The Chromosomal Proteins JIL-1 and Z4/Putzig Regulate the Telomeric Chromatin in Drosophila melanogaster

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Abstract

Drosophila telomere maintenance depends on the transposition of the specialized retrotransposons HeT-A, TART, and TAHRE. Controlling the activation and silencing of these elements is crucial for a precise telomere function without compromising genomic integrity. Here we describe two chromosomal proteins, JIL-1 and Z4 (also known as Putzig), which are necessary for establishing a fine-tuned regulation of the transcription of the major component of Drosophila telomeres, the HeT-A retrotransposon, thus guaranteeing genome stability. We found that mutant alleles of JIL-1 have decreased HeT-A transcription, putting forward this kinase as the first positive regulator of telomere transcription in Drosophila described to date. We describe how the decrease in HeT-A transcription in JIL-1 alleles correlates with an increase in silencing chromatin marks such as H3K9me3 and HP1α at the HeT-A promoter. Moreover, we have detected that Z4 mutant alleles show moderate telomere instability, suggesting an important role of the JIL-1-Z4 complex in establishing and maintaining an appropriate chromatin environment at Drosophila telomeres. Interestingly, we have detected a biochemical interaction between Z4 and the HeT-A Gag protein, which could explain how the Z4-JIL-1 complex is targeted to the telomeres. Accordingly, we demonstrate that a phenotype of telomere instability similar to that observed for Z4 mutant alleles is found when the gene that encodes the HeT-A Gag protein is knocked down. We propose a model to explain the observed transcriptional and stability changes in relation to other heterochromatin components characteristic of Drosophila telomeres, such as HP1α.

Introduction

Telomere elongation is needed in all eukaryotes with linear chromosomes due to the incapacity of cellular polymerases to proceed in 3' to 5' direction. Telomere length homeostasis is important for protecting the chromosomes from terminal erosion and the loss of important genetic information. Moreover, a defined telomere length is required for the proper assembly of the telomere-capping complex (shelterin in telomerase telomeres or terminin in Drosophila) [1–3]. When telomeres recess excessively, the disassembly of the protective cap leaves the telomere ends unprotected. Consequently, the telomeres are recognized by the DNA damage machinery, and upon repair are fused together resulting in genomic instability [4]. Eukaryote telomeres are dynamic structures that make up their telomere length from a balanced mechanism of gains and losses. The net result of this process is a telomere of the appropriate length to exert the different telomeric functions, as well as for protecting the genetic content [5,6].

Several proteins have been described with both positive and negative effects on telomere length regulation [1]. Some of these cellular components act regulating the different telomerase subunits either by directly activating their expression, or their biochemical function [7]. On the other hand, changes on the telomeric chromatin have also been related to changes in telomere length in several organisms pointing to an epigenetic component in telomere regulation in eukaryotes [8]. Thus, telomere length homeostasis is a complex cellular process that integrates signals from different regulatory mechanisms.

Drosophila is the telomerase exception better studied so far, having acquired a retrotransposition based mechanism whose prevalence along all the genus (120 MY) demonstrates its robustness [6,9,10]. The success of this mechanism is based in the targeted transposition of three different specialized non-LTR retrotransposons, HeT-A, TART and TAHRE [11–14]. Retrotransposons belong to Class I transposable elements (TE), and their mechanism of transposition involves an RNA intermediate implying that each new successful transposition will increase the copy number of the element. This, in the case of the telomeric transposons will translate in increased telomere length directly benefiting the host and indirectly incrementing the absolute copy number of the telomeric transposons, ensuring their survival.

Recent molecular studies demonstrate that telomeres in most eukaryotes are composed of two domains; the protective cap that lies at the very end and the distal (telomeric) domain. Flanking the telomere domain lays the proximal (subtelomeric) domain, which shows different chromatin characteristics [8,13,16]. The telomeric domain is composed of the telomerase repeats in telomerase
Drosophila telomeres constitute a remarkable exception to the general telomerase mechanism of telomere maintenance in eukaryotes. The essential role of the telomeric transposons HeT-A, TART, and TAHRE (HTT) in this organism contrasts with the strong conservation of their retrotransposon personalities. The particularities of this system add an extra layer of complexity to the control of telomere length in Drosophila; on one hand, telomere expression should be fine-tuned in order to achieve telomere function whenever needed; on the other, terminal transposition should be tightly controlled to guarantee genomic stability. Here, we report the dual role of the JIL-1-Z4 complex in regulating the HeT-A retrotransposon transcription (by the action of the JIL-1 kinase) and in guaranteeing the stability of telomeres (by the zinc finger protein Z4). We show how the loss of JIL-1 and Z4 causes major changes at the chromatin of the HeT-A promoter that can explain the phenotypes that we observe in JIL-1 and Z4 mutant alleles. Moreover, we give evidence of the involvement of the HeT-A Gag protein in the recruitment of Z4 to the HTT array, and we demonstrate how the disruption of this interaction has fatal consequences for telomere stability.

Author Summary

Drosophila telomeres constitute a remarkable exception to the general telomerase mechanism of telomere maintenance in eukaryotes. The essential role of the telomeric transposons HeT-A, TART, and TAHRE (HTT) in this organism contrasts with the strong conservation of their retrotransposon personalities. The particularities of this system add an extra layer of complexity to the control of telomere length in Drosophila; on one hand, telomere expression should be fine-tuned in order to achieve telomere function whenever needed; on the other, terminal transposition should be tightly controlled to guarantee genomic stability. Here, we report the dual role of the JIL-1-Z4 complex in regulating the HeT-A retrotransposon transcription (by the action of the JIL-1 kinase) and in guaranteeing the stability of telomeres (by the zinc finger protein Z4). We show how the loss of JIL-1 and Z4 causes major changes at the chromatin of the HeT-A promoter that can explain the phenotypes that we observe in JIL-1 and Z4 mutant alleles. Moreover, we give evidence of the involvement of the HeT-A Gag protein in the recruitment of Z4 to the HTT array, and we demonstrate how the disruption of this interaction has fatal consequences for telomere stability.

