similar requirement for a structure to distinguish natural ends of linear chromosome from chromosome breaks has been identified in other species with linear chromosomes, from viruses to eukaryotes, although the solution has varied widely (2). Here, the term telomere will refer to the collection of structures and functions that have been ascribed to chromosome ends, while cap will refer to the specific structure that distinguishes a natural chromosome end from a chromosome break.

In eukaryotes that have telomerase, the capping complex specifically recognizes the telomerase-generated terminal repeats, although some components, such as Ku70/80, may bind DNA independently of sequence. *Drosophila*, however, with an array of retrotransposons, each up to 12 kb in length, does not have a specific terminal sequence. The location of a cap, in fact, may be able to jump short distances, thus allowing the addition of HTT elements to the chromosome end (15), the elongation of chromosomes by gene conversion (16,17), and rapid telomere shortening of up to 50 kb that leaves at least some of the TAS sequence intact (18-20). *P* transposable element-mediated DSBs near the telomere may facilitate these rapid telomere shortening events, although telomere-linked DSBs may also occur spontaneously (20). However, when chromosome breaks are induced in somatic cells farther from the telomere, they either induce mitotic arrest and apoptosis or in some cases broken chromosomes may be transmitted through mitosis by undergoing a breakage-fusion-bridge cycle (21,22), in which a dicentric chromosome breaks at anaphase, and the resulting chromosome fragment fuses with its sister after replication and forms a new dicentric anaphase bridge, similar to that seen by McClintock (13).

After irradiation of females with a mutation in the mutator gene *mu2* it is possible to recover high frequencies of terminal deficiency chromosomes that have lost a telomere (23,24). These deficiencies do not appear to induce cell cycle arrest or end-to-end fusions and are stable over many sexual generations in the absence of a *mu2* mutation, even though they may have unique DNA sequence at the chromosome end, rather than the HTT retrotransposons (25). The broken ends may eventually acquire these transposable elements (15,26). Thus, it is clear that the capping function in *Drosophila* does not require a specific DNA sequence, but instead may act through an epigenetic mechanism similar to that seen for centromeres (27,28).

Stabilization of a broken chromosome end with a new cap, termed healing, appears to be cell type specific. McClintock noted that maize chromosomes broken during meiotic anaphase proceeded through a breakage-fusion-bridge cycle during pollen growth and endosperm development, but healed in the embryo (13,14). Similarly, chromosomes broken in *Drosophila* oocytes can remain unrepaired for days in a *mu2* mutant female, but are healed immediately after fertilization. Further, healing may be nucleus-specific, as chromosome breaks in the oocyte pronucleus after fertilization are healed, while breaks in the sperm pronucleus are not (24).

Proteins associated with the cap

HP1 and HOAP are major components of the *Drosophila* chromosome cap and are required for chromosome stability (Table 1), independent of the presence of HTT elements (29,30). HP1 is highly conserved, with three family members in both *Drosophila* and humans. The original HP1 (HP1a) is discussed here. HP1 may bind DNA directly in the formation of the chromosome cap, unlike its binding activity in repressive chromatin, where it interacts with histone H3 methylated at Lys9 (H3Me3K9) (31). HOAP is a DNA binding protein that resembles sequence-specific high mobility group (HMG) proteins (32,33). Loss of either results in telomere fusions, even when the retrotransposon array is still present at the chromosome end. Other proteins necessary for capping function are also major components of the DNA damage response (11), as has been found in mammals (34). In particular, members of the Mre11/Rad50/

Nbs (MRN) complex are required to maintain HOAP at the telomere and prevent telomere fusions (35-39), as are the ATM and ATR kinases (39-43). The ATM and ATR DNA damage response pathways are partially redundant. While loss of ATR does not affect chromosome stability, mutations in both pathways simultaneously results in a greater telomere-fusion mutant phenotype than loss of ATM by itself (40). ATM and MRN, however, appear to work in the same damage response pathway (35). In humans and other eukaryotes telomere protection depends on the length of the terminal array, thus it is difficult to determine whether ATM targets enzymes necessary for elongation of the terminal array or proteins that mediate telomere protection. In flies, however, ATM targets protection, not elongation.

