Coordination of transposon expression with DNA replication in the targeting of telomeric retrotransposons in *Drosophila*

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Abstract

In *Drosophila*, a group of retrotransposons is mobilized exclusively to telomeres in a sequence-independent manner. How they target chromosome ends is not understood. Here, we focused on the telomeric element *HeT-A* and characterized the cell cycle expression and cytological distribution of its protein and RNA products. We determined the timing of telomere replication by creating a single *lacO*-marked telomere and provide evidence suggesting that transposon expression and recruitment to telomeres is linked to telomere replication. The *HeT-A*-encoded ORF1p protein is expressed predominantly in S phase, particularly in early S phase. Orf1p binds *HeT-A* transcripts and forms spherical structures at telomeres undergoing DNA replication. *HeT-A* sphere formation requires Verrocchio, a putative homolog of the conserved Stn1 telomeric protein. Our results suggest that coupling of telomere elongation and telomere replication is a universal feature, and raise the possibility that transposon recruitment to *Drosophila* telomeres is mechanistically related to telomerase recruitment in other organisms. Our study also supports a co-adaptive relationship between the *Drosophila* host and *HeT-A* mobile elements.

Keywords CST complex; *Drosophila* telomere; telomere replication; telomeric transposon

Subject Categories DNA Replication, Repair & Recombination

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Introduction

Transposable elements (TEs) are ubiquitous in eukaryotes and can make up a significant portion of the genome. TE insertions may be inconsequential, but in some cases they can have detrimental effects on the host genome, for example, by altering the function of genes in their vicinity or by inducing genome rearrangement via ectopic recombination between copies of related elements. On the other

hand, there are instances in which genetic conflict between the host and TE has resulted in domestication of a selfish element to fulfill cellular functions that are essential for host survival. For example, TEs have been co-opted to regulate origins of DNA replication (Costas *et al*, 2011) and to regulate the expression of cell fate genes (MacFarlan *et al*, 2011). Thus, the impact of TE insertions is influenced by the insertion sites and can be either detrimental or beneficial to the host.

How a particular site of insertion is chosen is not well understood. Retrotransposons transpose through an RNA intermediate and in a non-random fashion. It has been suggested that the state of chromatin around the future insertion site may play a role (Mularoni *et al*, 2012). Interaction between host- and transposon-encoded factors has also been implicated in targeting transposons to specific genomic regions (Kirchner *et al*, 1995; Xie *et al*, 2001). Extreme cases exist in which a class of transposons inserts exclusively at one genomic region. The best-studied examples are the R2 elements that target the rDNA locus in animals, and the SART and TRAS elements that target the telomeric repeats in *Bombyx mori*. In these two cases, the target specificity is determined, at least partly, by a transposon-encoded endonuclease that cuts within a specific DNA sequence at the target loci (Xiong & Eickbush, 1988; Fujiwara *et al*, 2005).

Another case of targeted transposition occurs in *Drosophila* species, where a class of non-LTR retrotransposons populates the telomeric regions. These elements transpose exclusively to the ends of the chromosome (Biessmann *et al*, 1990) and serve as the predominant way to counteract incomplete end replication, an essential function that is fulfilled by the telomerase reverse transcriptase in many other organisms (for a general review on *Drosophila* telomere protection, see Pardue & DeBaryshe, 2011 and Raffa *et al*, 2013). How the *Drosophila* elements achieve precise end-targeting is not understood. Remarkably, ectopically expressed transposon proteins can be targeted to chromosome ends (Rashkova *et al*, 2002), providing the first line of evidence for interaction between host and transposon factors.

Interestingly, the presence of these elements is neither sufficient nor necessary for attracting new transposition events. An engineered telomeric element placed internally does not attract additional insertions of similar elements (our unpublished results). More

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importantly, a telomeric element can attach to a chromosome end that has been deprived of native transposons and instead consists of artificial DNA sequences (Beaucher *et al*, 2012 and references therein). These results suggest that the transposon machinery is likely using features of the *Drosophila* telomeric chromatin as a guide for transposition.

The targeting of *Drosophila* telomeric transposons is reminiscent of the targeting of telomerase activities to chromosome ends. In both yeast and mammals, telomere elongation by telomerase appears coupled to telomeric replication by the conventional DNA replication machinery (Diede & Gottschling, 1999; Marcand *et al*, 2000; Zhao *et al*, 2009; Wellinger & Zakian, 2012). In addition, the capping complex, which prevents the recognition of telomeres as DNA double-strand breaks (DSB), is required for telomerase recruitment. For example, the yeast Cdc13-Stn1-Ten1 (CST) complex is an integral component of the protective cap at telomeres, and the Cdc13 subunit also recruits the telomerase complex (Pennock *et al*, 2001; Wellinger & Zakian, 2012). In mammals, the Tpp1-Pot1 complex recruits telomerase and participates in end protection (Hockemeyer *et al*, 2007; Xin *et al*, 2007). So far, no host factor has been identified as involved in the recruitment of telomeric transposons in *Drosophila*. Nor do we know whether telomere elongation in *Drosophila* occurs during telomere replication, although the localization of transposon RNA has suggested that their expression is turned on during S phase (George & Pardue, 2003; Walter & Biessmann, 2004).

