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The epigenetic regulation of *Drosophila* telomeres

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Abstract

Drosophila telomere maintenance depends on the transposition of three specialized retrotransposons – *HeT-A*, *TART* and *TAHRE (HTT)*. Controlling the activation and silencing of these elements is crucial to maintain telomere length homeostasis without compromising genomic instability. In this thesis, I have identified the role of different chromosomal proteins involved in creating the correct chromatin environment to achieve telomere length homeostasis and stability. JIL-1, together with HP1a and Z4, acts as a boundary at the telomere-subtelomere frontier. The interplay of these proteins leads to an equilibrium in the activation/repression state of the telomere retrotransposons. Additionally, I have contributed to the finding that the HeT-A Gag protein is a key component to target different protein complexes to the telomeres and to guarantee genome stability. I have also been able to demonstrate that the Z4 partners DREF, TRF2 and KEN are also involved in the silencing of the HTT array, probably by mediating chromatin remodeling. Finally, I have identified a special subtelomere domain at the 4R telomere with different chromatin characteristics, and demonstrated that SETDB1, HP1a and POF are involved in the regulation of the telomeric retrotransposons in the 4th chromosome. These results provide important insights to better understand how, in *Drosophila*, the telomere retrotransposons are orchestrated to achieve a telomere function analogous to telomerase telomeres in other eukaryotes.

Key words: *Drosophila melanogaster*, telomeres, retrotransposons, *HeT-A*, chromatin, JIL-1 kinase, Z4

Resum

El manteniment dels telòmers de *Drosophila* depèn de la transposició especialitzada de tres retrotransposons, *HeT-A*, *TART* i *TAHRE* (HTT). El control de l'activació i la repressió d'aquests elements és crucial a l'hora de mantenir la llargada telomèrica sense comprometre'n l'estabilitat genòmica. En aquesta tesi jo he pogut identificar el paper de diferents proteïnes cromosòmiques involucrades a crear un estat de la cromatina adient per mantenir la longitud i l'estabilitat telomèrica. JIL-1, juntament amb HP1a i Z4, ajuda a crear el llindar entre la frontera dels dominis telomèric i subtelomèric. L'actuació conjunta d'aquestes proteïnes aconsegueix un estat d'equilibri d'activació/repressió dels retrotransposons telomèrics. A més a més, he contribuït a la descoberta de la implicació de la proteïna HeT-A Gag en el reclutament de diferents complexes proteics als telomèrs de *Drosophila* per poder garantir l'estabilitat telomèrica. També he pogut demostrar que altres membres dels complexes on participa Z4, com ara: DREF, TRF2 i KEN, estan també implicats en el silenciament dels retrotransposons telomèrics segurament per mitjà de la remodelació de la cromatina. Finalment, he pogut demostrar que el domini subtelomèric del telòmer 4R té una estructura cromatínica diferent a la resta dels dominis subtelomèrics dels altres cromosomes i he pogut demostrar que les proteïnes SETDB1, HP1a i POF estan implicades en la regulació de l'array HTT del cromosoma 4. Els resultats d'aquesta tesi ajuden de manera substancial a comprendre com els retrotransposons telomèrics estan orquestrats per tal de poder fer una funció anàloga als telòmers de telomerasa en altres eucariotes.

Paraules clau: *Drosophila melanogaster*, telòmers, retrotransposons, *HeT-A*, cromatina, JIL-1 quinasa, Z4

Prologue

"Drosophila telomeres are fascinating in their own right. They are also an important model for the study of more general questions. Cytological or genetic studies show that Drosophila telomeres perform the same roles as do other telomeres. Many proteins that affect the behavior of telomeres in other organisms affect telomere behavior in Drosophila. Because it is a variant that accomplishes the same functions as do other telomeres but uses a different mechanism, this telomere can also help us understand telomeres in other organisms. In addition, Drosophila telomeres reveal an unexpected relationship between telomeres and retrotransposons that may help us understand the evolution of both chromosomes and transposable elements."

Mary-Lou Pardue, Following the Chromosome Path to the Garden of the Genome (2007)

In this thesis we share our contribution to the *Drosophila* telomere field, giving a special emphasis to the epigenetic regulation of the telomeric retrotransposons *HeT-A*, *TART* and *TAHRE*. The epigenetic regulation of *Drosophila* telomeres is crucial in order to achieve the orchestrated dynamics of gene expression and silencing necessary for a correct telomere homeostasis.

This thesis is structured in four main sections. The first one includes the *Introduction*, covering the background information on telomerase and retrotransposon telomeres, with some subsections dedicated to the different telomeric domains. In the second section the main *Objectives* are described. Following, we present the *Results* obtained during this thesis in four different chapters. For each results chapter a brief introduction, material and methods, and discussion is presented. Chapters 1 and 2 have already been submitted

for publication to *Genetics* and *PLoS Genetics*, respectively, and the chapter 3 is being prepared to be submitted to *Mobile DNA* journal. The fourth section corresponds to a general *Discussion* relating the results obtained in this thesis together with other published works. Following the *Conclusions* section, I have included two *Annexes*; the *Annex 1* corresponds to the first results obtained by myself from a project that has been continued by Elisenda López-Panadès, another PhD student from the laboratory. *Annex 2* corresponds to a published book chapter entitled: "*Drosophila Telomeres: an Example of Co-Evolution with Transposable Elements*" on the volume: *Repetitive DNA* of the Book Series: *Genome Dynamics*, to which I have contributed as first author.

Abbreviations

ALT	Alternative Lengthening of Telomeres
Armi	Armitage
ATM	Ataxia Telangiectasia, Mutated
ATR	ATM and Rad3-Related
Aub	Aubergine
BEAF	Boundary Element Associated Factor
Brm	Brahma
CD	Chromo Domain
ChIP	Chromatin Immunoprecipitation
Chriz	Chromo domain protein interacting with Z4
Chro	Chromator
COMPASS	Complex of Proteins Associated with Set-1
CSD	Chromo Shadow Domain
CTD	C-Terminal Domain
Dnmt2	DNA methyltransferase 2
DREF	DNA Replication-related Element-binding Factor
DSB	Double Strand Break
E(tc)	Enhancer of terminal gene conversion
E(Z)	Enhancer of Zeste
EcR	Ecdysone Receptor
eff	*effete*
Elp3	Elongator protein 3
Exo1	Exonuclease 1
FL	Full Length
HeT-A	*Healing transposon*
HipHop	HP1-HOAP interacting protein
HOAP	HP1-ORC-associated protein
HP1	Heterochromatin Protein 1
HP2	Heterochromatin Protein 2
HTT	*HeT-A*, *TART* and *TAHRE*
IP	Immunoprecipitation
JAK	Janus Kinase
KDI	Kinase Domain I
KDII	Kinase Domain II
Ken	Ken & Barbie
Kr-H1	Kruppel Homolog 1
Kr-H2	Kruppel Homolog 2
LID	Little Imaginal Discs

LINES	Long Interspersed Nuclear Elements
LTR	Long Terminal Repeat
MOF	Males absent Of First
Moi	Modigliani
Mre11	Meiotic recombination 11
MRN	Mre11/Rad50/Nbs1
MSL	Male Specific Lethal
NAP-1	Nucleosome Assembly Protein 1
NTD	N-Terminal Domain
NURF	Nucleosome Remodeling Factor
OB	Oligonucleotide/oligosaccharide-Binding
ORF	Open Reading Frame
Pc	Polycomb
PcG	Polycomb Group
PEV	Position Effect Variengation
piRNA	PIWI-interacting RNA
Piwi	P-element induced wimpy testis
PNTR	Perfect Non-Terminal Repeats
POF	Painting Of Four
Pol II	RNA Polymerase II
POT1	Protection Of Telomeres 1
PROD	Proliferation Disrupter
RAP1	Repressor/Activator Protein 1
***rasi*RNA**	Repeat-associated small interfering RNA
rDNA	Recombinant DNA
RNAi	RNA interference
RT	Reverse Transcriptase
SINES	Short Interspersed Nuclear Elements
STAT	Signal Transducer and Activator of Transcription
SU(VAR)	Suppressor of Variegation
SuUR	Suppressor of Under-Replication
TAHRE	*Telomere Associated element HeT-A Related*
TART	*Telomere Associated Retrotransposon*
TAS	Telomere Associated Sequences
TE	Transposable Element
Tel	*Telomere elongation*
TERC	Telomerase template RNA Component
TERRA	Telomeric Repeat Containing RNA
TERT	Telomerase Reverse Transcriptase
TF	Telomere Fusions
TGC	Terminal Gene Conversion
TIN2	TRF1 Interacting Protein 2
TIR	Terminal Inverted Repeats
TPE	Telomere Position Effect
TPP1	TINT1/PIP1/PTOP
TPRT	Target-Primed Reverse Transcription
TR	Telomerase RNA