Results

In order to select candidate proteins that could have a role regulating the expression of the telomere retrotransposons, we took advantage of a study that characterizes the protein distribution along the different domains of D. melanogaster telomeres. Andreyeva and collaborators used the Tel stock, with telomeres ten times longer than in wild type flies, which offer a better resolution of the different telomeric domains [17], to perform immunocytochemistry experiments. These experiments demonstrated that the proteins JIL-1 and Z4 localize specifically at the HTT domain but neither at the capping nor at the TAS subtelomeric domain [15]. We have centered all analyses presented here on the HeT-A retrotransposon, which is the most abundant of the three telomeric retrotransposons in the HTT array of D. melanogaster [31].

HeT-A transcription is regulated by JIL-1

We analyzed the levels of mRNA of the HeT-A element in different JIL-1 and Z4 mutant alleles and, in order to contextualize our results, we compared them with the levels obtained from the mutant allele of one of the genes already known to influence HeT-A expression, the Su(var)2-5 gene which encodes for HIP1a (Figure 1A and 1B) [32,33]. Different mutations in the Su(var)2-5 gene, result in a pronounced increase in HeT-A transcription and severe problems of telomere stability [32,33]. Because the number of HeT-A copies may vary among stocks, we determined the number of copies of the HeT-A element for each of the analyzed stocks (Figure S1A and S1B). We used this data to normalize the level of HeT-A transcription per number of copies and understand if JIL-1 and Z4 mutants show a differential HeT-A transcription activity (Figure S2A and S2B, Figure 1A and 1B). Because in any given stock full length and truncated HeT-A elements coexist at the telomeres [31,34], we performed the quantitative Real-Time experiments with two different sets of primers separated more than 5 kb along the full length HeT-A transcript (see materials and methods for primer details).

To determine the level of HeT-A transcription we used whole third instar larvae to extract total mRNA. Some of the larval
tissues in this stage, like the brain and imaginal discs, are in
demand for active cell division and have been reported to show
active HeT-A expression [35]. We analyzed a JIL-1 hypomorph
allele, JIL-1z60 that contains a molecular lesion, which results in
low levels of functional protein [22], and also a null allele
JIL-1z2 [25]. In addition, we analyzed the JIL-1Su(var)3-1 allele, which has
been suggested to be a gain of function allele [36]. Homozygous
JIL-1z60 animals have only a 17% of eclosion rate [22], and
JIL-1z2/JIL-1z2 are homozygous lethal [25].

We obtained a significant reduction of HeT-A transcription with
both sets of primers (3’UTR and gag gene) compared to the control
strain (w1118) for all the alleles with the exception of the
JIL-1Su(var)3-1 allele (Figure 1A and 1B, left section of the graphics). In
this last case, we did not observe a decrease in HeT-A transcription
probably due to the ectopic phosphorylation activity of JIL-1 in this
allele [30]. The JIL-1 alleles show telomere lengths comparable to wild type (Figure S1A and S1B). The three JIL-1 alleles tested are in different genetic backgrounds, although for the
hypomorph and the null mutations we have obtained very similar
results, we crossed all the alleles with the w1118 strain in order to
minimize the contribution of the genetic background in these
measures.

Similarly, we analyzed three different Z4 mutant alleles, Z47.1,
Z42.1 and pzg66. The Z47.1 allele is a hypomorph allele that lacks the
promoter region of the Z4 gene and is lethal at the pupal stage
[18]. The Z42.1 and the pzg66 alleles are null alleles that result in
embryonic and early larval lethality [18,21]. We obtained a
substantial increase in HeT-A transcription in the case of the
hypomorph allele Z47.1 (Figure 1A and 1B). The results are similar
for both sets of primers used indicating that the increase affects
most HeT-A copies. The increased HeT-A transcription in the
Z47.1 allele is consistent with a major level of HeT-A transcripts in all the
allelic combinations where this allele is present (Figure 1A and 1B).
In addition, the number of copies of the HeT-A element in this
allele was substantially increased indicating longer telomeres in
this stock (Figure S1A and S1B). Note that the level of HeT-A
transcription in the Z47.1 allele is close to the HeT-A transcription in the Su(var)2-552 mutation of HP1a. The Z47.1 and
pzg66 alleles did not show a different level of HeT-A transcription
compared to our control strain (w1118) for neither the 3’UTR

Figure 1. HeT-A expression normalized to copy number in different mutant alleles. HeT-A Gag (A) and HeT-A 3’UTR (B) transcripts decrease in JIL-1 mutants (JIL-1z60JIL-1z2) and in Z4 mutants when combined with JIL-1z2 null allele (JIL-1z2/Z47.1, JIL-1z2/Z42.1 and JIL-1z2/
pzg66). Z47.1 allele also affects HeT-A Gag transcripts but in this case an increase in the expression is observed. The Su(var)2-552 mutant allele, was used in this experiments as a positive control. HeT-A transcript levels were normalized to actin transcripts and corrected for the respective HeT-A copy number in each analyzed stock. Error bars represent standard deviations of three independent experiments. Asterisks indicate statistically significant differences using the t-test (one asterisk, P<0.05 to 0.01; two asterisks, P<0.01 to 0.001; three asterisks, P<0.001) in HeT-A expression of each mutant compared to w1118.
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region nor the gag gene. As expected the number of HeT-A copies in these null Z4 alleles is not significantly different from the control strain (w1118). Only when the Z4\(^{7.1}\) allele is combined with the Z4\(^{2.1}\) or the pzg66 alleles the levels of HeT-A transcription increase above the ones in the w1118 strain. The levels of HeT-A expression concerning the HeT-A gag gene in the Z4\(^{7.1}/Z4\(^{2.1}\) and the Z4\(^{7.1}/pzg66\) genotypes are significantly different from the ones obtained by the Z4\(^{7.1}/Z4\(^{7.1}\) homozygous combination. For the 3' UTR region, the difference in expression in only significant from the one obtained for the Z4\(^{7.1}/Z4\(^{7.1}\) genotype for the Z4\(^{7.1}/pzg66\) allelic combination.

We also investigated if we could observe a genetic interaction between JIL-1 and Z4 by measuring the levels of HeT-A transcription of a total of seven allelic combinations between JIL-1 and Z4 (Figure 1A and 1B, Figure S2A and S2B). Although for the Z4\(^{2.1}\) and pzg66 alleles we had not detected levels of HeT-A transcription significantly above the ones of w1118, we found that the combinations JIL-1\(^{z60}/Z4\(^{7.1}\) and JIL-1\(^{z60}/Z4\(^{2.1}\) for the gag gene, and the JIL-1\(^{z60}/pzg66\) for both the 3' UTR region and the gag gene recover HeT-A transcription to w1118 levels (Figure 1A and 1B). Accordingly with the results obtained for the single mutation, in the combination JIL-1\(^{z60}/Z4\(^{7.1}\) we obtained levels of HeT-A gag transcription above the ones in w1118.