A few other proteins, including the heterochromatin proteins SUUR, HP2 and SU(VAR)3-7 and the transcription factor WOC (Table 1), have been identified at the extreme chromosome ends by immunocytochemistry (44,45), although their functions at the tips are not known. The first three may play a role in heterochromatin formation (Table 2). In addition to telomeres, where it is required to prevent telomere fusions, WOC is found at interband regions along the chromosome arms, where it colocalizes with the initiating forms of RNA polymerase II. *woc* mutants exhibit normal accumulations of HP1 and HOAP at telomeres. Likewise, mutations in *Su(var)205*, *cav*, *tefu* and *rad50* (Table 1) did not affect WOC localization to telomeres, suggesting that WOC and the HP1/HOAP complex act independently to control telomere stability (45).

The gene *eff* (synonym *UbcD1*) encodes a highly conserved class I ubiquitin (E2) conjugating enzyme. Mutations in this gene cause high frequencies of telomeric fusions independent of the presence of HTT elements (46,47). It is not known whether the UbcD1 protein causes poly-ubiquitination and degradation of a telomere component or mono-ubiquitination resulting in modified allosteric conformation and activity of its substrate, nor is the substrate known. *eff* mutations, however, do not appear to affect the telomeric localization of either HP1 or HOAP (11,47).

The capping complex appears to cover an extensive region of DNA. First, when a capped broken chromosome end is placed close to the yellow gene, it affects the ability of the tissue specific enhancers to activate yellow transcription. Specifically, when an enhancer upstream of the yellow promoter is located within 4 kb of the chromosome end the enhancer function is inhibited (17). Second, *P* transposable elements were unable to transpose when they were within 5 kb of the chromosome end (48). Third, transvection, the trans-activation of a promoter by somatic pairing between the gene in question and enhancer elements of the homologous gene, requires at least 6 kb of sequence upstream of the promoter in order to be effective (49); extensive homology, however, was not required. Thus, several chromosomal functions are inhibited when they are too close to a chromosome end.

The retrotransposon array and chromosome elongation

The three retrotransposons at *Drosophila* telomeres are unusual in a number of respects that may reflect their chromosomal position (50). All three elements have very long 3' untranslated regions (UTRs) that encompass half the element. The sequence of these UTRs, as well as the coding sequence, exhibit a strand bias in nucleotide content that resembles that seen in telomerase-generated telomere sequences in other organisms (51) and have the propensity to form G-quadruplex structures (52), suggesting strand-specific constraints on nucleotide content independent of any telomerase RNA template. *HeT-A* elements also lack a reverse transcriptase gene needed for their transposition.

Drosophilids elongate their telomeres by a combination of targeted transposition of HTT elements and homologous recombination between these elements (1,53,54). A transposition mechanism has been proposed (1,54-57) based on that of other non-LTR retroelements, with

the difference that HTT elements would not need a DNA cut for transposition. They instead could use the free 3' terminus at the chromosome end to prime reverse transcription. Although the details of this process remain unresolved, this transposition mechanism (Fig. 2) predicts that the HTT elements should form a telomeric head-to-tail arrangement, with their oligo(A) tails always facing towards the centromere. The stochastic nature of transposition coupled with the end replication problem predicts that individual elements in the array will be variably 5' truncated. Both of these predictions are observed (58-60). An important prerequisite in this telomere elongation mechanism is the generation of transcripts from the HTT elements in spite of 5' truncations. *HeT-A* elements contain promoter activity within the last 600 bp of their 3' UTRs, which is directed through their oligo(A) tails (16,61) and has the ability to transcribe downstream elements in the telomeric array (62).

The structure of the *Drosophila* telomere raises the question of how the transcriptional activity and targeting of the telomeric retrotransposons is controlled. It appears important to regulate the access of transposition intermediates to the chromosome end, which may be an important mechanism for telomere length control, similar to the telomere-telomerase interaction in other eukaryotes (6). HTT transcriptional activity may also play an important role in length maintenance, as both HTT transcript abundance and attachment to chromosome ends may be controlled by an RNAi-based mechanism in the germ line (63). HP1 also plays a critical role in limiting telomere elongation. Heterozygous mutations in the *Su(var)205* gene, which encodes HP1, cause an increase in both targeted retrotransposition of HTT elements and telomeric gene conversion (64). Interestingly, the *Su(var)205*⁰² mutation, which disrupts the chromodomain (65), does not affect HP1 localization to telomeres or telomere fusions when homozygous (29), although it does affect *HeT-A* transcript levels (31).