TRAL	Trailer hitch
TRF1	Telomeric Repeat binding Factor 1
dTRF1	TATA box binding protein (TBP)-Related Factor 2
TRF2	Telomeric Repeat binding Factor 2
Trr	Trythorax-related
Trx	Trythorax
TSE	*Trans*-Silencing Effect
UTR	Untranslated Region
Ver	Verrocchio
$\boldsymbol{w^{m4}}$	*white* molted 4
Woc	Without children
Zf30c	Zinc finger protein 30C

Contents

Introduction

Introduction

1. History of Telomere Biology

The telomere history starts in the 1920's, when Hermann Muller irradiated *Drosophila melanogaster* with X-rays and noticed that the resulting DNA double strand breaks were unstable and became attached to other broken parts of the chromosome. In a paper from 1938, Muller states that this attachment only occurs between the broken ends and not between the originally free ends of the chromosomes (Muller, 1938). He names these "free ends" of the chromosomes as telomeres, from the Greek terms for "end" (*telos*) and "part" (*meros*). At this time, Muller thought that the telomeres were an essential gene that the cell could not loose.

Around the same time Barbara McClintock, while working with mobile DNA elements in maize, discovered that X-ray broken chromosomes were reattached and formed ring chromosomes (McClintock, 1932). In a 1931 report, McClintock describes the importance of the "natural ends" of the chromosomes; stating that, unlike the broken chromosomes, the "natural ends" had distinct functions and properties since they never got attached with the broken ends (McClintock, 1931). She also demonstrated how, if left unprotected, the ends of chromosomes could fuse to each other and enter in a "breakage-fusion cycle" with deleterious consequences for the cell (McClintock, 1939).

Although the telomeres were discovered in the 1930s, not much was known until 1971, when James Watson and Alexey Olovnikov discovered the "end replication problem" (Olovnikov, 1971; Watson, 1972). While studying the mechanisms of DNA replication, these scientists observed that the cells were

not able to completely replicate the linear ends of the DNA, and consequently some DNA information was lost every cell cycle. Olovnikov related these findings with the ones of Leonard Hayflick's on the limited number of cell divisions (cellular senescence) (Hayflick and Moorhead, 1961), and suggested for the first time that telomere shortening was the mechanism that limited the number of times that cells can divide. Watson and Olovnikov believed that cells must have a mechanism to maintain the telomeres, but were unable to describe it.

In 1978, Elizabeth Blackburn and Joseph Gall, while working with the ciliated protozoan *Tetrahymena thermophila*, discovered that the telomeres were composed of approximately fifty tandem repeats of the DNA sequences CCCCAA/CCCCTT (Blackburn and Gall, 1978). Some years latter, when working with Jack Szostak, Blackburn demonstrated that *Saccharomyces cerevisiae* telomeres were efficiently maintained (Szostak and Blackburn, 1982). At this time, Blackburn and Szostak hypothesized that yeast telomeres were being lengthened by an unknown enzymatic mechanism. It was not until 1984, that the enzyme was isolated in *Tetrahymena* and named telomerase by Blackburn and her student Carol Greider (Greider and Blackburn, 1985). In 2009, Blackburn, Szostak and Greider were awarded with the Noble Prize in Medicine for their work discovering the structure of telomerase and the mechanisms of telomere maintenance.

More recently, several studies demonstrated that the telomeres act not only as a physical protection to the end of the chromosomes, but they are a much more dynamic and complex structure. Moreover, telomere dysfunction was associated with several key processes like cellular aging, senescence, and tumorogenesis (Blackburn, 2001). Nowadays the telomere field is still a subject of intense investigation with approximately 15,000 publications existing in PubMed.

In the next sections of this chapter I present a brief summary of telomerase telomeres, followed by a detailed description of *Drosophila melanogaster* telomeres, with a special emphasis on the telomeric chromatin.

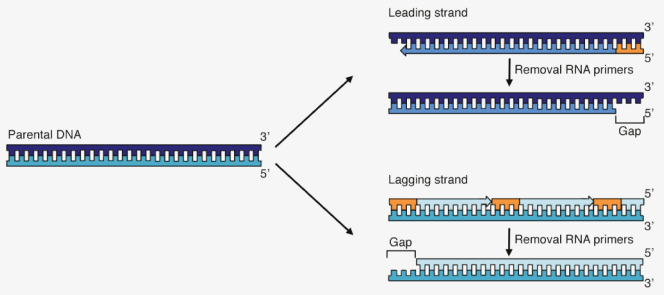
2. Telomeres and Telomerase

In eukaryotes, the natural ends of the chromosomes are linear. However, the exposure of linear DNA fragments resembles DNA breaks and is deleterious for the cell. Therefore, in order to prevent harmful DNA repair, the cells developed specialized structures to protect the natural ends of the chromosomes. These structures are called telomeres and can be defined as a complex of DNA and proteins that assembles at the end of the chromosomes. The telomeres help to stabilize the chromosomes and protect genes from being eroded through successive rounds of DNA replication (De Lange et al., 2006). Since the DNA polymerases are not able to completely replicate the end of the chromosomes - “end replication problem” (see Box 1) (Olovnikov, 1971; Watson, 1972), a special mechanism was developed to maintain an appropriate telomere homeostasis. In most eukaryotes, the telomerase is the enzyme enrolled in this process, while some insects and plants adopted alternative mechanisms after the loss of telomerase.

In general, telomeres consist of a 6-8 base pair sequence rich in guanines that is repeated hundreds or thousands of times. The size of the sequence and the number of repeats varies between species (Hathcock et al., 2002). Human telomeres, for example, range in size from 2 to 50 kilobases and consist of approximately 300 to 8,000 precise repeats of the sequence TTAGGG (sequence conserved in all vertebrates) (De Lange et al., 2006). By contrast, *S. cerevisae* telomeres are smaller and more heterogeneous in composition, consisting in 40 to 100 copies of the sequence C_{1-4}/TG_{1-3} (Förstemann and Lingner, 2001). Other typical examples are the mixed arrays of the sequences TTAGGG and TTTGGG in tomato telomeres, and TTAGG in silkworm (Craig, 2002). In all cases, the double stranded repeats

are followed by 50 to 300 nucleotides of single stranded DNA repeats. This strand is present at the 3'-end of the chromosomes and generates a G-tail or 3' overhang. The G-tail invades the double-stranded telomeric DNA, displaces one of the DNA strands and base pairs with the other strand forming a lariat like structure called "t-loop" (Figure 1A and 1B). The t-loop provides an architectural solution to the problem of telomere protection by sequestering the chromosome terminus, hiding this way the linear ends from end-to-end fusions and unregulated nuclease digestion (Griffith et al., 1999). The integrity of the t-loop is maintained by a specialized group of proteins known as the shelterin complex (Figure 1C) (de Lange, 2005; Hug and Lingner, 2006).