JIL-1 regulates transcription by chromatin changes in the HeT-A promoter

Because both JIL-1 and Z4 are proteins related with chromosome structure, we studied if the changes observed in the expression of the HeT-A retrotransposon were related to changes in the chromatin environment at the promoter of this retroelement. Therefore, we investigated by Chromatin immunoprecipitation (ChIP) experiments, changes in different chromatin marks and changes in the levels of the proteins of study, JIL-1 and Z4 (Figure 2), as well as of HP1a, a protein already known to localize at the HTT array and to affect HeT-A transcription (Figure 1A and 1C), [15,16,33].

To analyze the relative chromatin changes in the homozygous mutant alleles JIL-1\(^{z60}/JIL-1\(^{z60}\) and in the hypomorph Z4\(^{7.1}/Z4\(^{7.1}\) allele which had been the only one with increased HeT-A transcription, we measured trimethylation of both lysine 9 and 4 of Histone H3 (H3K9me3 and H3K4me3), the most characteristic histone modifications indicative of repressed and active chromatin. Figure 2A shows the relative changes, compared to wild type, in homozygous mutant alleles of JIL-1 (JIL-1\(^{z60}/JIL-1\(^{z60}\)) and Z4 (Z4\(^{7.1}/Z4\(^{7.1}\)). The increase observed for H3K9me3 in JIL-1\(^{z60}/JIL-1\(^{z60}\) mutants is in accordance with the decrease of HeT-A expression in the same allelic combination. In contrast, the
increase in HeT-A transcription of the $Z^f1/Z^f1$ allele has two different causes, a substantial decrease in H3K9me3 and a simultaneous increase in H3K4me3, indicative of active transcription.

Next, we quantified the presence of JIL-1 at the HeT-A promoter in mutant and wild type flies. Figure 2B shows the changes in JIL-1 occupancy at the HeT-A promoter. $Z^f1/Z^f1$ mutant flies show an increase in JIL-1, in accordance with a higher expression of He-T-A in this mutant allele. In contrast, Su(var)2-505/Cyo flies, heterozygous mutant for HP1a, show a moderate decrease of JIL-1 occupancy suggesting a subtle dependence between these two proteins. Interestingly, JIL-1$^{+60}$/JIL-1$^{+60}$ shows a substantial increase in the presence of HP1a at the HeT-A promoter, which could be in part responsible for the silencing of He-T-A expression in this same allele (Figure 2C). The $Z^f1/Z^f1$ allele on the other hand, shows HP1a levels comparable to Su(var)2-505/Cyo flies suggesting an interdependent relationship between these two chromosomal proteins. Finally, we observed that the presence of the Z4 protein decreases in JIL-1 and Su(var)2-5 mutants further interconnecting these three chromosomal proteins in their role of chromatin modulators at the HeT-A promoter (Figure 2D).

We did not perform ChIP analyses in the case of the $Z^f1$ and pZ$^{60}$ alleles for the HeT-A promoter because of two reasons; these alleles did not show a significant difference in HeT-A transcription, very few third instar larvae of the pZ$^{60}$/Z$^f1$ and $Z^f1/Z^f1$ genotypes are obtained from each cross, and for the combination pZ$^{60}$/Z$^f1$ no animals eclosed, making very difficult to perform this experiment with the adequate amount of material.

**JIL-1 interacts with Z4**

Because JIL-1 and Z4 localize similarly in polytene chromosomes and in the HTT array [13,18,22,23], and both of them had been found to directly interact with Chromator [27,28], we wondered if the two proteins could be directly or indirectly interacting. We thus performed a co-immunoprecipitation experiment with the endogenous proteins. Figure 3A shows how both proteins JIL-1 and Z4 are able to co-immunoprecipitate in Schneider S2 cells. Although the input lane of JIL-1 shows a very faint signal caused by the fact that a considerable amount of protein is not extracted and remains in the cell pellet, the protein is clearly detectable in the IP with the Z4 antibody, thus suggesting that a significative amount of soluble or extractable JIL-1 is part of a complex containing Z4. In the case of Z4, no substantial amount of the protein was detected in the pellet (not shown). The results of the co-immunoprecipitation experiments suggest that Z4 and JIL-1 belong to the same protein complex when they are at different genomic locations, such as at the HTT array.

**Z4 interacts with HeT-A GAG**

JIL-1 and Z4 specifically localize at the HTT array but not at the TAS or the cap domain, therefore a specific telomere targeting mechanism should be in place. One of the proteins that specifically localizes at Drosophila telomeres, is the Gag protein of the He-T-A element [37,38]. We therefore tested if He-T-A Gag could be involved in the targeting mechanism of Z4 and JIL-1 to the HTT array. We set a co-immunoprecipitation experiment with a recombinant form of He-T-A Gag fused to GFP together with the endogenous Z4 protein. Figure 3B shows that the He-T-A Gag-GFP protein co-immunoprecipitated with Z4, and that consequently Z4 co-immunoprecipitated with He-T-A Gag-GFP. Although we have not been able to detect co-immunoprecipitation of the endogenous He-T-A Gag with Z4 (we assume that due to low levels of expression of He-T-A Gag in most Drosophila tissues and cells), the overall data suggest that the Z4-He-T-A Gag interaction likely occurs in vivo. We did not detect a JIL-1-He-T-A Gag interaction (data not shown).

**Z4 and He-T-A gag mutants show telomere instability in mitotic cells revealed by the appearance of telomeric fusions**

Because changes in telomere length and telomere chromatin can result in telomere instability, we checked whether JIL-1 and Z4 mutant alleles showed any sign of genomic instability detectable by telomere fusions (TFs). We checked metaphase chromosome preparations from third instar larval neuroblasts of JIL-1 and Z4 mutants and compared them to a negative control ($w^{1118}$) and positive controls (mutant alleles of genes known to participate in telomere protection in Drosophila like woc and caravaggio, the gene encoding the HOAP protein [39,40].