Very few other genes have been tested for their effects on telomere elongation in *Drosophila*. Of those that have, the Ku70/Ku80 complex, a component of NHEJ, and the E(TC) protein limit chromosome elongation via gene conversion (53,66). PROD binds just upstream of the *HeT-A* promoter, and *prod* mutants exhibit increased *HeT-A* transcript levels, suggesting that PROD acts as a repressor of *HeT-A* transcriptional activity (67). However, given that *prod* mutants do not develop long HTT arrays, it appears that increased transcript levels are not sufficient to drive increased transposition of these elements.

Telomere growth does not continue indefinitely in stocks heterozygous for these mutants; rather HTT array length reaches a plateau (68), suggesting a mechanism for limiting telomere length. One candidate, telomere shortening, may occur in a mechanism resembling sporadic shortening described in yeast (69) and seen in some *Drosophila* stocks, especially those with *P* elements located in telomere regions within about 50 kb of the chromosome end (18-20). Alternatively, exceptionally long terminal arrays correlate with reduced fertility and fecundity in individual females, which is reflected in abnormal oocyte development (70), suggesting selection against long HTT arrays.

Insertions of other transposons into the HTT array are rare, and only one incident of a *roo* insertion into a *HeT-A* at the 3L telomere has been reported (71). The 4R telomere may be an exception in that it lacks TAS and, instead, has a 5.4 kb "transition zone" that consists of a scramble of HTT elements and other non-telomeric transposons between the uninterrupted HTT array and the last gene (58,60). These observations are not surprising given the rapid turnover of telomeric sequences near the chromosome terminus. *P* elements can also integrate into the HTT array (71). It is possible to directly compare the frequency of insertions into the HTT and TAS regions using the results of the *Drosophila* Gene Disruption Project in which >30,000 *P* element insertions were generated. After accounting for the different array lengths and the fact that many of the insertions noted as being in either HTT or TAS were not in fact telomeric, we calculate that there may have been roughly 8 fold more frequent *P* element

insertions into TAS than HTT. It has been noted, however, that TAS is a strong hot spot for P element insertions (72-74). Thus, the HTT array does not appear to be refractory to transposition by other elements.

Proteins associated with the HTT array

To begin to unravel the mechanisms that regulate the transcriptional activity of the telomeric retrotransposons, it is important to understand the structure of the telomeric chromatin. Proteins associated with the HTT array, however, are difficult to identify, because no genetic screen can be performed for HTT-binding proteins owing to the absence of a predictable mutant phenotype. Therefore, immunohistological staining for potential candidate proteins is one of the few options, which so far has identified three proteins at the HTT array: Z4, JIL-1 and PROD (44,67). All three proteins are also associated with a multitude of other sites in the *Drosophila* genome, suggesting general and widespread functions (Table 2). These studies used extended HTT arrays generated by the *Tel* mutant that are easily distinguishable from the cap region. It is possible that these long HTT arrays have a chromatin structure that differs from shorter arrays.