Box 1: The *end replication problem*



When cells divide DNA replicates but the end of the linear chromosomes and the inability of DNA polymerases to extend DNA in the 3' to 5' direction lead to a major problem in DNA replication. DNA polymerases require short RNA primers with a free 3'-OH group to initiate replication in a 5' to 3' direction. From the 3' end of a linear chromosome, the DNA polymerase is able to synthesize continuously the leading strand. However, the lagging strand is synthesized discontinuously through short fragments known as Okazaki fragments, each of them with its own RNA primer. The RNA primers of each Okazaki fragment are removed and substituted by DNA. Nevertheless, a problem arises at the end of the chromosome because the DNA polymerase is unable to fill the gap left by the terminal RNA primer. Consequently, the new DNA molecule is shorter than the parent DNA molecule by at least the length of one RNA primer. Without a solution to this *end-replication problem*, chromosomes would progressively shorten each cell division and essential genes would be eroded, a process that would bring catastrophic consequences to the cell (Olovnikov, 1971; Watson, 1972).

2.1 Shelterin complex

Shelterin is a complex of six proteins that assembles at the end of the chromosomes. The role of the shelterin complex is to mark the telomeres as

the natural chromosome ends and to prevent them from being recognized as double-strand breaks by the DNA repair machinery, avoiding this way inappropriate telomere fusions. Furthermore, shelterin is involved in the formation of the t-loop structure, and regulates the telomere maintenance by telomerase (De Lange et al., 2006).

In mammals, the shelterin complex is composed by six well-characterized telomeric proteins (Figure 1C). This complex assembles at the end of the chromosomes through the binding of the proteins TRF1 (Telomeric Repeat binding Factor 1) and TRF2 to the double stranded DNA repeats. A third protein, POT 1 (Protection Of Telomeres 1), binds to the single-stranded G-tail acting as a "locker" for the closed structure of the t-loop. These three proteins will then recruit RAP1 (Repressor/Activator Protein 1), TIN2 (TRF1 INteracting protein 2), and TPP1 (TINT1/PIP1/PTOP 1). Both TRF1 and TRF2 are able to control telomere size by inhibiting telomerase elongation (Martínez and Blasco, 2011).

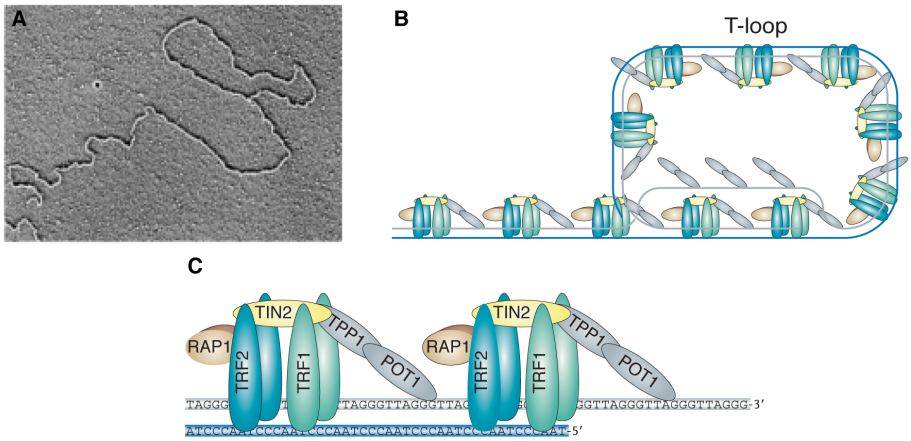


Figure 1: Mammalian t-loop structure and shelterin complex. (A) Electron microscopy image of the t-loop structure obtained from telomeric DNA of HeLa cells. (B) Schematic representation of the t-loop structure with the associated shelterin proteins. (C) Shelterin complex proteins bind to double and single-stranded telomeric DNA. The proteins TRF1, TRF2, RAP1, POT1, TIN2 and TPP1 associate at the telomeres forming the shelterin complex. Adapted from (Griffith et al., 1999; Martínez and Blasco, 2011).

The shelterin complex is known to repress the DNA damage response; depletion or loss of function of shelterin components leads to the activation of the ATM (ataxia telangiectasia, mutated) or ATR (ATM and Rad3-related) DNA damage responses, cell cycle arrest and chromosome instability. In conclusion, although only composed by six proteins, the shelterin complex has a very important role in telomere length regulation, protection against enzymatic attack and recruitment of telomerase (de Lange, 2005).

2.2 Telomerase

In most eukaryotes, telomerase is the enzyme responsible for the *de novo* addition of the telomeric repeats (Morin, 1989), compensating this way the terminal deterioration that occurs every cell cycle. In humans, telomerase is composed by a catalytic subunit, TERT (TElomerase Reverse Transcriptase), an RNA subunit TR or TERC (TElomerase template RNA Component), and dyskerin. TERT is the reverse transcriptase domain and is responsible for the addition of the six nucleotide repeat TTAGGG onto the ends of the telomeres (Morin, 1989; Harrington et al., 1997; Counter et al., 1997). TERC contains the template RNA region that is complementary to the human telomere sequence (Feng et al., 1995). Finally, dyskerin is the protein that binds and stabilizes TERC (Cohen et al., 2007).

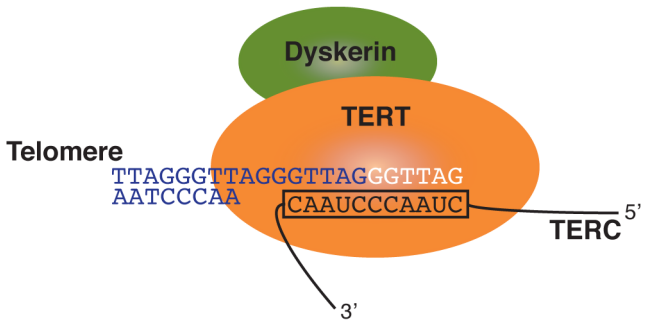


Figure 2: Schematic representation of the telomerase subunits. The enzyme telomerase is composed of a catalytic subunit (TERT), an RNA subunit (TERC) and dyskerin, a stabilizing protein.

Telomerase is highly expressed during early embryogenesis, germ cells and stem cells, but in somatic cells its expression is very low or absent (Wright et al., 1996; Masutomi and Hahn, 2003) and each time one of these cells divides the telomeres get shorter due to the “end replication problem”. As a consequence of telomere shortening, somatic cells can only undergo a limited number of cell divisions before the telomeres get critically short and the fewer units of telomere binding proteins loose their functionalities of telomere protection, leading to end-to-end chromosome fusions, cell cycle arrest (replicative senescence) and/or apoptosis (replicative senescence) (Schoeftner and Blasco, 2009).

Telomere maintenance by telomerase plays an important role in human aging and cancer. In most human cancers telomerase expression is upregulated and cells are able to maintain telomeres and divide indefinitely (Shay and Wright, 2006). Accordingly, telomerase *knockout* mice reveal damage in organs with highly proliferative tissues and exhibit premature aging associated with decreased telomerase activity and short telomeres (Blasco et al., 1996). Therefore, telomerase activity needs to be tightly regulated in order to maintain telomere homeostasis while protecting from cell immortality.

2.3 Mammalian telomere regulation

The telomeric chromatin structure has been shown to be important for telomere regulation and function. Mammalian telomere chromatin is enriched in epigenetic marks that are characteristic of constitutive heterochromatin, such as certain histone modifications and DNA methylation. Alterations of histone modifications in the telomeric chromatin or DNA methylation of the subtelomeric domain are associated with changes in telomere length; indicating that the telomeric chromatin is involved in telomere length homeostasis (Blasco, 2007).

Due to their heterochromatic properties, the telomeres were thought to be restrictive to transcription. However, recent studies report that the telomeric

C-rich strand (complementary to G-rich strand) is frequently transcribed by the RNA polymerase II, giving rise to a UUAGGG-repeat containing a non-coding RNA named TERRA (Telomeric Repeat containing RNA) (Feuerhahn et al., 2010). Current models propose a role for TERRA controlling telomerase activity, presumably by base pairing with the template region of the RNA component of telomerase (TERC) (Redon et al., 2010). Additionally, TERRA was also found to promote telomere shortening by facilitating the nuclease activity of the enzyme Exo1 (Exonuclease I) at the telomeres (Pfeiffer and Lingner, 2012). Therefore, the expression of TERRA needs to be tightly regulated in order to avoid excessive telomere shortening. Recent data show that telomere lengthening represses TERRA expression by an increase of H3K9me3 and HP1α presence at the telomeric DNA (Arnoult et al., 2012).