We could observe TFs involving the same chromosome (intrachromosomal) and different chromosomes (inter-chromosomal) in all the Z4 mutant alleles present in this study $Z^f1$, $Z^{f1}$, and pZ$^{60}$, (Figure 4A, 2nd, 3rd and 4th column). Similarly, TFs were observed in neuroblasts from the positive control, woc$^{964}/woc^{R111}$ mutant allele (Figure 4A 5th column). No TFs were observed in neuroblast preparations of the negative control stock ($w^{1118}$) (Figure 4A 1st column).
We further investigated whether the observed TFs in the Z4 mutant alleles could be resolved during the next anaphase with no other consequences for the cell, or in contrast could cause asymmetric heredity of the genomic content and initiate genomic instability. We analyzed anaphase neuroblasts of Z4\(^{1}/Z4^{1}\) third instar larvae and compared them again with a positive (woc\(^{964}/\)woc\(^{111}\)) and a negative control (w\(^{111}\)). Figure 4B (2nd column) shows different anaphases of the Z4\(^{1}/Z4^{1}\) neuroblasts where chromatin bridges (1st and 3rd panel), and aberrant DNA content (2nd and 3rd panels) can be observed. Similarly, different chromatin bridges were observed for neuroblast preparations of woc\(^{964}/\)woc\(^{111}\) larval brains (3rd column Figure 4B). No abnormal
anaphases were observed for the w1118 neuroblast preparations (1st column Figure 4B).

In order to rule out a possible unrelated effect of the genetic background in the z4 mutant alleles, we knocked down z4 by RNA interference in S2 cells. Intra and inter-chromosomal TFs were also detected after the preparation of metaphase chromosomes of the interfered cells for the z4 gene (Figure 4C, 2nd column and Figure 4D). Again TFs were detected in the positive control (S2 cells interfered for the caranagro gene, encoding the HOAP protein) (Figure 4C 4th column and Figure 4D) and no TFs were observed when the S2 cells were interfered for an unrelated RNA (Figure 4C 1st column, and Figure 4D, see materials and methods for details).

As we had observed an interaction between Z4 and HeT-A Gag, we decided to test if the lack of the latter could also result in telomere instability. Due to the impossibility to obtain mutant alleles for HeT-A in D. melanogaster (many copies of the HeT-A element exist in any given stock, [31]), we decided to interfere for the HeT-A retrotransposon mRNA in S2 cells by RNAi. Figure 4C, 3rd column and Figure 4D show how a decrease on HeT-A mRNA and, as a consequence, on the levels of HeT-A Gag protein results in different TFs in metaphase chromosomes, involving chromatids from the same chromosome and from different chromosomes. Obtaining similar TF phenotypes when interfering for HeT-A Gag and Z4 reinforces the relationship of these two proteins at the HTT array.

In order to investigate other possible causes for the telomeric instability observed in the z4 mutant alleles, apart from the changes in the telomeric chromatin in these mutants, we tested two alternative hypothesis; 1) disturbance of the loading of the telomere-capping complex and 2) the possible involvement of the non-homologous end-joining DNA repair complex in fusing the telomeres after being recognized as a double strand break by the non-homologous end joining DNA repair complex in fusing the telomere-capping complex and 2) the possible involvement of the Ligase IV enzyme in the observed TFs in the z4 mutant alleles [41], by combining the z4 and Z4 mutant alleles (z4/f1/JIL-1c50/Z47.1 and z4/f1/JIL-1c50/Z47.1/HOAP-GFP) and we found no significant difference between the single z4 mutant alleles (Figure 4E), indicating that the partial increase in heterochromatin marks is not sufficient to compensate the lower levels of Z4 in a JIL-1/z4 mutant background. Therefore, the role of Z4 in the structure of the telomere chromatin is key to guarantee telomere stability in Drosophila.

### Discussion

JIL-1 is the first positive regulator of telomeric expression described in Drosophila

Much effort has been put forward to study the negative regulation of the telomeric retrotransposons [42] (for a review, see [6]) as these elements have been able to maintain their personalities or individual characteristics as transposable elements while fulfilling a cellular role [9,43–45].

HeT-A is a retrotransposon with the essential function of telomere elongation, and therefore a fine-tuned regulation capable of achieving both, telomere replication and avoiding putative harmful transpositions and consequently genomic instability.

We also inspected the possibility of TFs in the trans-heterozygous combination JIL-1c50/JIL-1c50 and found no result significantly different from the w1118 strain (Figure 4F).

Next, we decided to test if a JIL-1 mutant in a z4 mutant background could rescue the TF phenotype. Because in a JIL-1 mutant background some heterochromatin marks increase their presence in the HeT-A promoter, (H3K9me3 and HP1a, Figure 2A and 2C and Figure 3) it is possible that they are enough to compensate the lower amount of Z4 in the JIL-1 mutation (Figure 2D). With this purpose we tested the double mutant combinations (z4/f1/JIL-1c50, z4/f1/JIL-1c50, z4/f1/JIL-1c50, z4/f1/JIL-1c50) and we found no significant difference between the single z4 mutant alleles (Figure 4F), indicating that the partial increase in heterochromatin marks is not sufficient to compensate the lower levels of Z4 in a JIL-1/z4 mutant background. Therefore, the role of Z4 in the structure of the telomere chromatin is key to guarantee telomere stability in Drosophila.

![Figure 5. Model of the chromatin environment at the HeT-A promoter.](image)

(A) Wild type: Z4 defines a boundary at HeT-A promoter that protects from the action of HP1a and other heterochromatin markers. JIL-1 guarantees a certain level of euchromatin inside the HeT-A promoter in order to allow gene expression. (B) JIL-1 mutants: destabilization of the Z4 boundary and the heterochromatin spreads into the HeT-A promoter (enrichment in HP1 and H3K9me3). (C) Z4 mutants: Disappearance of the Z4 boundary, increase in euchromatin marks (H3K4me3) and decrease in heterochromatin marks (HP1 and H3K9me3). Subtle increase in JIL-1 and in euchromatinization of the HeT-A promoter. (D) In Su(var)2-5 mutants: The lack of HP1a allows relaxation of the Z4 boundary causing a JIL-1 and Z4 spread along the HTT array and a relative decrease of these proteins inside the HeT-A promoter. Although the levels of JIL-1 inside the HeT-A promoter are lower than in wild type, the release of silencing caused by loss of HP1a results in increased HeT-A expression.