In order to delve deeper into the mechanism that governs the precise telomere-targeting process in *Drosophila*, we have characterized the *HeT-A* element, the most abundant telomeric transposon in *Drosophila*, and focused on the endogenous behavior of its RNA and

protein components. Our results suggest that the coupling of telomere elongation with replication also occurs in *Drosophila* and might be universal, as we show that the *HeT-A* components are targeted to telomeres either during or immediately after their replication. Our results also support a universal connection between end-capping and end-elongation functions, as the Verrocchio protein, essential for telomere protection in *Drosophila*, is critical for the assembly of *HeT-A* machinery at telomeres.

Results

In vivo assembly of “*HeT-A* sphere” at telomeres

We chose to study *HeT-A* as a representative telomeric element. *HeT-A* has a single open reading frame (Orf) that encodes a predicted RNA-binding protein (Khazina & Weichenrieder, 2009), which we designate as Orf1p. Using antibodies generated against Orf1p, we performed immunoprecipitation (IP) experiments using whole extracts from embryos. This antibody recognizes a 110-kD protein (Fig 1A), suggesting that *HeT-A* is expressed at a low level in embryos. We obtained similar results when we used extracts from ovaries or cultured S2 cells.

To provide a positive control for the specificity of our antibodies, we took advantage of known genetic backgrounds in which *HeT-A* is de-repressed. Retrotransposons are silenced by piRNA and related pathways in the germline (Juliano *et al*, 2011; Pek *et al*, 2012). In mutants defective for these pathways, *HeT-A* expression is greatly de-repressed (Shpiz *et al*, 2011). When we performed Western blot analyses on ovary extracts from the *spindle E* (*spnE*) mutant, we

Figure 1. Orf1p is normally expressed at a low level.

- A Orf1p IP from embryonic extracts. IP was performed with a rabbit Orf1p antibody or its corresponding pre-immune serum (Pre-Im). Proteins on Western blots were detected with a guinea pig anti-Orf1p antibody. The 110-kD band indicated by the arrow is present in “Orf1p IP,” but absent in “Pre-Im IP” lanes. The same band is also undetectable in lanes in which input extracts were loaded. The positions for the 150- and 100-kD markers are indicated to the left of the picture.
- B Orf1p is overexpressed in *spnE* mutant ovaries. Extracts were taken from ovaries of the indicated genotypes. The 110-kD band is present only in the mutant.
- C, D Orf1p immunostaining of *spnE* mutant ovaries, with anterior to the left. In (C), a pair of mid-stage egg chambers was stained with DAPI for DNA and anti-Orf1p. In the merged picture, Orf1p signals are in red. The greatest concentration of Orf1p molecules is in the oocyte (arrowheads). Signal at the nuclear periphery of nurse cells is indicated by an arrow. In (D), an early egg chamber was similarly stained. Orf1p forms foci (arrowheads) in the developing cystoblasts.

observed a vast overproduction of the 110-kD Orf1p in comparison with the heterozygous control (Fig 1B). A similar overproduction of Orf1p was observed in ovaries from the *aubergine* mutant defective in piRNA biology. This overproduction of Orf1p offers an opportunity to test whether our antibody can be used to immunolocalize Orf1p. Consistent with Western blot results in Fig 1B, we only observed Orf1p staining in *spnE* ovaries. In mid-stage egg chambers, Orf1p concentrated in the perinuclear space of the nurse cells and the developing oocyte (Fig 1C). Interestingly, Orf1p can first be observed at the 4-cell cystoblast in the *spnE* mutant (Fig 1D), suggesting that stem cells and early differentiating cystoblasts lack *HeT-A* expression and that repression is not mediated by the *spnE*-controlled pathway.

We next characterized the cellular distribution of Orf1p in proliferating tissues. In whole-mount staining of wild-type larval brains, our antibody detected Orf1p foci in a subset of cells (Fig 2). We estimated that 11% of the nuclei are positive for Orf1p ($n = 1,206$), with an average of 5.5 foci per cell. Upon closer examination, we discovered that Orf1p was mostly confined to the nucleus, for which the periphery can be labeled with lamin antibodies (Fig 2). In addition, Orf1p foci often appeared as circles. In whole-mount staining, the angle at which a cell could be observed is random. Therefore, a 2-dimensional Orf1p circle translates to a 3-D sphere, which we named “*HeT-A* sphere.”

HeT-A spheres appear “hollow” by anti-Orf1p staining, suggesting that they are filled with other components of the transposon machinery. Since Orf1p is likely a RNA-binding protein and *HeT-A* RNA itself is the transposition intermediate, we hypothesized that the sphere is filled with *HeT-A* RNA molecules. We performed FISH with sense or antisense riboprobes under non-denaturing conditions to minimize hybridization to *HeT-A* DNA, followed by immunostaining of Orf1p. *HeT-A* RNA was detectable in 95% of the spheres ($n = 107$). We observed a strong hybridization signal localized to the inside of the sphere (Fig 3A and B) only with the antisense probe, suggesting the presence of sense *HeT-A* transcripts. However, we cannot exclude the possibility that some of the fluorescent

signals came from the probes hybridizing to telomeric ssDNA. Quantification of the fluorescent signals from Orf1p and *HeT-A* nucleic acids revealed that the signal peaks near the center of the sphere (Supplementary Fig S1). Moreover, *HeT-A* spheres can have diameters exceeding 1 micron, up to one-fifth of the diameter of the host nucleus (Supplementary Fig S1). Therefore, a *HeT-A* sphere is very large and likely contains all the components encoded by *HeT-A*.