3. Telomeres and Transposable Elements

Although in most eukaryotes the telomeres are elongated by the telomerase holoenzyme, this is not the only mechanism used to maintain chromosome length. Telomerase has been lost a few times in the evolution of plants and insects. In plants, the telomeric sequence TTTAGGG appears to be highly conserved. Nevertheless, the canonical telomere elongation mechanism was lost in three genera of the family Solanaceae (Peska et al., 2008; Watson and Riha, 2010). In the onion family for example, the telomeres are maintained by the transposition of a *Ty1-copia*-like retrotransposon and *En*/*Spm* transposable element-like sequence, and through recombination between satellite sequences (Pich and Schubert, 1998). During insect evolution telomerase was lost at least six times. In the case of the silkworm, *Bombyx mori,* the telomeres are composed of mixed arrays of the telomerase repetitions and two non-LTR (Long Terminal Repeats) retrotransposons named *SART* and *TRAS*. *SART* and *TRAS* actively transpose into the TTAGG telomeric sequences, likely compensating the low enzymatic activity

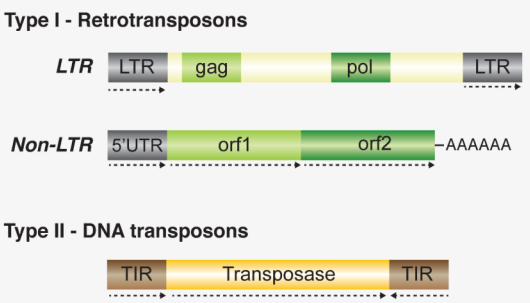
of telomerase in this organism (Fujiwara et al., 2005). Therefore, the silkworm telomeres could be an evolutionary intermediate in transition from a mechanism where the telomere elongation is performed by telomerase to a mechanism operated by the specific transposition of two non-LTR retrotransposons (Fujiwara et al., 2005; Tatsuke et al., 2010). Additionally, telomerase has not been found in any of the dipteran species, where homologous recombination has been proposed as the possible mechanism of telomere maintenance (Cohn and Edström, 1992a; 1992b; Nielsen and Edström, 1993).

As other dipterans, *Drosophila* does not have telomerase, neither its telomeres are formed by short repeats of G-rich nucleotides. Instead, *Drosophila* chromosome ends are maintained by the specific transposition of three non-LTR retrotransposons named *HeT-A* (Healing Transposon), *TART* (Telomere Associated RetroTransposon) and *TAHRE* (Telomere Associated element *HeT-A* RElated) (Silva-Sousa et al., 2012). The telomeric retrotransposons arrange at the end of the chromosomes in head-to-tail arrays, with their poly(A) tails pointing towards the centromere (Biessmann et al., 1993). Telomere length in *Drosophila* varies from stock to stock but is generally comprised between 75kb and 100kb (George et al., 2006).

Drosophila telomere retrotransposons also constitute an exception in the transposable elements (TEs) field. Retrotransposons are known to have both beneficial and detrimental effects on the host genome; these events are thought to be incidental due to the selfish activities of the TEs (Roy-Engel, 2012). By contrast, the telomere retrotransposons *HeT-A*, *TART* and *TAHRE* are completely dedicated to their role at the telomeres (George et al., 2006). *Drosophila* telomeres are therefore an example of how the genome and the transposable elements adapt to each other to the benefit of both: 1) The genome of *Drosophila* recognizes the telomeric retrotransposons as a mechanism that performs an essential role, allowing their transposition in a tightly regulated manner (Pardue and DeBaryshe, 2008); 2) The telomeric retrotransposons, while maintaining their transposon characteristics, have

adapted to a telomeric role by developing unusual features that are conserved across *Drosophila* species (Casacuberta and Pardue, 2005).

Box 2: Types and structures of transposable elements



Transposable elements (TEs) are pieces of DNA that have the ability to move from one chromosomal location to another (McClintock, 1950). In an oversimplified classification, TEs can be divided in two main groups accordingly to their mechanisms of mobilization; "copy and paste" (for type I TEs) and "cut and paste" (for type II TEs). Type I TEs or retrotransposons, require an RNA intermediate to transpose and undergo duplicative transposition, therefore their total number increases after each transposition leading to genome expansion. Retrotransposons can be subdivided in two sub-types, the long terminal repeat (LTR) and the non-LTR. The LTR retrotransposons contain LTRs flanking the element and encode Gag and Pol proteins similar to the ones of retroviruses, essential for their transposition. The second type of retrotransposons are the non-LTR retrotransposons, these TEs lack LTRs. Non-LTR can be either autonomous (LINEs, Long Interspersed Elements) with two ORFs (open reading frames), which encode all the machinery needed for transposition, or non-autonomous (SINEs, Short Interspersed Elements) with only one ORF, these elements use the machinery of autonomous TEs to efficiently transpose. By contrast, type II TEs or DNA transposons, do not require an RNA intermediate for their transposition. Instead, they encode a protein called transposase that binds to the terminal inverted repeats (TIR) that flank the element, excises the TE from its genomic location and inserts it in a new location in a "cut and paste" mechanism. In this situation the total number of copies for each TE in the genome does not increase with a new transposition (Slotkin and Martienssen, 2007).

The telomere retrotransposons have several characteristics of non-LTR retrotransposons with some specific adaptations probably linked to their telomeric role. *HeT-A*, *TART* and *TAHRE* are composed of either one or two open reading frames (ORFs) flanked by 5' and 3' untranslated regions (UTRs). The three elements have an oligo(A) sequence at their 3' end characteristic of non-LTR retrotransposons, as these retrotransposons transpose by poly(A)$^+$ RNA intermediates (Silva-Sousa et al., 2012). Moreover, the telomeric retrotransposons have truncated 5'UTRs like most non-LTR retrotransposons. However, while in non-LTR retrotransposons this characteristic is due to a failure to complete reverse transcription, in the

telomere retrotransposons there is a second reason, the terminal erosion while being at the end of the chromosome.

Special features, not shared with other non-LTR retrotransposons, are also found in the telomere retrotransposons. *HeT-A*, *TART* and *TAHRE* only successfully transpose at the telomere domain and not to different places in the genome (George et al., 2006). Nevertheless, few *HeT-A* defective copies have been found outside the telomeres, more specifically in the heterochromatic region of the 3rd and Y centromeres (Agudo et al., 1999; Abad et al., 2004; Losada et al., 1999; Mendez-Lago et al., 2009). However, these copies show very low expression, indicating that the expression of the *HeT-A* retrotransposon comes almost exclusively from the telomeres (Piñeyro et al., 2011). In fact, it is not clear if they belong to copies that transposed directly to the centromeric heterochromatin or if they reached these locations by chromosome recombination or reorganization events. Finally, the three telomere retrotransposons have very long 3'UTRs that correspond to more than half of the genome of the element. These long 3'UTRs may serve as a platform for the binding of specific proteins related with the establishment of telomere chromatin (Danilevskaya et al., 1998) .

3.1 Telomeric retrotransposons - *HeT-A*, *TART* and *TAHRE*

HeT-A

HeT-A is the main component of *Drosophila* telomeres. Although *HeT-A* is the most successful of the three telomeric retrotransposons, it is a non-autonomous element that only encodes the structural protein Gag but not the enzymatic proteins (RT and endonuclease) that allow the transposition (Pardue et al., 1996) (Figure 3A).