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should be in place to ensure a normal telomere structure. During development, all the tissues that undergo active cell division such as the brain or the imaginal discs need certain levels of telomere replication. Naturally, these are the tissues where the telomeric retrotransposons are more expressed [35]. Here, we demonstrate that the JIL-1 kinase is important to achieve wild type levels of HeT-A transcription in larval tissues, being the first positive regulator of telomere transcription described in Drosophila.

Although in Drosophila the role of JIL-1 in activating transcription has remained controversial [46,47], at least in the HTT array it could act as a positive regulator of transcription for three different reasons: 1) When telomere elongation is needed, a fast activation of HeT-A transcription should be expected. Accordingly, the mammalian JIL-1 orthologous MSK1/2 have been shown to rapidly induce gene expression on the face of stress or steroid response [48]. 2) HeT-A is embedded into the HTT array, a domain that needs to be protected from the influence of the repressive heterochromatin of the neighboring TAS domain [16]. JIL-1 has been suggested to protect the open chromatin state from the spreading of neighboring repressive chromatin at certain genomic positions [49,50]. 3) The decrease in expression that we have observed in the JIL-1 mutants is moderate (Figure 1C). Recent data at genomic level revealed that JIL-1 function agrees with a reinforcement of the transcriptional capability of a particular genomic domain rather than net activation [51]. In summary, the telomeric role of JIL-1 at the HTT array is in agreement with all of the above.

Phalke and co-workers [52] suggest that JIL-1 has a role in retrotransposon silencing in general and has no effect on telomere transcription. A possible explanation for this discordance with our results and hypothesis is that the mutant allele of JIL-1 assayed by Phalke and co-workers, the JIL-1<sup>1<sup>10<sup>10<sup>/nm-3<sup>/alleles, corresponds to a C-terminal deletion of the JIL-1 protein that causes the protein to miss-localize and phosphorylate ectopic sites [30,36]. The ectopic phosphorylation caused by the JIL-1<sup>1<sup>10<sup>10<sup>/nm-3<sup>/alleles would activate the expression above wild type levels in those genes that normally are not targeted by JIL-1, as it happens to be the case for the Invader<sup>4 retrotransposon. In our study, we have assayed the JIL-1<sup>1<sup>10<sup>10<sup>/nm-3<sup>/alleles obtaining similar result than for the wild type stock, likely for similar reasons (Figure 1A and 1B). Supporting this, in addition of the JIL-1<sup>1<sup>10<sup>10<sup>/nm-3<sup>/alleles, we present here data from two more JIL-1 alleles (Figure 1), JIL-1<sup>1<sup>10<sup>10<sup> and JIL-1<sup>1<sup>10<sup>12<sup>, that correspond to loss of function alleles and, in both cases, result in a substantial decrease in HeT-A transcription (Figure 1A and 1B). Moreover, the changes in telomere transcription that we report here have been assayed directly on the major component of the HTT array, and not through a reporter [52]. Our data demonstrates that JIL-1 is necessary to maintain active transcription of the telomeric retrotransposon HeT-A or, what is the same, transcription from the telomeres in Drosophila.

Although we have demonstrated that JIL-1 is necessary to maintain transcription from the HTT array, we have not detected a decrease in telomere length in the JIL-1 mutant alleles. A reasonable explanation for this observation is that the JIL-1 mutant alleles here analyzed (JIL-1<sup>1<sup>10<sup>10<sup> and JIL-1<sup>1<sup>10<sup>12<sup>) have been maintained as heterozygous. It is therefore possible that one copy of JIL-1 is enough to promote enough HeT-A transcription to elongate significantly the telomeres when needed.

**Z4 is necessary to guarantee telomere stability in Drosophila**

Although in the case of the hypomorph mutation Z4<sup>1<sup>10<sup>12<sup> we have observed an increase in HeT-A transcription and HeT-A copy number significantly above the control strain (JIL-1<sup>1<sup>10<sup>12<sup>), the null alleles Z4<sup>2<sup>1<sup> and puz<sup>66<sup> do not show an up-regulation of HeT-A transcription or an increase in its copy number (Figure 1A and 1B, Figures S1 and S2). Although we have crossed all the stocks to the w<sup>1118<sup> strain to minimize the effects of the genetic background, it could still have a certain influence when comparing the puz<sup>66<sup> allele with the Z4<sup>1<sup>10<sup>12<sup>. Nevertheless the Z4<sup>1<sup>10<sup>12<sup> and Z4<sup>2<sup>1<sup> alleles come from the same genetic background [18]. A possible explanation could rely on the fact that the Z4<sup>1<sup>10<sup>12<sup> mutation is a hypomorph mutation where a small amount of Z4 protein is still present. By ChIP analyses we have detected an increase of JIL-1 protein at the HeT-A promoter above control levels, which could explain in part the major transcription of HeT-A in this mutant background (Figure 2B). Because Z4 and JIL-1 interact (Figure 3A), it is possible that although low, the amount of Z4 present in the Z4<sup>1<sup>10<sup>12<sup> allele is enough to recruit JIL-1 to the HeT-A promoter. In the puz<sup>66<sup> and the Z4<sup>1<sup>10<sup>12<sup> null alleles, JIL-1 cannot be recruited towards the HeT-A promoter and there is no increase in transcription. Nevertheless, with our current data we cannot conclude that Z4 directly controls the level of HeT-A transcription.

We have detected a phenotype of telomere instability in all three Z4 mutant alleles Z4<sup>1<sup>10<sup>12<sup>, Z4<sup>2<sup>1<sup> and puz<sup>66<sup> (Figure 4), suggesting a role of this chromosomal protein in guaranteeing telomere stability in Drosophila. Although a number of genes involved in the capping function in Drosophila still remain unidentified [3], we do not have evidences that Z4 directly participates in the protection of the telomeres. Mutant alleles of genes directly involved in the capping function, such as zoc or carangio (HOAP), show multiple and more numerous TFs in larval neuroblasts (Figure 4A, 4C and [39,40]) than the ones that we have observed in the Z4<sup>1<sup>10<sup>12<sup> mutant alleles. Moreover, we have been able to detect staining for one of the major capping components, the HOAP protein, in the TFs of Z4<sup>1<sup>10<sup>12<sup> mutant neuroblast cells (Figure 4D), indicating that the telomere-capping complex is still loaded to a certain degree. Instead of directly participating in the capping, our hypothesis is that the major chromatin changes caused by the lack of Z4 at the HTT array result in a secondary loss of necessary chromatin and capping components like HP1a (Figure 2C).