For the attachment of *HeT-A* to telomeres, a *HeT-A* sphere has to be targeted to the ends of chromosomes. We investigated whether *HeT-A* spheres are telomere-bound by using the HOAP protein as a marker for telomeres. HOAP has been shown to predominantly, if not exclusively, localize to telomeres (Rashkova et al, 2002; Cenci et al, 2003; Rong, 2008). *HeT-A* spheres associate with HOAP foci very well, as 68% of spheres are HOAP-positive ($n = 112$, Fig 3C), suggesting that many if not all of the *HeT-A* spheres are targeted to chromosome ends. In addition, HOAP signals are not found in the center of the sphere, but are rather limited to the “ring” portion of the sphere (Fig 3D and E). In 62% of the HOAP-positive spheres, multiple HOAP signals can be associated with a single sphere (Fig 3C–E).

In summary, Orf1p is produced in a subset of the proliferating cells, forms a sphere filled with *HeT-A* sense transcripts, and often associates with multiple telomeres. A schematic representation summarizing our findings with regard to *HeT-A* assembly at telomeres is shown in the insert in Supplementary Fig S1.

Orf1p expression during the cell cycle

In whole-mount stained larval brains, such as those shown in Fig 2, we observed Orf1p signals in only a subset of the cells. We observed a similar non-uniform pattern in other proliferating tissues, such as imaginal disks that give rise to adult structures, and in cultures of *Drosophila* S2 cells (Supplementary Fig S2). Orf1p signal was undetectable in early embryos and in non-dividing cells such as the polytene cells from the larval salivary glands.

Previous results showed that *HeT-A* RNA is most abundant in S-phase cells (George & Pardue, 2003; Walter & Biessmann, 2004).

Figure 2. Orf1p is expressed in a subset of cells.

Whole-mount staining of larval brain. In the merged image, Orf1p signals are in red. A 5-μm scale bar is given.

Figure 3. Orf1p forms spheres at telomeres.

A, B Orf1p immunostaining combined with *HeT-A* RNA FISH. In these two examples, *HeT-A* RNA signals are associated with every *HeT-A* sphere and fill the interior of the spheres. In the merged images (A'' and B''), Orf1p signals are in green and RNA signals are in red. A 5-μm scale bar is given. C–E Double staining of Orf1p and HOAP. In the merged image (C''), HOAP signals are in red. Arrowheads in (C') and (C'') indicate instances in which multiple HOAP signals are associated with a single Orf1p sphere. A 5-μm scale bar is given in (C). (D) and (E) are images of Orf1p and HOAP double staining at 2× magnification compared to the images in (C). They highlight two other examples of multiple HOAP signals attached to single Orf1p spheres.

We wondered whether the protein product from these transcripts, that is, Orf1p, has a similar distribution. To address this question, we co-stained larval brains with antibodies against Orf1p and known markers for various phases of a typical cell cycle (a diagram showing the relevant markers is shown in Fig 4B). We did not observe co-localization of Orf1p with phospho-H3, suggesting that Orf1p-expressing cells are not in M phase (Supplementary Fig S3). We did observe overlapping signals of Orf1p and CycE (Fig 4A), a marker for the G1/S boundary. Interestingly, this overlap was only partial, with Orf1p-positive cells showing opposite CycE patterns and vice versa. We estimated that 31 % of Orf1p-positive cells are also CycE-positive ($n = 116$).

We hypothesized that these CycE-positive cells, and perhaps some CycE-negative cells that express Orf1p, may be at the G1/S boundary or in early S phase. To test this, we treated brains with the nucleotide analog EdU to label cells in the bulk of S phase. Interestingly, we observed essentially no overlap between Orf1p and EdU signals (Fig 4A), as only 3 of 86 Orf1p-positive cells displayed weak EdU signals. Therefore, a significant portion of Orf1p-positive cells appears to be at the G1/S boundary or in early S phase. However, some of the CycE-negative but Orf1p-positive cells could be in late S or G2 phases and would be EdU-negative as they might not have accumulated labeled nucleotides sufficiently during the EdU pulse. We used the CycB marker, which is broadly expressed in S, G2 and M phases in larval brains (May et al, 2005). Nevertheless, CycB enters the nucleus in G2 and thereby provides an excellent G2 marker. We observed a partial overlap of Orf1p with CycB (Supplementary Fig S3) with 28 % of Orf1p-positive cells also scored as CycB-positive ($n = 199$). However, only one of these 56 double-positive cells displayed nuclear CycB staining, suggesting that only a minor fraction of these cells were in G2. Nevertheless, a significant

number of cells that are double-positive for Orf1p and cytoplasmic CycB may still be in late S phase. In summary, our results suggest two possible phases of Orf1p expression during the cell cycle in larval brains: a window of Orf1p production at the G1/S boundary and one at the S/G2 boundary. We performed the same series of experiments to co-localize Orf1p with CycE and EdU in S2 cells and obtained essentially identical results (Supplementary Fig S2). These results thus extend those from previous reports on cell cycle regulation of *HeT-A* transcription.

The above results suggest the exciting possibility that at least some telomeres in *Drosophila* may replicate early in S phase and that retrotransposon machinery is recruited to the telomeres as they are undergoing replication. Since neither staining for a telomeric protein such as HOAP nor staining for the natural telomeric DNA (retrotransposon arrays) could unambiguously identify sister telomeres, we set out to create a single, molecularly marked telomere in order to monitor its state of replication.