The amino-termini of the four core histones can harbor a variety of post-translational modifications, and such modified histones can serve as epigenetic marks in chromatin. For example, histone H3 trimethylated at lysine 4 (H3Me3K4) is associated with transcriptionally active chromatin, while H3Me3K9 and H3Me3K27 are markers for inactive chromatin, and acetylation of several lysine residues of histones H3 and H4 serves to mark actively transcribed regions. Histone modifications in the pericentric heterochromatin of *Drosophila* and their involvement in gene silencing are well characterized (75), but analyses of the telomeric regions are just beginning. Andreyeva et al. (44) have localized some characteristic histone modifications at the telomeres of polytene chromosomes by immunohistochemistry and found H3Me3K9 at HTT but not at TAS, while H3Me3K27 was absent from HTT but present at TAS and at the cap. The acetylated histone, H4AcK12, which is often associated with heterochromatin, was not found at telomeres. However, H3Me3K4, a euchromatin mark was present in the HTT array. This limited set of modified histones indicates that the HTT array carries both heterochromatic and euchromatic marks similar to the kinetochore region (76). Even though H3Me3K9, which usually binds the heterochromatin protein HP1, is found at the HTT region, HP1 was not seen by immunocytochemistry. Recent chromatin immunoprecipitation experiments, however, show HP1 distributed along the HTT array outside the cap, and disruption of HP1 function increases *HeT-A* transcription and transcription of reporter genes at several sites in the HTT array (R Capkova Frydrychova, TK Archer and JM Mason, unpublished). Thus, HP1 may play different roles at the cap and along the HTT array outside the cap.

While these studies point to a unique pattern of histone modifications and chromosomal proteins in the chromatin of the HTT array, genetic analysis has clearly shown that *white* reporter genes inserted into the HTT array exhibit characteristics of euchromatin (71). Many more histone modifications need to be tested and the proteins interacting with these epigenetic imprints need to be identified to gain a clear picture of HTT chromatin. An interesting candidate is the phosphorylated histone H3PhS10, which is generated by the JIL-1 kinase and is influenced by several signaling cascades (77).

Telomere associated repeat sequences

Telomeres in *Drosophila* and in other organisms have been considered heterochromatic for at least two reasons: they contain repetitive DNA sequences and have the ability to repress the activity of genes inserted into telomeric regions (50). The repressed and variegated expression of telomeric transgenes is phenotypically similar to position effect variegation (PEV), the

clonal inactivation of a euchromatic gene positioned close to or within pericentric heterochromatin (78,79).

Detailed genetic analysis of telomeric transgenes revealed that the HTT array is not responsible for telomeric silencing (71). Instead, the source of TPE was localized to TAS, which is a region adjacent to the HTT array (Fig. 1). The TAS region consists of several kilobases of complex satellite sequences, which varies among telomeres, although there are sequence similarities. The repeat unit is 460 bp on the 2L telomere (80), 1 kb on 3R, and two different repeats (400 and 800 bp) are interspersed on XR (72).

Transgenic experiments with *P*-element constructs bearing a 2L TAS array adjacent to a mini-*white* reporter gene revealed that TAS silencing is array-length and orientation dependent (81). Similarly, a 1.2 kb fragment from the X-TAS is also able to silence an adjacent transgenic mini-*white* gene (82). Silencing is inversely related to distance from TAS based on expression of transgenes located in the HTT array (Fig. 3). *white* reporter genes inserted into the HTT array more than 5-10 kb from TAS did not exhibit repression or variegation, but inserts located closer to TAS were repressed and showed variegated expression (Fig. 3). Spreading of silencing varied, possibly depending on the structure of the transposable element, the telomere affected, or other factors in the genetic background (71). This supports the notion that telomeric silencing is mediated by TAS rather than by the HTT domain and that distance from TAS is a factor that determines the degree of repression.

Screens of eye color phenotypes in experiments with $P\{w^{var}\}$, a *white* eye color reporter transgene precisely inserted between the terminal retrotransposon array and TAS of the 2L telomere (20,83), revealed TAS-mediated silencing in trans on telomeric transgenes located in both homologous and non-homologous telomeres (20,62,83). Flies with a heterozygous 2L TAS deficiency showed a significant increase in the level of eye pigment in comparison to their siblings bearing a wild type 2L telomere (Fig. 4). PCR analyses showed that transcription of the $P\{w^{var}\}$ transgene can be initiated from a promoter of an upstream *HeT-A* element, and that the level of this *HeT-A* $\{P\{w^{var}\}$ read-through transcript is elevated in the presence of a 2L TAS deficiency (62).