Results from the ChIP experiments (Figure 2) suggest a relationship between JIL-1, Z4 and HP1a in fine-tuning the chromatin structure at the HTT array. HP1a has a dual role at the telomeres explained by its participation in both the capping function and the repression of gene expression that also exerts in other genomic domains [33,53]. In the HP1a Su(var)2-5<sup>3<sup> allele, which is known to have a major transcription of HeT-A and problems of telomere stability, we have observed a pronounced decrease in Z4 and JIL-1 (Figure 2B and 2D). In the Z4<sup>1<sup>10<sup>12<sup> allele the decrease in Z4 protein is accompanied by a similar decrease in H3K9me3 and HP1a at the HeT-A promoter (Figure 2A, 2C and 2D). Finally in the JIL-1<sup>1<sup>10<sup>12<sup> allele the increase in silencing epigenetic marks like H3K9me3 and HP1a is also accompanied by a decrease in Z4 (Figure 2). In particular, the pronounced dependence of the presence of HP1a and Z4, points toward the loss of HP1a and H3K9me3 to a possible cause for telomere instability in the Z4 mutant alleles here studied. Interestingly, in the Su(var)2-5<sup>3<sup>/Su(var)2-5<sup>3<sup> heteroallelic combination (considered a null mutation) [33]), 15% of telomeres involved in telomere associations are still able to recruit the HOAP protein [40]. Therefore our data on HOAP localization in the Z4 mutant alleles is still consistent with the TFs being caused by the decreased availability of HP1a in these cells (Figure 2C). The above results demonstrate that Z4 in a coordinated manner together with JIL-1 and HP1a is an important component of the telomere chromatin in Drosophila, which upon its reduction causes significant changes in the chromatin of the HTT array, which are the cause of the
observed telomere instability in all the Z4 mutant alleles here studied (discussed below, Figure 5).

**HeT-A Gag targets the Z4-JIL-1 complex to the HTT array**

We have been able to detect a biochemical interaction between JIL-1 and Z4, and our data suggests that these two proteins can be components of the same protein complex (Figure 3A). This interaction had been previously suggested because both proteins have been found co-localizing in different genomic locations, but no direct proof existed to date [15,18,22,23,27,28]. In each genomic location where the Z4-JIL-1 complex is needed, a special mechanism of recruitment should exist. Importantly, we have shown how Z4 specifically interacts with HeT-A Gag (Figure 3B). HeT-A Gag is the only protein encoded by the HeT-A element and has been shown to specifically localize at the telomeres [37,38]. HeT-A Gag has been shown to be in charge of the targeting of the transposition intermediates for the HeT-A element and also for its telomeric partner the TART retrotransposon [37]. Interestingly, when we studied the consequences for telomere stability after knocking down the HeT-A gag gene by RNAi, we also observed similar TFs than when knocking down the Z4 gene, further relating the action of both genes in telomere stability. Z4 is known to participate in different protein complexes with roles in different genomic locations [19,21,27]. Because it has been demonstrated that Z4 is able to associate with a variety of proteins in these complexes, we think that the description of a mechanism for its specific targeting to telomeres through one of the telomeric retrotransposon proteins is especially relevant.

**A model for the role of JIL-1 and Z4 in Drosophila telomeric chromatin**

Integrating information from previous literature and the results exposed by this study, we propose a possible model to describe the state of the chromatin at the HTT array in each of these three mutant scenarios: JIL-1, Z4 and Su(var)2-5, as well as in wild type (Figure 5).

We should take into account that 1) HP1α has been shown to spread along the HeT-A sequence [16]. 2) The structure and the phenotypes of the different Z4 mutant alleles suggest a possible role of this protein in setting and maintaining the boundaries between heterochromatin and euchromatin in polytene chromosomes [18,28]. 3) JIL-1 has been extensively shown to be important to counteract heterochromatinization and, when missing, causes a spreading of heterochromatin markers such as H3K9me2, HP1α and Su(var)3-7 [49,50,54,55]. 4) JIL-1 has been found to co-localize with Z4 at the band-inter-band transition in polytene chromosomes and also to co-purify with Z4 in different protein complexes [18,27,28]. In addition to this, we have been able to detect a biochemical interaction between JIL-1 and Z4 (Figure 3A), as well as, a certain dependence on the presence of JIL-1 for the proper localization of Z4 (Figure 2D), suggesting a possible role of JIL-1 upstream of Z4. Finally, 5) The ChIP analyses in this study suggest a certain dependence of Z4 on HP1α or onto similar chromatin requirements for the loading of both proteins at the HTT array, more specifically at the HeT-A promoter (Figure 2A, 2C and 2D). Summarizing all of the above, we propose that the chromatin at the HeT-A promoter could have the following structure:

In a wild type situation (Figure 5A), the HeT-A promoter contains intermediate levels of HP1α, JIL-1 and Z4. HP1α would be spread along the HTT array, JIL-1 would be concentrated at the promoter region of HeT-A guaranteeing certain level of expression and Z4 would be important to set the boundary between these two opposite modulators.

In a JIL-1 mutant, (Figure 5B), the lack of JIL-1 would disturb the Z4 boundary causing a slight decrease in the Z4 presence. This result is in agreement with a Z4-JIL-1 partial interaction (Figure 3A and [20]). The decrease in JIL-1 presence and the disturbance of the boundary causes a spreading of HP1α into the HeT-A promoter, increasing its presence and repressing transcription from the HTT array (Figure 1 and Figure 2).

In a Z4 mutant (Figure 5C), the disappearance of the boundary together with the significant decrease in H3K9me3 causes a decrease in HP1α binding and a substantial modification of the chromatin at the HTT array (Figure 2). The lack of sufficient HP1α at the HTT array causes a destabilization of the chromatin at the gap domain triggering telomere instability as a result (Figure 4). This scenario applies to the three Z4 mutant alleles present in this study, the hypomorph Z4Δ1, and the nulls pzd16 and Z4Δ2. On one hand the loss of some Z4 in Z4Δ1/Z4Δ2 genotype produces overexpression of HeT-A because in addition to a relaxation of the chromatim, part of JIL-1 is still recruited to the HeT-A promoter (Figure 2A and 2B) and activates transcription in a more effective way than in a wild type situation.