Creation of a single marked telomere using the “telomere by design” scheme

Drosophila cells have the remarkable ability to accomplish *de novo* establishment of a functional telomere on a chromosome end with essentially any DNA sequence (Beaucher et al, 2012 and references therein). In the most common *de novo* telomere formation assay, a DNA double-strand break (DSB) is induced in the germline. Progeny are recovered that have inherited a terminally deleted target chromosome with a protective cap assembled on the proximal end of the DSB. We previously used the rare cutting I-SceI endonuclease to induce a site-specific DSB and achieved an average frequency of 50 % for *de novo* telomere formation on the I-SceI-induced DSB (Gao et al, 2010; Beaucher et al, 2012). However, our prior success relied on the fortuitous insertion of a transposon construct carrying the I-SceI cut site near one of the telomeres. We could better utilize the high efficiency of *de novo* telomere formation if we could pre-select the desired DNA sequence for the future telomere.

We recently developed the SIRT gene targeting method (Gao et al, 2008), in which an attachment site for the phage phiC31 integrase can be inserted at any position by homologous recombination. Subsequently, plasmids carrying essentially any DNA sequence can be directed to the targeted site via phiC31-mediated site-specific integration. Using the same principle, we developed the “telomere by design” scheme in which the *attP* attachment site is first targeted to the most distal essential gene, *modulo* (*mod*), on chromosome 3R (Supplementary Fig S4). Gene targeting also introduces a *white*⁺ (*w*⁺) eye marker gene distal to the *attP* site. The integrating plasmid carries the complementary *attB* attachment site, a *yellow*⁺ (*y*⁺) bristle marker gene, a cut site for I-SceI, and additional sequences for the future telomere. These DNA elements are arranged in a way that places the I-SceI cut site right next to and distal to the future telomere. These two elements are in turn flanked by *y*⁺ (proximal) and *w*⁺ (distal) markers on the chromosome (Supplementary Fig S4). Upon induction of I-SceI in the germline, progeny with a potential *de novo* telomere formed at the *mod* region would appear as white-eyed (*w*[−]) animals with wild-type bristle pigmentation (*y*⁺). Using this scheme, we placed a 240-copy tandem repeat of the lacO site at the very end of 3R and

Figure 4. Orf1p expression during the cell cycle.

- A** Whole-mount double staining of cell cycle markers (CycE and EdU) and Orf1p. In the left column, Orf1p and CycE show a partial overlap. In the merged image (CycE in green and Orf1p in red), the arrowheads point to four Orf1p-positive nuclei, only two of which are CycE-positive. The left column shows the lack of overlap between Orf1p and EdU signals.
- B** A schematic representation of a typical cell cycle with different phases indicated below the horizontal line. The cell cycle markers and their presumed distribution are indicated above the line.

used this unique molecular marker to test our hypothesis that Orf1p production marks cells undergoing telomere replication. The molecular characterization of this telomere is shown in Supplementary Fig S4.

Telomere replication coincides with Orf1p recruitment

To monitor the replication status of our lacO model telomere in Orf1p-expressing cells, we performed FISH on whole-mount brains with an oligonucleotide probe corresponding to a single lacO unit (either the plus or minus strand) and subsequently immunostained the tissue for Orf1p. Both lacO probes gave similar results. In Orf1p-positive cells, we observed three classes of lacO foci (Fig 5A). In the first class (defined as “Twin” in Fig 5), lacO exists as two foci in close proximity, which we interpret as representing two sister telomeres. In the second class (“Stronger Single” in Fig 5), although lacO exists as a single focus, it is clearly stronger in fluorescence intensity than surrounding lacO foci in Orf1p-negative cells (for fluorescence quantification procedure, see Supplementary Fig S5). We imagine that during its replication, the lacO array would be more accessible to the oligonucleotide probes. The increase in fluorescence intensity that we observed could be due to an increase in probe accessibility associated with replication, or the lacO array having undergone various degrees of replication, or the combination of the above two reasons. Therefore, we suggest that in these cells with a “Stronger Single” lacO focus, the 3R telomere is also in a replicative state. In the last class (“Single”), the single lacO focus appears just as bright as the lacO foci in surrounding cells. Quantification of the three classes of foci showed that in nearly 90% of the Orf1p-positive cells, the 3R telomere has undergone different degrees of replication. Interestingly, essentially all lacO foci in Orf1p-positive cells are associated with a *HeT-A* sphere, suggesting that the recruitment of transposon machinery to telomeres highly coincides with telomere replication.

Our lacO-marked telomere also provides a tool to further test our earlier proposition that some *Drosophila* telomeres replicate early in S phase. If some of the Orf1p-expressing cells are in early S phase as our results suggest, the lacO-marked telomere would be more likely to exist in a replicative state than another non-telomeric region in the same Orf1p-expressing cell. To test this, we repeated the immuno-FISH experiment on animals carrying a lacO array at three individual non-telomeric locations: two at internal regions of chromosome 3R and one at the subtelomere of 3R. This last control is the precursor line for the telomeric lacO stock prior to *de novo* telomere formation at the *mod* region. For all three controls, we observed a significantly smaller percentage of Orf1p-positive cells with replicated or replicating lacO arrays ($P < 0.0001$, Fig 5B). This result therefore supports our previous proposition that some of the Orf1p-expressing cells are in early S phase. Interestingly, we frequently observed subtelomeric lacO foci in close proximity to a *HeT-A* sphere (an example is shown in Fig 5A), consistent with lacO’s close proximity to the 3R telomere.