A large number of dominant mutations that modify PEV have been characterized (78). These mutations are frequently found in genes encoding chromosomal proteins or enzymes that modify histones (84). In spite of phenotypic similarities, however, TPE appears to be qualitatively different from PEV. Suppressors and enhancers of PEV do not modify TPE, which suggests different sets of chromosomal proteins as limiting factors in these two types of silencing (74,82,85). As part of a systematic search for trans-acting TPE modifiers, Mason et al. (85) screened a collection of stocks containing autosomal deficiencies covering 77% of the major autosomes for dominant suppressors of silencing of a telomeric transgene. A large portion of stocks that showed TPE suppression were identified as carrying a 2L TAS deficiency, confirming that 2L TAS plays a significant role in the suppression of telomeric silencing towards both homologous and non-homologous telomeres. Although the screen potentially identified 18 sites of TPE suppressors, these sites did not correspond to the positions of genes known to control chromatin structure, with the exception of one site corresponding to the location of *gpp*, which was identified as a dominant suppressor of TPE by Schedl's group (86). *gpp* is the *Drosophila* homologue of the yeast *DOT1* gene, encoding a histone methyltransferase responsible for methylation of Lys 79 of histone H3. There are several possibilities that may explain the low number of potential sites that have been revealed by the deficiency screen, such as the localization of suppressors of TPE primarily on the X or fourth chromosome, the possible recessive character of TPE suppressors (in contrast to dominant PEV modifiers), or the simple chromatin structure in the TAS containing relatively few components

(85). In any case, despite intensive efforts in a number of labs, the genetic factors playing a role in telomeric silencing remain unidentified.

PcG proteins and their role in telomeric silencing

Two Polycomb-group (PcG) repressors, PC and E(Z), have been immunolocalized to TAS of all telomeres, except 2R (44), where these proteins were not detected despite the presence of TAS. Consistent with the methyltransferase activity of E(Z) toward histone H3 at Lys 27, H3Me3K27 was also enriched at TAS. While genetic data on silencing of transgenes inserted into TAS support the idea that TAS arrays form heterochromatic domains (71), immunolocalization on polytene chromosomes (44) revealed that TAS regions lack two well-established marks of heterochromatin: HP1 and H3Me3K9. Both proteins are believed to play a crucial role in heterochromatin formation. Their absence in TAS supports the view that TPE and PEV are under different genetic control.

Three similarities, however, exist for transgenes inserted into TAS and near a Polycomb response element (PRE), which is a region that binds PcG proteins. First, both exhibit variegated expression. Second, long-range interactions may occur. Capkova Frydrychova et al. (62) observed genetic interactions between nonhomologous telomeres, while Vazquez et al. (87) reported that the PcG gene *mcp* can mediate physical interactions between remote chromosomal sites. Transgenes inserted into TAS can silence other genes with the same sequence at many other sites in the genome (88) possibly using an RNAi-based mechanism (89). Third, pairing sensitivity, defined as the lower expression of a flanking reporter gene in a homozygous state than in a hemizygous one, has been observed at telomeres (82) and at PRE-containing transgenes (90). Although these parallels may suggest that PcG proteins are significant players in *Drosophila* TPE, the deficiency screen for dominant modifiers of TPE (85) did not identify deficiencies of PcG genes as dominant TPE suppressors. Thus, the exact role of these proteins in telomeric silencing remains to be elucidated.

Evolution of *Drosophila* telomeres

How might telomeres with retrotransposons at the terminus have arisen? One might imagine a simple sequence of events, each with a reasonable probability of occurrence (91). The first departure from a canonical telomere composed of simple repeat sequences generated by telomerase might have been the loss of telomerase. This has happened numerous times during evolution in species from the dipteran insects, including *Drosophila*, to plants, such as onion (92). There are several examples, from arthropods in general and insects in particular, of independent lost telomerase (93,94).

After the loss of telomerase, the simple telomeric repeat sequence might still be maintained by recombination between these repeats. When human cancer cells become immortalized, for example, the majority of them have reactivated telomerase to maintain chromosome length, but a number of immortalized cancer lines have not. Instead, they use recombination as an alternative mechanism for maintaining chromosome length (95). Some yeast cells that survive after having lost telomerase also show recombination between simple telomeric repeats (96).