Finally, in a Su(var)2-5 mutant background (Figure 5D), the lack of HP1α along the HeT-A sequence allows a relaxation of the boundary causing a spread of JIL-1 and Z4 from the HeT-A promoter towards the rest of the array and creating as a consequence, permissive chromatin environment releasing HeT-A silencing (Figure 1, Figure 2, and [33]).

Our model does not completely explain the complex relationships that regulate telomere chromatin, likely because other important components are yet to be described or associated with the ones presented here. For example, other chromatin regulatory components that have been associated with Drosophila telomeres are such as: the deacetylase Rpd3, with a regulatory role on chromatin structure [56], and the histone methyltransferase SetDB1 and the DNA methyltransfer Dnmt2 [52,57] which by acting in the same epigenetic pathway repress transcription of HeT-A as well as of retroelements in general [52]. Future in depth studies on additional chromatin components will allow us to complete and detail even more the description of the chromatin at the HTT array, and allow a better understanding of the mechanism of retrotransposon telomere maintenance and the epigenetic regulation of eukaryote telomeres in general. In the meantime, here we describe a plausible scenario in the view of our transcription and ChIP data.

The results shown here demonstrate the role of JIL-1 as the first described positive regulator of telomere (i.e. HeT-A) expression in Drosophila. Because HeT-A is in charge of telomere maintenance in Drosophila, these results are key to understand how telomere elongation is achieved in retrotransposon telomeres. We also demonstrate that Z4 is necessary to guarantee telomere stability. The data presented here strongly suggest that JIL-1 and Z4 exert these functions by maintaining an appropriate telomere chromatin structure by a coordinated action together with other known telomere components such as HP1α. Moreover, we show that JIL-1 and Z4 interact biochemically. Last, and importantly for understanding how the specific role of the Z4-JIL-1 complex at the telomeres is defined and differentiated from its role in other genomic regions, we show that Z4 might interact with the HeT-A Gag protein, providing evidence for a targeting mechanism that specifically retrieves this complex to the telomeres.

**Materials and Methods**

**Fly stocks and crosses**

Fly stocks were maintained and crossed performed at 25°C on standard Drosophila corn meal medium. w1118 strain was used as...
control. JHL-1<sup>wt</sup>/TM6, JHL-1<sup>gfp</sup>/TM6 and JHL-1<sup>Sativa</sup>/TM6/1
TM38/Tb stocks were provided by Kristin M. Johansen. Z4<sup>gfp</sup>/
TM38b and Z4<sup>wt</sup>/TM38 came from Harald Eggert and Harald
Saumweber. pEG<sup>3</sup>/TM6 from [21] was a kind gift of Anja Nagel.
The stocks l1<sup>S vg</sup>/TM6 and HOAP-GFP were obtained from Yi Kang
Rong. The use of TM6 and use of l1<sup>S vg</sup>/TM6 alleles were provided
by Maurizio Gatti. Sat<i>via</i> /<sup>2</sup>-<sup>9</sup>/CyO was obtained from Bloom-
ington Stock Center.

Genomic DNA extraction
Genomic DNA was extracted from adult flies to quantify the
number of HeT-A copies in each strain. Ten third instar larvae
without salivary glands were homogenized in 200 µl solution A
(0.1 M Tris-HCl pH 9.0, 0.1 M EDTA and 1% SDS) and
incubated at 70°C for 30 min. 28 µl 8 M KAc were added and
the samples incubated for 15 min on ice. Cell debris were harvested
at maximum speed for 15 min at 4°C. The supernatant was
transferred to a new tube and the DNA precipitated by adding 0.5
volumes isopropanol and centrifuging at 15,000 rpm for 5 min. Pelleted DNA was washed with 1 volume 70% ethanol and
centrifuged. Finally, the DNA pellet was air-dried, and resuspended in 50 µl 1x TE by rotating o/n at 4°C. After genomic DNA extraction, the number of copies was determined by
quantitative Real-Time PCR using 2 ng of DNA per reaction. Primers used for real time HeT-A_F (CCCCGCCAGAAGGACGGA) and HeT-A_R (TGTTGCAAGTGGCGCGCA)
for the 3'UTR region, He-T-A Real Time Gag F (ACAGATGCGCAAGGCTTCAGG) and He-T-A Real Gag Time R (GCCAGCGCATTTCATGC) for the Gag gene, Actin_F (GCACGCTTTACTCTTTCACGAGCATTCAGCAGGACGAT)
and Actin_R (ATGTGACGCGPACGTTCAGCAG)

RNA extraction and cDNA synthesis
Total RNA was isolated from ten whole third instar larvae and extracted using RNeasy Mini Kit (Qiagen) according to manu-
facturer’s protocol. RNase Free DNase Set (Qiagen) was used to remove genomic DNA contaminations as follows: one on column during the extraction accordingly to manufacturer’s protocol, and two in solution for 2 hours at 37°C. RNA was cleaned by precipitation and its quality was assessed using NanoDrop spectrophotometry.

One microgram of RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) with
oligo(dT) primers, and the expression of the different transcripts analyzed by quantitative Real-Time PCR. For each fly strain, two independent RNA extractions were prepared and analyzed three independent times. Primers used for real time PCR: He-T-A_F (CCCCGCCAGAAGGACGGA) and He-T-A_R (TGTTGCAAGTGGCGCGCA) for the 3'UTR, He-T-A Real Time Gag F (ACAGATGCGCAAGGCTTCAGG) and He-T-A Real Gag Time R (GCCAGCGCATTTCATGC) for the Gag gene. Actin_F (GCACGCTTTACTCTTTCACGAGCATTCAGCAGGACGAT) and Actin_R (ATGTGACGCGPACGTTCAGCAG)

Chromatin immunoprecipitation experiments (ChIPs)
Brains and imaginal discs from third instar larvae were dissected in 1x PBS with protease inhibitors and incubated in 0.5 mg/ml colcemid (Roche) for 2 hours. A hypotonic shock was applied by incubating brains in 0.5% sodium citrate for 10 min. Brains were fixed in 60% acetic acid and squashed. For anaphase preparation, brains were dissected as before, the hypotonic shock was omitted, and the brains were successively immersed in 45% and 60% acetic acid, DNA was stained with DAPI in mowiol medium. Mitotic chromosome preparations were analyzed on a Zeiss Imager Z2 fluorescence microscope using the AxioVision software.