In summary, our results indicate first that at least some of the telomeres replicate early in S phase, and second that the recruitment of the transposon machinery, and quite possibly the act of transposition, coincides with telomere replication.

Figure 5. Orf1p-positive cells have replicated or replicating telomeres.

- A** Whole-mount Orf1p staining combined with DNA FISH to detect lacO arrays at different genomic locations. In each vertical pair of images, Orf1p staining is shown at the top and lacO FISH at the bottom. The insert is a 2× magnification of the merged image showing the relative positions between the lacO focus (in red) and the *HeT-A* sphere (in white). The *HeT-A* sphere and lacO focus of interest are indicated by arrowheads. For the telomeric lacO array, three classes of lacO foci are shown with their classification indicated at the top.
- B** The distribution of the three classes of lacO foci according to the location of the lacO arrays is shown as color-coded vertical bars. The sample sizes for FISH experiments are: 109 for telomeric lacO; 78 for subtelomeric lacO; 105 for internal lacO #1; and 121 for internal lacO #2. $P < 0.0001$ for all pairwise comparisons between telomeric versus non-telomeric lacO arrays.

Verrocchio is required for the appearance of *HeT-A* spheres at telomeres

Telomeric DNA sequences have been ruled out as a determinant for the targeted transposition of telomeric retroelements. We took a candidate approach to identify host factors important for this process by performing Orf1p immunostaining in mutants known to cause telomere dysfunction. We initially focused on mutants that disrupt telomere transcription and telomere capping.

The heterochromatin protein 1 (HP1), encoded by the *su(var)205* gene in *Drosophila*, is essential for transcriptional silencing of telomeric retroelements. In *su(var)205* homozygous mutants, *HeT-A* transcription is greatly de-repressed (Savitsky et al, 2002; Perrini et al, 2004). We observed strong Orf1p staining accompanied by very large *HeT-A* spheres (Fig 6B and Supplementary Fig S6). We suggest that this size increase might be related to the highly elevated *HeT-A* transcription in the mutant. RNA FISH revealed abundant *HeT-A* transcripts inside these large spheres, with *HeT-A* RNA present

in at least 80% of the spheres ($n = 69$). Since our HOAP staining is highly variable in the *su(var)205* mutant background, we cannot discern whether the large *HeT-A* spheres in the mutant cells are still targeted to chromosome ends. Nevertheless, we conclude that HP1 is at least not essential for the assembly of *HeT-A* spheres.

Another prime candidate for Orf1p recruitment, due to its predominant and continuous localization at telomeres, is the HOAP protein encoded by the *cav* locus. In the *cav*¹ mutant, there appeared to be a reduction in the overall abundance of *HeT-A* spheres (Fig 6C and lower magnification images in Supplementary Fig S6) with an average of 2.2 foci per cell ($n = 76$), which is significantly reduced from 5.5 foci per cell in wild-type animals. Nevertheless, wild-type sized spheres were observed in the mutant, suggesting that HOAP is at least not required for the assembly of the transposon RNP. In addition, we consider that the reduction in *HeT-A* sphere abundance may not be suggestive of a role for HOAP in recruiting Orf1p, rather it might be a secondary effect of severe uncapping defects in the mutant. Mutants of *cav* have the strongest defect in telomere

Figure 6. Orf1p distribution in telomere mutants.

A–E Whole-mount staining of Orf1p in three mutants with a wild-type control (*wt*). Genotypes are given for each mutant. For both *ver¹* and *ver³⁴²*, the animals were trans-heterozygous for the indicated *ver* allele and a deficiency of the *ver* locus. For the *su(var)205* (HP1) mutant, note the large size of Orf1p spheres. For both *ver¹* and *ver³⁴²*, note a prominent nuclear background of Orf1p that lacks spheres.
F–H Mitotic chromosome squashes from neuroblasts with the indicated genotypes. In the *ver¹* nucleus, most chromosomes engage in telomere fusion. In the *ver³⁴²* nucleus, only one pair of chromosomes is end-fused.

capping as measured by telomere fusion frequency among all known telomere-uncapping single mutations (Cenci *et al*, 2003; Bi *et al*, 2005). It is likely that Orf1p recruitment requires a DNA end, of which the number should be greatly reduced in *cav*-mutant cells, which might then result in fewer *HeT-A* spheres. Therefore, we favor the proposition that HOAP is not directly required for Orf1p recruitment.

Recently, Raffa *et al* (2010) reported that the Verrocchio (Ver) protein, essential for telomere protection in *Drosophila*, shares significant sequence and structural similarities with the Stn1 protein, which is a component of the conserved Cdc13-Stn1-Ten1 (CST, Ctc1-Stn1-Ten1 in higher eukaryotes) complex that is essential for telomere maintenance. Supplementary Fig S7 shows sequence similarities between Ver and Stn1 proteins from other organisms. Strikingly, Orf1p foci are essentially absent in brain cells from the *ver¹* null mutant (Fig 6D). In wild-type animals, 83% of the Orf1p-positive cells ($n = 181$) contained at least one *HeT-A* sphere. This number drops to 1% in *ver*-mutant cells ($n = 134$). The fact that *ver¹* cells also experience massive telomere fusion could confound our interpretation of the Orf1p results, as we discussed previously for the *cav* mutant. We fortuitously recovered a hypomorphic allele of *ver*, *ver³⁴²* (see Supplementary Materials and Methods). Mutants homozygous for *ver³⁴²* or trans-heterozygous for *ver³⁴²* and *ver¹* survived as fertile adults, in contrast to absolute lethality of *ver¹* mutants. Dividing cells of the hypomorphic mutant displayed rather mild uncapping defects. Mitotic cells trans-heterozygous for *ver¹* and a deficiency covering the *ver* locus had an average telomere fusion frequency of 5.4 fusions per cell, with 100% of the cells showing at least one fusion ($n = 37$). On the other hand, cells trans-heterozygous for the hypomorphic *ver³⁴²* allele and the same deficiency had an average of 1.5 fusions per cell, with only 44% of the