Simple telomeric repeats are required at chromosome ends as long as the cap binds to the terminus in a sequence-dependent manner. Loss of sequence specificity would allow the complex repeats of TAS to become positioned at the chromosome ends. Species found to have lost telomerase have also lost simple telomeric repeats. *Drosophila* also appears to have lost a TRF2-like protein that would stabilize a t-loop. *Chironomus* telomeres lack the long 3' overhangs needed for t-loop formation (97). Recombination between these complex repeat sequences has been proposed as a mechanism for chromosome length maintenance in these species. It should be noted that 'lower' dipterans, such as the midge *Chironomus* and the

mosquito *Anopheles*, which may resemble a *Drosophila* ancestor, fall into this category (55, 98,99).

This pattern of chromosome length maintenance by recombination may be augmented by the occasional insertion of transposable elements. In the silkworm, *Bombyx mori*, two retrotransposons target telomere regions, but insert into the terminal array rather than attach to the chromosome tip (100,101). In addition, mammalian LINE elements have the capability of endonuclease-independent transposition and can attach themselves directly to chromosome ends with disrupted telomeres (102).

Targeting of retrotransposable elements might be achieved by two mechanisms. Loss of the endonuclease gene, such as is seen in *Drosophila HeT-A* elements, would prevent the transposons from inserting into interstitial regions and allow attachment to the chromosome end, which resembles a permanent DSB. This mechanism would require that the proteins translated from a specific RNA intermediate preferentially bind to that RNA and return with it to the nucleus (Fig. 2). This has been seen for mammalian LINE transposons (103), and there is evidence for this type of mechanism in *Drosophila HeT-A* transposition (104). A second targeting mechanism may be the homing of the GAG protein from these elements to the chromosome end (105). The HTT arrays found in *Drosophila* chromosomes could have arisen from canonical telomeres by a series of plausible steps. In fact many of the intermediates proposed here have counterparts in the telomeres of non-*Drosophila* species. The ‘unusual’ telomeres of *Drosophila* may represent merely one end of a continuum of possible telomere structures (91), but they provide valuable information on telomere function. In eukaryotes with canonical telomeres, end protection and chromosome elongation both depend on the simple repeats generated by telomerase. *Drosophila* telomeres show that the two vital functions of telomeres, capping and length maintenance, can be separated.

Conclusions

Drosophila telomeres are functionally equivalent to those from eukaryotes that use telomerase to extend their chromosome ends. Ultimately, it is function that defines the telomere. Natural chromosome ends in *Drosophila* are required for chromosome stability, as they are in other species, while DSBs induce cell cycle delay and are subject to DNA repair. *Drosophila* telomeres maintain chromosome length and have several other activities that are less well understood. They form heterochromatin and silence neighboring genes, as do telomeres in yeast and humans. While physical associations among *Drosophila* telomeres have not been found, other than those that arise from anaphase movements, telomere-telomere communication has been noted in the regulation of TPE.

While at first glance telomeres in *Drosophila* appear to be physically very different from those in other species, the differences are not as stark as they appear. In yeast, mammals and *Drosophila*, most of the proteins necessary for telomere stability, including ATM and the MRN complex, perform double duty in the DNA damage response pathways. The terminal DNA array in *Drosophila* is very different from the canonical telomeric DNA sequence, but numerous other plant and animal species also lack telomerase and the simple telomerase-generated repeats. The complex and variable TAS arrays are not unique to *Drosophila*. Although the complete chromatin structure of the *Drosophila* telomere remains to be determined, it appears to be a hybrid of euchromatic and heterochromatic domains. Much needs to be learned, but the small overall size of the *Drosophila* telomere makes it an excellent region to study complex epigenetic interactions.