Direct visualization of GFP-fusion proteins on mitotic chromosomes
Third instar larval brains were dissected in 1x PBS with protease inhibitors and incubated in 0.5 mg/ml colcemid (Roche) for 2 hours. A hypotonic shock was applied by incubating brains in 0.5% sodium citrate for 10 min. Proteins were fixed by incubating with Brower’s Fixation Buffer (0.15 M PIPES, 3 mM MgSO4, 1.5 mM EDTA, 1.5% NP-40, and 2% formaldehyde, pH 6.9) for 3 min. Brains were washed in 1x PBS-Triton (0.1%) for 3 min and allowed to soak in 50% glycerol for 5 min. Brains were squashed in a drop of glycerol, immersed in liquid nitrogen, and mounted in DAPI-mowiol medium. Mitotic chromosome preparations were analyzed on a Zeiss Axio Imager.Z2 fluorescence microscope using the AxioVision software.

RNAi knockdown in S2 cells
Fragments of the Z4, HeT-A gag, hoap, and Sar1 (non-LTR retrotransposon from Bombyx mori) coding sequences were amplified

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by PCR and cloned in pSTBlue-1 vector to produce dsRNA. Single stranded RNAs were synthesized by using SP6 and T7 RNA polymerases (Promega), both strains were incubated for 5 min at 90°C and annealed by slowly cooling to room temperature to obtain the dsRNA. dsRNA was then precipitated and treated with DNase (Qiagen) and RNase A (Roche) for 15 min at 37°C. A phenol-chloroform extraction was performed followed by precipitation and quantification of dsRNA with NanoDrop spectrophotometer ND1000. 50 µg of dsRNA were diluted in 1 mL of supplemented Schneider medium, added drop-wise to a total of 1.5×10^6 cells, and incubated at 25°C. The same protocol was repeated at 24 h and 48 h after seeding the cells. An aliquot of cells was collected at 24 h, 48 h, and 72 h after seeding. For description of cytology experiments, see next section S2 cells metaphase chromosome preparation. Gene fragments of about 530 bp were amplified using the primers: HeTGag-RNAi-F (CTAGCGGCAAACAACATCG) and HeTGag-RNAi-R (GGGATTGCAGATTCTTGGC) to amplify the 

HeT-A sequence with accession number: X68130 from nt 3046 to nt 3383, HOAP-RNAi-F (GCCGAGACTAAAGCGCAGAC) and HOAP-RNAi-R (CTGTAGTCGTCAGGTCCTTG) to amplify the 

transcript of the gene acc num: CG7752 from nt 1943 to nt 2432. All amplified regions were checked for off-site targets.

S2 cells metaphase chromosome preparation

500 µl collected cells were treated with 500 µl colcemid (10 µg/mL Roche) during 2–3 h in the dark. Cells were centrifuged 3 min at 1500 rpm and the pellet was washed with PBS. Cells were centrifuged again and the pellet resuspended with 500 µl 0.5% sodium citrate. After 10 min r.t. incubation, cells were centrifuged, resuspended in 1 mL fixation solution (methanol:acetic, 3:1) and sodium citrate. After 10 min r.t. incubation, cells were centrifuged, re-suspended in 50 µL Fixation Solution, and cells were dropped onto a microscope slide. Slides were air dried and mounted in DAPI-containing Mowiol medium. Images were obtained using the Zeiss Axio Imager.Z2 fluorescence microscope.

Cell transfection

Drosophila S2 cells were seeded at 3×10^5 cells/mL and transfected with one microgram of plasmid DNA using Effectene Transfection Reagent (Qiagen), accordingly to manufacturers protocol. Cells were incubated for 48 hours at 25°C and collected by centrifugation at 2000 rpm for 5 min, washed twice in 1× PBS and frozen at −80°C. HeT-A Gag-GFP plasmid was used in cell transfection [37].

Protein co-immunoprecipitation assays

Protein extracts from S2 cells were prepared in 1 ml lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% TritonX-100, 1 mM EDTA, 1 mM EGTA, and Complete EDTA-free protease inhibitor cocktail from Roche), incubated on ice for 20 min, and centrifuged at 13 000 rpm for 15 min at 4°C. Fresh lysates were incubated with 50 µl PureProteome Protein A and Protein G Magnetic Beads (Millipore) coated with specific antibodies, for 4 hours at 4°C with rotation. The magnetic beads were previously incubated with the respective antibodies in 500 µl lysis buffer for one hour at 4°C with rotation and washed 5 times with 500 µl lysis buffer. Immunocomplexes were washed 3 times with lysis buffer and eluted from the beads with 50 µl 1 x sample buffer. Samples were boiled for 10 min, loaded on a SDS-PAGE gel and analyzed by Western Blot. Anti-GFP (Invitrogen, A11120), anti-Z4 [58], anti-JIL-1 (mouse, gift from Kristen Johansen), and control mouse IgG (Santa Cruz Biotechnology, sc-2025) were used for protein immunoprecipitation, and anti-HeT-A Gag, anti-Z4 and anti-JIL1 [59] were used in Western Blot experiments.

Supporting Information

Figure S1 HeT-A copy number of jil-1 and z4 mutants. The genomic content of the HeT-A retrotransposon of each stock was measured in HeT-A gag (A) and HeT-A 3’UTR (B) regions. Z4.7 and Su(var)2-505 2-505 mutant alleles have more HeT-A copies than control flies. Error bars represent standard deviations of three independent experiments. Asterisks indicate statistically significant differences using the t-test (one asterisk, *P<0.05 to 0.01; two asterisks, **P<0.01 to 0.001; three asterisks, ***P<0.001) in HeT-A copy number of each mutant compared to w1118. (TIF)

Figure S2 HeT-A expression in jil-1 and z4 mutants. Absolute expression of HeT-A gag (A) and HeT-A 3’UTR (B) in the analyzed stocks. HeT-A transcription was normalized to actin transcription. Error bars represent standard deviations of three independent experiments. (TIF)

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Author Contributions

Conceived and designed the experiments: RS-S EC. Performed the experiments: RS-S EL-P DP. Analyzed the data: RS-S EL-P EC. Contributed reagents/materials/analysis tools: RS-S EL-P EC. Wrote the paper: RS-S EC.