cells showing fusions ($n = 105$) (see Fig 6G–H for examples of telomere fusion). Remarkably, even in the presence of abundant telomere ends, the overall abundance of *HeT-A* spheres was still vastly reduced in the hypomorphic mutant, as only 8% of the Orf1p-positive cells ($n = 276$) contained at least one *HeT-A* sphere versus 83% in wild-type (Fig 6E and Supplementary Fig S6). The reduction in the abundance of Orf1p foci in *ver* mutants was accompanied by a decrease in Orf1p protein level (Supplementary Fig S9). However, we do not favor the interpretation that reduced production of Orf1p directly causes the loss of Orf1p foci. Even with a reduction in the steady-state Orf1p level as measured by Western blot analyses, Orf1p is nevertheless produced sufficiently for Orf1p-positive cells to be easily identified in *ver¹* mutant brains (Fig 6 and Supplementary Fig S6). Therefore, there are ample Orf1p molecules in the nucleus, yet they are not being recruited to form telomeric spheres. This is more consistent with the proposition that Ver is required for Orf1p recruitment to chromosome ends and that in the presence of insufficient amounts of Ver, Orf1p is not recruited to telomeres and might be subsequently degraded. Interestingly, the absence of *HeT-A* spheres in *ver*-mutant cells was accompanied by the disappearance of clustered *HeT-A* transcripts in RNA FISH experiments (Supplementary Fig S10) along with an approximately threefold reduction in the steady-state level of *HeT-A* transcripts as measured by qRT-PCR (see Materials and Methods). The *ver* mutation did not affect the partial overlap between Orf1p and CycE expression, suggesting that the defect in *HeT-A* sphere formation is not likely due to a general disruption of the cell cycle.

In summary, we have identified the potential *Drosophila* Stn1 homolog Ver as an important factor for regulating the end-targeting of *HeT-A* spheres.

Discussion

Drosophila telomeric transposons have the remarkable ability to target chromosome ends for transposition. In this study, we have characterized the components of the *HeT-A* machinery and their relationship with host factors and cellular programs. Our results reveal a multifaceted regulation of the end-targeting process, which highlights the co-adaptive relationship between the selfish element and its *Drosophila* host.

Mechanisms that ensure end-targeting of *HeT-A*

Orf1p oligomerization may facilitate end-targeting

We discovered that *HeT-A* Orf1p localizes in the nucleus and forms spherical structures that encapsulate its transcripts. The *HeT-A* sphere, in the most general view, resembles virus-like particles produced by other retroelements in *Drosophila*, such as copia and gypsy elements (Shiba & Saigo, 1983; Song *et al*, 1994), except that *HeT-A* spheres are orders of magnitude larger and that *HeT-A* spheres are confined to the nucleus. *HeT-A* sphere formation can potentially serve two functions, first by concentrating factors essential for transposition and delivering them to the target site and second by protecting the components in the sphere from the cellular degradation machinery of the host. Orf1p oligomerization is likely a prerequisite for *HeT-A* transposition, and if this critical process were limited to the site of telomeres, *HeT-A*

transposition to other parts of the genome would be greatly inhibited. This is likely true based on the observation that Orf1p-positive cells have a generally uniform Orf1p nuclear signal background in addition to the prominent foci of *HeT-A* spheres, which appear mostly associated with telomeres (Fig 3). This suggests the interesting possibility that telomeres contain a nucleation signal for Orf1p oligomerization.

Telomeric transcription may recruit *HeT-A* spheres

We consider the possibility that ongoing *HeT-A* transcription at telomeres might be a nucleation event for Orf1p multimerization and that this may serve to recruit the transposon machinery. Since Orf1p binds its transcripts, we envision that nascent *HeT-A* transcripts could attract nuclear Orf1p molecules to telomeres. As the local concentration of Orf1p increases, polymerization results. The supporting evidence for this model is as follows. First, the *HeT-A* sphere appears restricted to telomeres, as discussed above. Second, the size of *HeT-A* spheres increases in *su(var)205* mutants, possibly correlating with a higher level of *HeT-A* transcription (Fig 6).

Although highly attractive, this hypothesis seems inconsistent with our results in which a telomere, consisting entirely of lacO repeats and hence lacking the ability to generate nascent *HeT-A* transcripts, is still able to efficiently attract *HeT-A* spheres. Our recent results suggest that multiple telomeres cluster in *Drosophila* interphase cells and that the same lacO telomere can cluster with transposon-capped telomeres (Wesolowska et al, 2013). Therefore, it is possible that the *HeT-A* sphere attached to the lacO telomere was actually recruited from nascent transposon transcripts that originated from other telomeres in the same cluster. Nevertheless, *HeT-A* spheres could be recruited to a “*HeT-A*-less” telomere via other mechanisms. For example, a sphere could become detached from a telomere and be subsequently recruited to the lacO telomere via protein-protein interaction between Orf1p and the capping complex.