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Abbreviations

DSB	double strand DNA break
HTT	telomere-specific <i>HeT-A</i> , <i>TART</i> and <i>TAHRE</i> retrotransposons
LTR	long terminal repeat
MRN	MRE11/RAD50/NBS protein complex
NHEJ	nonhomologous DNA end joining
PRE	Polycomb response element
PcG	Polycomb group
PEV	position effect variegation
RNAi	RNA interference
TAS	telomere associated DNA sequence
TPE	telomeric position effect
UTR	untranslated region

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Drosophila

Mammalian

Figure 1. Schematic comparison of the overall structure of the telomere region in *Drosophila* and in human and their associated proteins. In *Drosophila*, the terminal capping complex (blue square) consists of proteins that are primarily associated with heterochromatin and bind in a sequence-independent manner to the end of the retrotransposon (HTT) array, which replaces the telomerase-generated repeats found in other eukaryotes. This array consists of tandem non-LTR retrotransposons of irregular length with their oligo(A) tails (indicated by A) facing towards the centromere. It is associated with proteins that are otherwise found in either heterochromatin or euchromatin (green rectangle) and has euchromatic properties. The subtelomeric (TAS) repeats (alternating light and dark blue small rectangles) assemble a

distinct silencing complex (orange oval), which spreads into the proximal region of the HTT array. Thus, the capping functions at the chromosome end are clearly separated from the silencing domain of TAS, which has heterochromatic properties. In human cells, the telomerase-generated TTAGGG repeats (alternating black and grey bar) may form a t-loop structure, which is stabilized by specific proteins (red oval). Proteins binding to the telomeric repeats assemble a heterochromatic silencing complex (telosome or shelterin; purple ovals), which may spread into the TAS region. Thus, capping and silencing are centered on the telomeric repeats. Associated proteins are shown below each structure. Not shown: the cap and shelterin are maintained by DNA damage response proteins, ATM and the MRN complex.

Figure 2.

Schematic of the nuclear and cytoplasmic events in *Drosophila* telomere elongation by retrotransposition. Transcripts are generated from a telomeric retrotransposon using promoter activity located in the 3' UTR of an upstream *HeT-A* element (black arrows). The transcripts leave the nucleus to serve as mRNA for the translation of the element-encoded GAG-like protein (red squiggle), which contains three nuclear localization signals. GAG binds the RNA and facilitates re-entry into the nucleus. After docking to a chromosome end, perhaps mediated by a protein-protein interaction between the GAG-protein and the terminal-capping complex, a reverse transcriptase uses the free 3' hydroxyl group at the chromosome end as primer to copy the RNA intermediate into the first DNA strand. Second strand synthesis occurs by DNA repair and completes the addition of a new *HeT-A* retrotransposon. It is conceivable that HTT transcripts that have not left the nucleus may also be used as templates for reverse transcription at the chromosome ends. However, sequence analyses of very recently transposed *HeT-A* elements (15) and of several in native telomeric arrays (58) suggest that there is a selection for the incorporation of elements with a functional GAG open reading frame that have previously been translated in the cytoplasm. Such a positive selection would prevent a gradual inactivation

of telomeric transposons due to the accumulation of mutations. Other symbols are as in Fig. 1.

Figure 3.

Cis-acting silencing at the telomeres of *Drosophila* as deduced from expression levels of *P* element insertions carrying a *white* (*w*) reporter gene in various telomeric domains. Silencing effects (orange oval) emerge from TAS and spread a short distance into the HTT array. Two different insertion constructs were used. The *EPgy2* element lacks the eye-specific enhancer and expresses *w* at a relatively low level; the *SuPor-P* element carries the eye-specific enhancer and expresses *w* more strongly. Insertions in TAS and in HTT close to TAS are repressed, while those in HTT far from TAS are not. Other symbols are as in Fig. 1.

Figure 4.

Trans-effect of TAS deletions on the expression of telomeric transgenes. *w* transgene expression at the 2L telomere occurs primarily from the *w* promoter (orange bent arrow) with a small contribution from the adjacent *HeT-A* promoter (blue bent arrow). This expression is repressed by the adjacent TAS in cis (orange oval) in the presence of a full length TAS array (red line). Deletion of 2L TAS in trans (*Df{2L}M26*) causes higher expression (green round arrow) of the *w* transgene. Others symbols as in Fig. 1