A typical non-LTR promoter is located at the 5'UTR but in the case of the *HeT-A* retrotransposon this domain only contributes slightly to the strength of transcription (Danilevskaya et al., 1997). Most of the *HeT-A* promoter sequence is located at the 3'UTR terminal, driving the transcription of the

HeT-A element immediately downstream (Danilevskaya et al., 1997; Pardue and DeBaryshe, 2008). Therefore, the transposition of a *HeT-A* element depends on the successful transposition of another *HeT-A* element immediately upstream, probably selecting for multiple transpositions at the same time. These findings are supported by a study that demonstrates that *HeT-A* can drive the expression of a promoter-less *yellow* gene at the end of a terminally truncated chromosome (Kahn et al., 2000). Interestingly, if a complete *HeT-A* element, together with its promoter (3'UTR of upstream element), is taken from the telomeric array, the resulting sequence is identical to the structure of an LTR retrotransposon with its two identical terminal repeats. This special feature of *HeT-A* makes this retrotransposon resemble a possible evolutionary intermediate between the promoters of typical non-LTR elements and those of retroviruses and LTR retrotransposons (Danilevskaya et al., 1997). Furthermore, *HeT-A* also has a 3'UTR antisense promoter, which drives the transcription of antisense non-coding RNAs probably involved in the regulation of telomere function and protection (Shpiz et al., 2009; Piñeyro et al., 2011).

TART

TART is the telomeric retrotransposon that most resembles a canonical non-LTR retrotransposon. This element has two ORFs that encode the Gag and Pol (endonuclease and reverse transcriptase (RT) domains) proteins essential for transposition (Figure 3B). In *D. melanogaster*, *TART* can be classified in three different subfamilies – *TART A*, *TART B* and *TART C* – accordingly to its UTR sequences (Fuller et al., 2010). Unlike *HeT-A*, the *TART* promoter is located at 5'UTR of the element. Nevertheless, *TART* also has a promoter at the 3'UTR, this promoter is very active but it drives only antisense transcription. *TART* antisense RNA is several-fold higher than the sense RNA (Danilevskaya et al., 1999), this special feature is conserved

across *Drosophila* species (Casacuberta and Pardue, 2003), but to date no role has yet been elucidated for this antisense RNA.

An unusual feature of *TART* is the presence of perfect non-terminal repeats (PNTRs); the very end of the 5'UTR sequence and the first codons of the *gag* gene are perfectly repeated near, but not at, the 3'UTR. PNTRs are 100% identical within each subfamily, and around 70% among subfamilies (Pardue and DeBaryshe, 2008). The perfect conservation of both PNTR sequences in a *TART* copy suggests a mechanism of second strand synthesis similar to the one of LTRs sequences of LTR retrotransposons. Other non-LTR retrotransposons like *TRE5-A* in *Dictyostellium* and *TOC1* in *Chlamydomonas* also have PNTRs (Craig, 2002). Like *TART*, these elements also produce substantial antisense transcripts. Further studies in the transposition mechanism of these three elements are needed in order to describe the importance and functionality of the PNTRs.

TAHRE

The third and last telomeric retrotransposon to be discovered has only few copies available in *D. melanogaster* genome and only one could be potentially active. This element was named *TAHRE* (Telomere Associated element *HeT-A* RElated) because it shares features with *HeT-A* like the 5'UTR, ORF1 and the end of the 3'UTR sequences. Moreover, *TAHRE* shares the presence of a second ORF with *TART.* Like *TART*, *TAHRE* ORF2 encodes the endonuclease and RT domains (Abad et al., 2004) (Figure 3C). It is surprising that the telomeric retrotransposon that seems to combine the best characteristics of its two partners has not been more successful in transposing. However, the finding that the *TAHRE* Gag protein is not able to enter the cell nucleus by it self may offer a clue on why the unsuccessful transposition (Fuller et al., 2010).

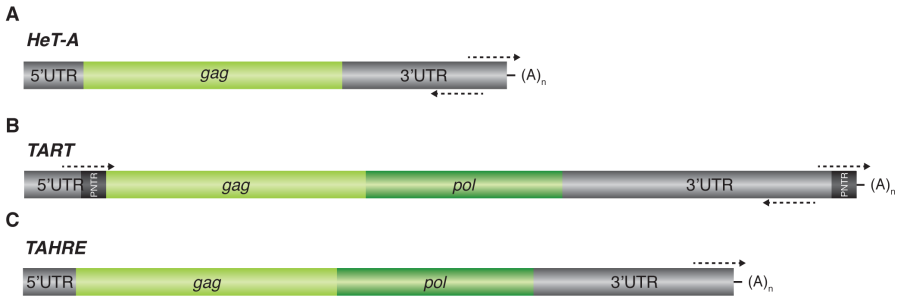


Figure 3: The telomeric retrotransposons *HeT-A*, *TART* and *TAHRE*. (A) *HeT-A*, the most abundant telomeric retrotransposon in *D. melanogaster*, is a defective element that only encodes the structural Gag protein. (B) *TART* element structure resembles a canonical non-LTR retrotransposon with 5' and 3'UTRs. *TART* encodes the structural Gag and the enzymatic endonuclease and reverse transcriptase proteins. (C) *TAHRE* is the less abundant element at *D. melanogaster* telomeres and has a high sequence homology with *HeT-A*. Bright grey boxes, non-coding 5' and 3' UTR sequences; light green boxes, Gag ORF; dark green boxes, Pol ORF; dark grey boxes, PNTR; $(A)_n$, 3' poly(A) tail; Dashed arrows, transcription start sites for full length sense and anti-sense RNA.

The similarities of *TAHRE* with *HeT-A* and *TART* allowed the proposal of a potential common ancestor for the telomere retrotransposons. Abad and collaborators suggested that an ancestral telomeric retrotransposon evolved to optimize its role in telomere maintenance giving origin to *TART* and *TAHRE* (Abad et al., 2004). *HeT-A* would appear latter from a processed copy of *TAHRE* without RT.

The reverse transcriptase (RT) domain of *pol* genes is the most conserved region in retroelements and it was used to analyze the evolutionary relationships of the telomeric retrotransposons (Malik et al., 1999; Morse, 1994). This analysis placed *TART* in the *jockey* clade of non-LTR retrotransposons, which includes elements as *jockey*, *Doc*, *F* and *X*. Since *HeT-A* does not encode RT, the Gag amino acid sequence was used in evolutionary studies. Although Gag proteins undergo much more rapid sequence change than RT domains (McClure et al., 1988), the amino acid sequence of HeT-A Gag showed significant similarities to sequences from

non-LTR retrotransposons belonging to the *jockey* clade (Pardue et al., 1996).

3.2 *HeT-A*, *TART* and *TAHRE* relationship

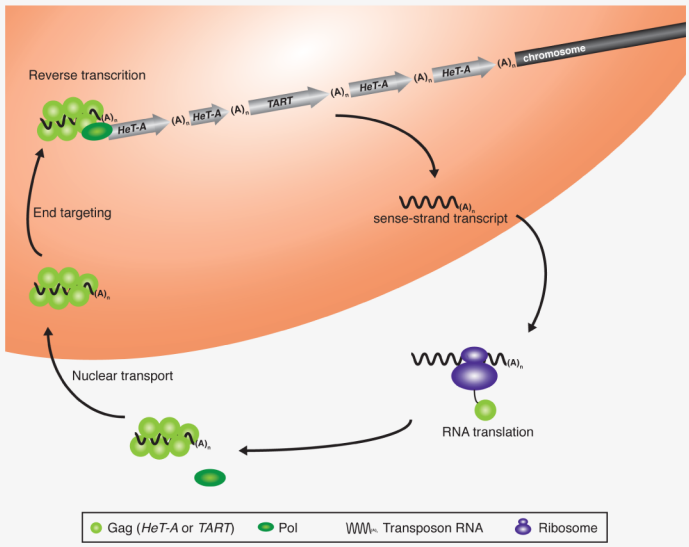
The fact that *Drosophila* needs three different retrotransposons to maintain its telomeres and why one of the three has not been able to overcome the other two is quite intriguing. Maybe the specific characteristics of *HeT-A*, *TART* and *TAHRE* are needed for a successful transposition of each of them. *HeT-A*, a defective element that needs to rely on a RT source for transposition, is the one that most benefits from this relationship since it is by far the most abundant of the three elements. Maybe the explanation relies on the fact that the Gag protein of *HeT-A* is the only of the three telomeric Gags with the ability to localize at the telomeres (Pardue and DeBaryshe, 2008), this property of HeT-A Gag has been conserved across *Drosophila* species (Casacuberta et al., 2007). As a result, *TART* and *TAHRE* need the Gag protein of *HeT-A* for a proper telomere targeting. Additionally, for a successful *HeT-A* transposition the RT needs to be provided in *trans*, probably by *TART* (Levis et al., 1993; Rashkova et al., 2002) or by *TAHRE* (Abad et al., 2004). Due to their genome similarities, pattern of transcription in germline cells and their control by the *rasi*RNA pathway, *TAHRE* seems to be the perfect partner for *HeT-A* in the germline (Shpiz et al., 2007). Nevertheless, *TAHRE* is present only in few copies in most analyzed stocks, while *TART* is present in several functional copies in all *Drosophila* stocks analyzed, being this way the most likely RT source for *HeT-A* (George et al., 2006). In summary, the three telomeric retrotransposons have an interdependent relationship where *HeT-A* provides telomere targeting for *TART* and *TAHRE*, and uses their RT for transposition. Depending on the cell type or development stage, *HeT-A* would choose *TART* or *TAHRE* as transposition partner (Pardue and DeBaryshe, 2011b). The fact that *HeT-A* is much more abundant than its telomere partners must be due to a most successful transposition or