Mechanisms that prevent spurious *HeT-A* transposition

The length of the telomeric transposon arrays varies among different stocks (Abad et al, 2004b; George et al, 2006), suggesting that *HeT-A* transposition happens sporadically. The rate of transposon attachment to *de novo* telomeres can also be low in the germline (Biessmann et al, 1992; Beaucher et al, 2012). On the other hand, our cytological results suggest that Orf1p, along with the RNA intermediate, is efficiently targeted to multiple telomeres in a cell. Therefore, many *HeT-A* spheres targeted to telomeres must not have resulted in actual transposition. Since *HeT-A* does not encode the reverse transcriptase (RT) needed for its transposition, we propose that the rate-limiting step in *HeT-A* attachment is the inclusion of an RT activity in *HeT-A* spheres. *HeT-A* was proposed to have evolved by losing the RT function from an ancient element (Abad et al, 2004a). This loss might have been evolutionarily advantageous to the host as it effectively limits rampant transposition of a selfish element.

The host also developed defense mechanisms regulating the selfish elements. First, to prevent overexpression, host proteins such as HP1 heterochromatinize telomeric transposons. Second, host proteins that specifically bind telomeres have developed the ability to potentially interact with Orf1p and direct *HeT-A* to chromosome ends. In

addition, other host proteins might also interact with Orf1p. For example, when overexpressed in *Drosophila* cultured cells, Orf1p has been shown to interact with the Z4 protein (Silva-Sousa et al, 2012). Through genetic analysis, we identified the Ver protein as being essential for *HeT-A* sphere formation. This defect seems specific to Orf1p's targeting to telomeres, as Orf1p is still able to enter the nucleus and the timing of Orf1p production appears unchanged in the mutant. The absence of *HeT-A* spheres in *ver* but not *cav* mutants seems inconsistent with prior results reporting the lack of Ver protein on *cav*-mutant telomeres in polytene cells (Raffa et al, 2010). It is possible that sufficient Ver remains on telomeres for recruiting *HeT-A* spheres in diploid cells of the *cav* mutant. Conceivably, the reduction in the average number of *HeT-A* spheres per cell from 5.5 to 2.2 in *cav*-mutant brains may reflect a partial loss of Ver on *cav*-mutant telomeres. Alternatively, Ver could be essential for sphere assembly independent of its association with telomeres. Currently, we are unable to distinguish these two possibilities.

The coupling of end-elongation and end replication is universal

Although Muller coined the word “telomere” based on experiments performed in *Drosophila*, the fly is not one of the popular systems for the study of telomere functions due to the fact that *Drosophila* lacks a telomerase. However, studies in *Drosophila* have contributed significantly to our understanding of fundamentally conserved features of telomeric functions. In this study, our results reveal that the coupling of end-elongation and end replication is likely universal.

In *S. cerevisiae*, telomeres replicate late in S phase (McCarroll & Fangman, 1988), and telomerase action has been suggested to couple with replication (Diede & Gottschling, 1999; Marcand et al, 2000). In cultured human cells, telomeres replicate throughout S phase (Ten Hagen et al, 1990), and yet telomere elongation occurs within 30 min of replication (Zhao et al, 2009). The coupling of telomere elongation and replication is thought to be important in that it facilitates the conversion of the single-stranded DNA, a result of telomerase action, into double-stranded DNA by conventional lagging-strand DNA synthesis. This function is perhaps more important for *Drosophila* telomeres, since reverse transcription of a single *HeT-A* element adds over 6 kb of ssDNA to the end.

We now have evidence to suggest that telomere replication is also accompanied by the recruitment of telomere-elongating activities in *Drosophila*. Results from our lacO marking of a single telomere suggest that Orf1p expression and telomere replication can be intimately connected. We found a high incidence of Orf1p-positive cells in which the lacO array exists in a replicative state. In addition, we observed association between lacO arrays with *HeT-A* spheres in all the Orf1p-positive cells that we imaged. If the 3R telomere appropriately represents the behavior of most telomeres, we can deduce that most replicating telomeres are associated with a *HeT-A* sphere. Certain telomeres, such as those associated with genomic regions that are mostly heterochromatic (e.g., telomeres on the Y chromosome), might behave differently from the 3R telomere in terms of the timing of replication. These “specialized” telomeres might replicate during late S phase when the bulk of heterochromatin is replicated. Nevertheless, our cell cycle profiling of Orf1p expression suggests that Orf1p may also be present during late S phase, making it possible that even for these “specialized” telomeres, Orf1p might be recruited during end replication.

The relationship between end-capping and end-elongation functions

In telomerase-maintained organisms, telomeric repeats added by the telomerase also serve as binding sites for capping proteins. This contributes to an intimate connection between the capping and elongation functions of telomere in these organisms. The Cdc13 subunit of the yeast CST complex and the Tpp1-Pot1 complex in mammals are examples of proteins serving both functions. Since end-capping can be sequence-independent in flies, *Drosophila* has been labeled as an organism in which end-capping and end-elongation are naturally uncoupled. Nevertheless, the two functions can also share common players in *Drosophila*, as we now identify Ver as such a candidate protein.