Drosophila Genes involved in telomere stability

Table 1

Mutant phenotype						
Gene	Protein	HOAP, HP1 mislocalization	Telomere fusions	Increased transposition to telomeres	Increased telomeric gene conversion	Human homologue
<i>Su(var)205</i>	HP1	Yes	Yes	Yes	Yes	HP1a (11,29,64)
<i>cav</i>	HOAP	Yes	Yes			-- (39,40,47)
<i>tefu</i>	ATM	Yes ^{*,†}	Yes			ATM (35,39-43)
<i>eff</i>	UbcD1	No	Yes			UBE2D2 (46)
<i>mre11</i>	MRE11	Yes	Yes			Mre11 (35,36,39,40)
<i>rad50</i>	Rad50	Yes	Yes			Rad50 (36-38)
<i>nbs</i>	Nbs	Yes	Yes			NBS1 (37,39,40)
<i>woc</i>	WOC	No	Yes			ZMYM4 (45)
<i>mei-41</i>	ATR	Yes [*]	Yes [*]			ATR (39,40)
<i>mus304</i>	ATRIP	Yes [*]	Yes [*]			ATRIP (39,40)
<i>Irbp</i>	Ku70			Yes	Yes	Ku70 (53)
<i>Ku80</i>	Ku80			Yes	Yes	Ku86 (53)
<i>aub</i>	AUB			Yes		PIWIL1 (63)
<i>spn-E</i>	SPN-E			Yes		TDRD9 (63)
<i>E(tc)</i>	E(TC)			No	Yes	-- (66)

* The ATM and ATR pathways are partially redundant. The strongest phenotypes are for double mutants with defects in both.

† Mislocalization is observed in polytene chromosomes where telomere fusions had not been found. Mislocalization was not observed in diploid chromosomes.

Table 2

Other functions of telomere-associated proteins.

Protein	Full Name	Proposed function outside of telomeres
The Cap*		
HP1	Heterochromatin Protein 1	Forms heterochromatin and silent euchromatin
HOAP	HP1-ORC Associated Protein	None known
Required to Prevent Telomere Fusions		
ATM	Ataxia Telangiectasia Mutated	kinase; DNA damage response
UbcD1	Ubiquitin conjugating enzyme D1	Degradation of inhibitor of apoptosis protein 1
MRE11	Meiotic Recombination 11	DNA endonuclease; DNA damage response
RAD50	Radiation Sensitive 50	DNA exonuclease; DNA damage response
NBS	Nijmegen Breakage Syndrome	DNA damage response
WOC	Without Children	Transcription factor
ATR	Ataxia Telangiectasia Related	kinase; DNA damage response
ATRIP	ATR Interacting Protein	DNA helicase; DNA damage response
Required to Maintain Telomere Length		
Ku70	Ku subunit 70	Nonhomologous end joining
Ku80	Ku subunit 80	Nonhomologous end joining
AUB	Aubergine	RNA interference
SPN-E	Spindle-E	RNA helicase; RNA interference
E(TC)	Enhancer of telomeric gene conversion	None known
TEL	Telomere elongation	None known
Terminal Binding		
SUUR	Suppressor of under-replication	Formation of interstitial heterochromatin
SU(VAR)3-7	Suppressor of variegation 3-7	Heterochromatin formation, condensation of male X
Retrotransposon Array		
JIL-1	JIL-1	Phosphorylates histone H3 at serine 10; maintains open chromatin

Protein	Full Name	Proposed function outside of telomeres
PROD	Proliferation disrupter	Heterochromatin formation; chromosome segregation
Z4	Z4	Separates chromatin compaction domains
HP1	Heterochromatin Protein 1	Forms heterochromatin and silent euchromatin
H3Me3K9	Histone H3 trimethylated at lysine 9	Heterochromatin formation
H3Me3K4	Histone H3 trimethylated at lysine 4	Mark for active chromatin
Telomere Associated Sequence		
PC	Polycomb	Cellular memory; transcriptional repressor
E(Z)	Enhancer of zeste	histone methylation; gene silencing
PH	Polyhomeotic	gene silencing
PSC	Posterior sex combs	chromatin remodeling; gene silencing
SCM	Sex comb on midleg	transcription factor; gene silencing
H3Me3K27	Histone H3 trimethylated at lysine 27	Mark for active chromatin

* The cap is defined operationally as preferential binding to the chromosome terminus and requirement for the prevention of telomere fusions.