alternatively, telomeres with more *HeT-A* elements might provide a better telomere function and may be positively selected.

3.3 Mechanisms of telomere elongation

In order to counteract the terminal erosion that occurs every cell cycle and maintain their optimal length the telomeres must be elongated. *Drosophila* telomeres are mainly elongated by the specific transposition of the telomeric retrotransposons *HeT-A*, *TART* and *TAHRE* onto the end of the chromosomes (see Box 3). However, like in telomerase organisms, recombination and gene conversion (non-reciprocal recombination) can also act as a backup mechanism of telomere lengthening (De Lange et al., 2006). When immortal human cancer cells fail to reactivate telomerase, recombination is often used as alternative lengthening of telomeres (ALT). Terminal gene conversion (TGC) is another mechanism to maintain telomere length, consisting in the replication of the end sequence when a template from the same or homologous chromosome is available. In *Drosophila*, this mechanism was observed when the uncharacterized *E(tc)* mutants showed a telomere length double than wild type strains without affecting the expression level of the telomere retrotransposons or its transposition rate (Melnikova and Georgiev, 2002). Additionally, experiments using broken chromosome ends with the *yellow* gene (without the promoter region) inserted close to the terminus revealed that the expression of that *yellow* gene could be activated by the transposition of *HeT-A* to the end of the broken chromosome. Moreover, approximately 20-30% of the *yellow* gene activation was induced by recombination events with a wild type chromosome, reintroducing this way the *yellow* promoter and enhancers in the broken end (Mikhailovsky et al., 1999; Kahn et al., 2000).

Box 3: Telomere retrotransposon life cycle



In *Drosophila*, the telomeres are maintained by the specific transposition of the telomeric retrotransposons *HeT-A*, *TART* and *TAHRE*. However, the specific mechanism of telomere elongation is not yet completely understood. Whenever telomere elongation is needed, the telomeric retrotransposons are transcribed into a sense-strand RNA that will serve both as mRNA and transposition intermediate. Once in the cytoplasm, the poly(A)+ sense-strand RNA is translated into Gag and Pol proteins. The newly synthesized proteins and the translated RNA associate forming a ribonucleoprotein that is transported to the nucleus. Once in the nucleus, the HeT-A Gag proteins probably target the ribonucleoprotein to the end of the chromosomes and the RNA serves as a template for reverse transcription, giving rise to new telomere additions. Since for most non-LTR retrotransposons the reverse transcription is primed by a 3' hydroxyl exposed at a nick in chromosomal DNA (Luan et al., 1993; Eickbush, 2002), it is thougth that *HeT-A*, *TART* and *TAHRE* are primed for reverse transcription by the free 3' hydroxyl group present at the end of the chromosome, a mechanism named target-primed reverse transcription (TPRT). (Mason et al., 2008; Pardue and DeBaryshe, 2011a; Zhang and Rong, 2012).

3.4 Telomere domains

Drosophila telomeres can be divided in two different domains accordingly to their position, function and characteristics – the capping and the telomeric domains (Figure 4). The *capping domain*, present at the very end of the telomere, is necessary to protect the end of the chromosome from being recognized as a double strand break (DSB) by the DNA repair machinery (Raffa et al., 2011). The *telomeric domain*, exclusively composed by the *HeT-A*, *TART* and *TAHRE* (HTT) retrotransposon array, regulates the telomere length and determines the stability of the capping domain (Biessmann et al.,

2005). Additionally, located between the HTT array and the euchromatic genes lies the *subtelomeric domain*, also called TAS (Telomere-Associated Sequences). This domain does not strictly belong to the telomeres, but its vicinity influences their function. TAS sequences are composed by complex satellite repetitions located in the vicinity of the euchromatic genes (Figure 4) and has been suggested to have a role mediating the silencing of the telomeric retrotransposons (Biessmann et al., 2005).

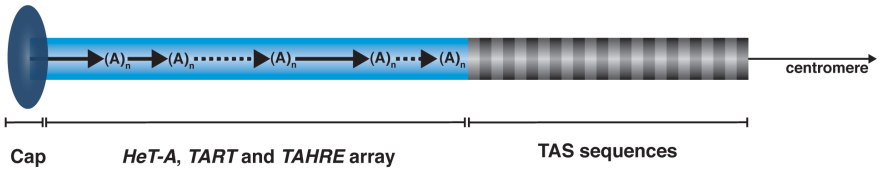


Figure 4: Different domains at *Drosophila* telomeres. *Drosophila* telomeres are constituted by two different domains: the capping domain (dark blue oval) that act as a physical cap; and the telomeric domain (light blue rectangle) formed by head-to-tail arrays of the telomere retrotransposons (HTT). Additionally, proximal to the HTT array is the subtelomeric domain (light and dark grey rectangles) constituted by complex satellite sequences. Black arrows represent *HeT-A* and dashed arrows *TART*.

3.4.1 Capping Domain

In addition to their role counterbalancing the incomplete DNA replication, the telomeres play an important function in chromosome end capping. The telomere capping function is essential to protect the chromosome ends from degradation and fusion events, allowing this way the cells to distinguish the natural chromosome ends from DNA breaks. As mentioned before, the shelterin complex is responsible for telomere capping in mammals. This complex is constituted by six proteins that assemble at the telomeres by recognizing the telomerase repeats (De Lange, 2002). Likewise, *Drosophila* telomeres are protected by the terminin complex (Raffa et al., 2011). However, the terminin complex assembles at the end of the chromosomes in a sequence independent manner (Levis, 1989; Biessmann et al., 1990; Raffa et al., 2009). This unique characteristic of terminin is not surprising since *Drosophila* telomeres are composed by a random distribution of the elements

HeT-A, *TART* and *TAHRE*. Several studies show how telomeres with non-telomeric sequences are able to remain stable for several generations (Biessmann et al., 1992; Ahmad and Golic, 1999) and recruit capping proteins (Fanti et al., 1998; Cenci et al., 2003). With time these deficient telomeres acquire telomere specific sequences (*HeT-A*, *TART*, and *TAHRE*), suggesting that telomere capping and telomere elongation are two independent processes (Craig, 2002). The ability of the capping complex to assemble independently of a particular sequence suggests that structural or chromatin determinants define the end of the chromosome and point towards an epigenetic mechanism for telomere protection in *Drosophila*.