Drosophila Ver shares sequence homology to Stn1 proteins from a variety of organisms. However, the functional relationship between Ver and Stn1 is currently not clear. Both Ver and Stn1 are required for telomere capping. However, although our results suggest that Ver might regulate transposon recruitment positively, Stn1 has been shown to counteract telomerase action in budding yeast and mammals (DeZwaan et al, 2009; Chen et al, 2012). Further functional dissection of Ver is needed to uncover any mechanistic links between telomerase and transposon recruitment to telomeres. Such links are implicated by numerous natural cases in which retrotransposon-based and telomerase-based mechanisms function cooperatively to maintain telomere length in a single organism (e.g., Arkhipova & Morrison, 2001; Gladyshev & Arkhipova, 2007).

Materials and Methods

Drosophila stocks

All stocks were obtained from the Bloomington *Drosophila* stock center unless noted otherwise. All stocks are described in flybase (flybase.net) unless noted otherwise. The mutant stocks for *spnE* and *aub* were kindly provided by Dr. Elissa Lei of NIH.

Antibodies

Rabbit and guinea pig (GP) anti-ORF1p antibodies were raised against an antigen consisting of residues 99–340 of ORF1p (GenBank sequence U06920), and affinity-purified using the same antigen. The coding region for the antigen was amplified with primer pair: Gag_HeT_5.1_BamH1 and Gag_HeT_3.1_SacI and cloned into the BamHI and SacI sites in the QIAexpress pQE30 vector. The HIS-tagged peptide was purified under denaturing conditions per manufacturer's (Novagen, His•Bind Kits) instructions. A rabbit serum was used to perform immunoprecipitation and Western blots at 1:500. A guinea pig serum was used at 1:2,000 on Western blots and 1:1,000 in immunostaining experiments.

HOAP antibody was used as previously described (Gao et al, 2010). CycE antibody from Santa Cruz Biotechnology was used at 1:500 in immunostaining. Lamin labeling was performed with a mixture of antibodies (clones ADL84.12, ADL67.10, and Lc28.26 from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa, Iowa City, IA). CycB antibody (clone F2F4, DSHB) was used at 1:100.

Extract preparation from ovaries and embryos

For the preparation of extracts for Western blots, ovaries were dissected in PBS and homogenized in 1× Laemmli buffer (Bio-Rad), boiled for 5 min and stored at −20°C until use. For immunoprecipitation (IP), ovaries or embryos were collected in 1.5-ml tubes, flash-frozen in liquid nitrogen and stored at −80°C until use. IP was performed as previously described (Gao et al, 2010, CHIP protocol), except with no cross-linking or reverse cross-linking. After IP, samples were boiled immediately in 1× Laemmli buffer and stored at −20°C until further analysis.

Immunohistochemistry

Ovaries and larval tissues were dissected in fresh PBS and fixed with freshly diluted 3.7% formaldehyde in PBS for 20 min at room temperature. RNA fluorescent *in situ* hybridization (FISH) was performed as previously described (Zhang & Ward, 2009), except that antidigoxigenin-rhodamine (Roche) was used to detect the RNA signal. Primers for generating RNA probes are listed in Supplementary Table S1. Following FISH, immunolabeling of proteins was performed following the standard protocol.

EdU labeling was performed as per the manufacturer's instructions (Invitrogen, Click-iT Alexa Fluor 488 Imaging kit). Larval tissue was cultured in 1× PBS with 1× EdU for 30 min prior to fixation and detection.

LacO FISH

Probe sequences are listed in the primer table (Supplementary Table S1). Immuno-FISH using LacO probes and guinea pig anti-ORF1p was performed as described (*Drosophila* Protocols, CSHL, protocol 2.7, Formaldehyde fixation onto slides for whole-mount FISH, and protocol 2.9, hybridization to tissue on slides or coverslips).

LacO foci fluorescent intensity was quantified using Astronomical Image Processing (AIP4WIN) software and single star photometry tool. More details are described in Supplementary Fig S5.

qRT-PCR to measure *HeT-A* transcript level

Larval brains were dissected in PBS from wandering third-instar larvae. RNA was extracted following standard TRIZOL (Life Technologies) RNA isolation protocol, and treated with Turbo DNA-free (Life Technologies) to remove genomic DNA contaminants. Each sample contains total RNA from approximately 100 brains. RNA amount in each sample was determined by quantitative RT-PCR using iScript one-step RT-PCR kit with SYBR green (Bio-Rad). Control RT-PCRs with no reverse transcriptase added gave negative results without signal, indicating that the amount of genomic DNA is undetectable. Relative Het-A expression levels were determined using threshold cycle (CT) values and normalized to *rp49* levels. Three independent tests were performed for each genotype. The primer used for *rp49* are *rp49_for* and *rp49_rev*. Those used for HeT-A are Het-A_3317F paired with Het-A_3496R, and HeT-A_2838F paired with HeT-A_2953R.

Experiments with primer pair of 3317F and 3496R indicated that *HeT-A* transcript level in *ver*¹ mutants is 26 ± 2% when compared with the *ver*¹/+ control. Experiments with primer pair of 2838F and

2953R indicated that *HeT-A* transcript level in *ver¹* mutants is $21 \pm 3\%$ when compared with *ver¹/+* animals.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

LZ and YSR conceived and designed the experiments. LZ, MB, YC, and YSR performed the experiments. LZ and YSR analyzed the data. LZ and YSR wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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