The terminin complex is composed by a complex of four proteins that assembles exclusively at the end of the chromosomes and prevents telomere fusions (TFs) (Raffa et al., 2011). These proteins are named HOAP (HP1-ORC-associated protein), HipHop (HP1-HOAP interacting protein), Moi (Modigliani) and Ver (Verrocchio). All of them are encoded by fast evolving genes for which no homologues have yet been found outside *Drosophila* species. In the case of Ver, which contains an OB fold domain, some similarities have been found to the human Rpa2/Stn1 proteins (Raffa et al., 2010). Rpa2/Stn1 proteins, together with Cdc13, form the CST complex that protects human telomeres together with shelterin (Wellinger, 2009).

HOAP, Moi and Ver directly interact with each other and mutations in their genes (*caravaggio*, *modigliani* and *verrocchio*, respectively) produce a high frequency of telomere fusions. Furthermore, HOAP and Moi directly interact with HP1a (Heterochromatin Protein 1) (Raffa et al., 2009) and HOAP interacts with HipHop, the fourth protein that forms the terminin complex (Gao et al., 2010). HOAP is known to bind double stranded DNA (Shareef et al., 2001), and Ver, through its OB-fold domain, binds single stranded DNA without sequence specificity (Raffa et al., 2011), (Figure 5). In conclusion, HOAP and HipHop bind primarily to double-stranded telomeric DNA recruiting Moi and Ver, which associate with the single stranded overhang. It is still not clear if *Drosophila* telomeres terminate with a 3' single-stranded overhang or

if this putative 3' overhang folds in a t-loop structure. The fact that *Drosophila* telomeres do not terminate in a specific DNA sequence makes it complicated to understand how they can form a stable t-loop.

In addition to the terminin complex, there are other proteins involved in *Drosophila* telomere capping. These proteins are not considered part of the terminin complex because they do not localize or function only at telomeres. To date, seven proteins were identified to be required for telomere stability: HP1a, ATM, ATR, NBS, Mre11, Woc and UbcD1.

As mentioned before, HP1a interacts with the terminin proteins HOAP and Moi (Cenci et al., 2003). However, this protein does not localize exclusively at the telomeres and has other functions in the cell (Vermaak and Malik, 2009). HP1a is a conserved chromosomal protein encoded by the suppressor of position effect variegation *Su(var)2-5* gene (Eissenberg et al., 1990). This protein was first described in *Drosophila* and related with heterochromatin formation (James and Elgin, 1986). HP1 is composed of a chromo domain (CD) and a chromo shadow domain (CSD) linked by a hinge domain (Eissenberg and Elgin, 2000). In *Drosophila*, there are three isoforms of HP1 – HP1a, HP1b and HP1c (Smothers and Henikoff, 2001). The isoform present at the telomeres is HP1a (from now on only HP1) (Cryderman et al., 1999). At *Drosophila* telomeres HP1 has two main functions; the capping function, where it controls telomere stability and end accessibility, and telomere retrotransposon silencing. The capping function is due to the direct binding of HP1 to the telomeric DNA, while the telomere silencing function is related to the binding of HP1 to H3K9me3 (Perrini et al., 2004).

The checkpoint kinases ATM and ATR also play a role in telomere protection in *Drosophila*. Mutations in *atr* do not lead to telomere fusions, however when both ATM and ATR are removed the phenotype of ATM depletion is enhanced. These results show that ATM and ATR have overlapping functions in telomere protection (Bi et al., 2005). Mutations on the MRN complex proteins NBS and Mre11 also result in telomere protection problems, these

proteins were shown to belong to the same pathway as ATM on telomere protection (Bi et al., 2005; Gao et al., 2009).

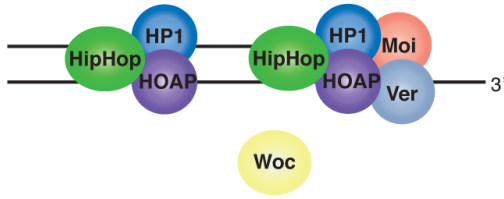


Figure 5: The capping domain. In *Drosophila*, the telomeres are capped by a complex of proteins called terminin. The terminin complex is formed by the proteins HOAP, HipHop, Moi and Ver. HOAP and HipHop primarily bind to double-stranded telomeric DNA, recruiting Moi and Ver, which associate with the single stranded DNA. HOAP and Moi directly interact with HP1, and HOAP interacts with HipHop. Moreover, the transcription factor Woc is also a component of the capping complex. Adapted from (Raffa et al., 2011).

Mutations in the gene *woc* (*without children*) also produce a high frequency of telomere fusions in larval brain cells (Raffa et al., 2005). *woc* is a transcription factor composed of eight zinc-fingers with a well-described role in gene regulation and ecdysone biosynthesis (Warren et al., 2001; Font-Burgada et al., 2008) . The role of Woc in telomere capping seems to be independent of HP1 and HOAP (Figure 5) since mutations in these proteins do not affect Woc localization at the telomeres and *vice versa* (Raffa et al., 2005).

Mutations in the gene *eff* (*effete* or *UbcD1*) also result in telomere capping problems (Cenci et al., 1997). The *eff* gene encodes a highly conserved protein that belongs to the class I ubiquitin-conjugating enzymes (E2) (Treier et al., 1992). The fact that UbcD1 is necessary for telomere protection suggests a mechanism of telomere capping related with protein ubiquitination. However, no Ubcd1 substrates have been found in *Drosophila* telomeres to date.

Finally, other proteins like HP2, SuUR (suppressor of underreplication), SU(VAR)3-7, and the Ku70/80 heterodimer also localize at the telomere cap

(Andreyeva et al., 2005; Melnikova et al., 2005). Mutants of these proteins do not cause telomere fusions (Perrini et al., 2004; Cenci et al., 2005; Shaffer et al., 2006). However, the Ku70/80 heterodimer regulates the telomere length by controlling the accessibility to the end of the chromosome (Cenci et al., 2005).

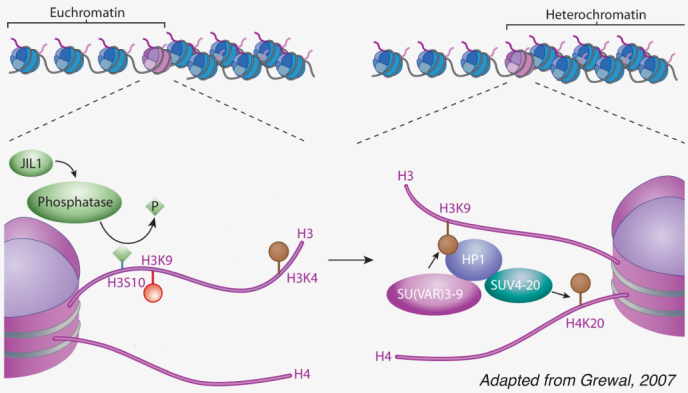
3.4.2 Telomere Domain – *HeT-A*, *TART* and *TAHRE* array

The telomeric domain is composed exclusively by head-to-tail arrays of *HeT-A*, *TART* and *TAHRE* (HTT) with their poly(A) tails facing towards the centromere. The telomeric domain is involved in the regulation of telomere length since the genes necessary for telomere elongation are embedded in this domain. The chromatin characteristics of the telomeric domain remain unclear; while some stocks show compacted DNA at the very end of their polytene chromosomes, others are composed of decondensed DNA (Zhimulev, 1998). The regulation of the telomeric chromatin at *HeT-A*, *TART* and *TAHRE* promoters is most likely one of the major mechanisms used to control telomere length, since it will influence the amount of transcripts and transposition intermediates available.

To better understand the chromatin characteristics and retrotransposon regulation in *Drosophila* telomeres, Andreyeva and co-workers took advantage of *tel1* mutants (strain that has ten times longer telomeres than a wild type strain (Siriaco et al., 2002)) to obtain a better resolution of the telomeres (Andreyeva et al., 2005). To carry out this study, they performed immunostaining experiments with several candidate proteins and compared their localization in the different telomere domains. This work demonstrated that the capping domain recruits the proteins HP1, HP2, SuUR and SU(VAR)3-7; the HTT array contains markers of both euchromatin (JIL-1, H3K4me3 and Z4) and heterochromatin (H3K9me3); and that the subtelomeric domain recruits polycomb (PcG) repressive proteins (E(Z), Pc and H3K27me3).

Recent studies using ChIP experiments demonstrated that HP1 is not exclusively located at the capping domain but is also present in the HTT array and TAS (Frydrychova et al., 2008). The fact that HP1 was found at the HTT array is not surprising since mutations in the *Su(var)2-5* gene lead to an increase in *HeT-A* expression and also longer telomeres (Perrini et al., 2004).

Box 4: Euchromatin *vs* Heterochromatin



Adapted from Grewal, 2007

The DNA is a very large polymer that interacts with histones forming nucleosomes and chromatin fibers, this way the DNA is arranged in a highly compacted form and can fit inside the cell nucleus. The chromatin structure in the vicinity of a gene directly influences its expression. When a gene needs to be expressed or repressed its chromatin structure can be modified by histone modifications.

There are two types of chromatin accordingly to the degree of compaction - euchromatin and heterochromatin. Euchromatin is a lightly packed form of chromatin, rich in active genes. This type of chromatin is characterized by the presence of specific histone marks like H3K4me3, H3S10ph, and H3K9Ac. On the other hand, heterochromatin is a tightly packed form of DNA that silences the expression of genes in two different ways. The constitutive chromatin acts primarily as genome stabilizer, preventing genome rearrangements between repetitive DNA sequences. As a consequence, genes located near repetitive DNA are indirectly silenced through the spreading of the silent chromatin by a phenomenon know as position effect variegation (PEV, see box 5) Additionally, the facultative chromatin is usually located at gene promoters, ensuring this way the epigenetic silencing of genes in certain cell types or tissues. The establishment of this form of heterochromatin depends on transcriptional repressors, which will recruit histone-modifying enzymes and structural proteins that maintain a silent state. The most characteristic marks of heterochromatin are H3K9me3 and H4K20Ac (van Steensel, 2011).

The binding of HP1 (through its chromo domain) to the HTT array is probably driven by H3K9me3, which implies the previous action of a histone methyltransferase. In *Drosophila*, the methylation of H3K9 is accomplished by the histone methyltransferases SU(VAR)3-9, dSETDB1 and dG9a, orthologous to the human SUV39H1, SETDB1 and EHMT2/G9a, respectively

(Schotta et al., 2004; Seum et al., 2007). SU(VAR)3-9 and dG9a have overlapping functions in heterochromatin formation and gene silencing (Mis et al., 2006). SU(VAR)3-9 is mainly associated with H3K9 methylation at the chromocenter. Furthermore, *Su(var)3-9* mutations do not affect the silencing of the telomeric retrotransposon *HeT-A* (Perrini et al., 2004). These findings indicate that another methyltransferase should be responsible for H3K9me3 at the telomeres. During the course of this thesis, it was discovered that mutations in the *dSetdb1* gene lead to an increase in *HeT-A* expression, indicating that dSETDB1 is involved in the methylation of H3K9 at *Drosophila* telomeres (Phalke et al., 2009; Gou et al., 2010). Additionally, dSETDB1 was found to interact with DNA methylase 2 (Dmnt2). Mutations in both proteins lead to the loss of DNA methylation at the telomeric domain, suggesting that dSETDB1 mediated DNA methylation also leads to *HeT-A* silencing (Phalke et al., 2009; Gou et al., 2010).

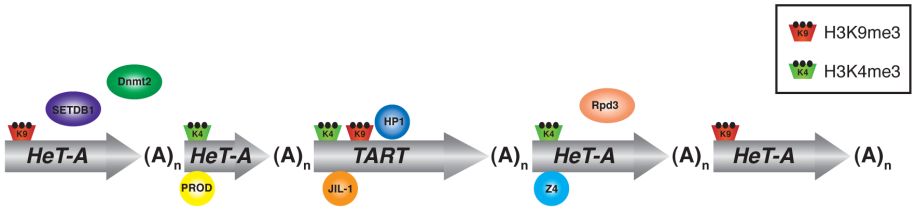


Figure 6: The telomeric domain. In *Drosophila*, the telomeric domain is composed by head-to-tail arrays of the retrotransposons *HeT-A*, *TART* and *TAHRE* (grey arrows). Several proteins were found to localize at this domain: JIL-1, Z4, PROD, dSETDB1, Dnmt2, HP1 and Rpd3, as well as the histone marks H3K9me3 and H3K4me3.

The PROD protein, encoded by the *prod* (*proliferation disrupter*) gene, is also involved in the regulation of *HeT-A* expression. PROD binds upstream the *HeT-A* promoter, which corresponds to the 3'UTR of the element near the poly(A) tail. Mutations in the *prod* gene lead to an increase on *HeT-A* transcripts but have no effect on telomere length, suggesting that PROD does not control telomere end accessibility (Török et al., 2006). Finally, the deacetylase Rpd3 was recently shown to deacetylate the *HeT-A* promoter

leading to changes in the heterochromatin structure, and bring stability to the telomeres (Burgio et al., 2011).

As mentioned before, JIL-1 and Z4 are the two other proteins associated with the HTT array. The specific role of these proteins at *Drosophila* telomeres was still not described at the beginning of this thesis and is one of its principal objectives, therefore, a detailed description of both will be presented in chapter 3.7.

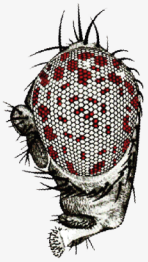
3.4.3 Subtelomeric Domain – TAS

Although not part of the telomere, immediately adjacent to the HTT array and before the first euchromatic gene there is the subtelomeric domain, a region composed of complex satellite repetitions called telomere associated sequences (TAS). This domain is present in several species from yeast to humans and is composed by highly polymorphic and dynamic sequences without any clear relation between species (Pryde et al., 1997). In *Drosophila*, TAS repeats can be divided in two families: the TAS-L family, found in the left arms of the 2nd and 3rd chromosomes (*2*L and *3*L), and the TAS-R family found at the right arms of the 2nd and 3rd chromosomes and the left arm of the X chromosome (*2*R, *3*R and *X*L). TAS-L and TAS-R do not show significant homology at the sequence level. The TAS-L family is composed of 40 to 60 tandem repeats of a canonical 458bp sequence, while the TAS-R family is composed by two different classes of repeats: a 440bp unit derived from the 3'UTR of the *Invader 4* retroelement and a telomere-specific unit (Mason et al., 2008).

The role of TAS is mainly associated with epigenetic regulation and heterochromatin formation. Particularly, TAS sequences contribute to the silencing of genes inserted in their vicinity, however, this silencing is unidirectional towards the end of the chromosome (Kurenova et al., 1998). By analogy with position effect variegation (PEV) (see Box 5), the telomeric silencing was named telomere position effect (TPE) (De Lange et al., 2006).

Despite their phenotypic similarities, TPE does not respond to *Su(var)* mutations as PEV does (Cryderman et al., 1999; Mason et al., 2004). The level of TPE related gene silencing depends on the proximity to TAS repeats; *white* reporter genes inserted into the HTT array close to TAS are repressed and show variegated expression but when the same reporter genes are inserted 5-10 kb from TAS no repression or variegation is observed (Mason et al., 2008). This findings support the idea that the telomeric silencing is mediated by TAS and not by the HTT array. However, we have observed that this rule does not always apply (see Results Chapter 1) and the silencing of the telomeric retrotransposons is not exclusively mediated by the TAS domain but also by epigenetic changes in in HTT array.

Box 5: Position Effect Variegation



Position effect variegation (PEV) is a variegation phenomena caused by the inactivation of a gene in some cells due to heterochromatin spreading. Additionally, PEV can also result from *P*-element insertions that place euchromatic genes into a heterochromatic environment, and chromosome rearrangements that position euchromatic chromosomal regions into heterochromatic compartments.

The classical example of PEV is the *Drosophila* w^{m4} (*white*-motled-4) translocation. In this mutation, an inversion on the X chromosome places the *white* gene next to pericentric heterochromatin. In a normal situation, the *white* gene is expressed in all cells of the adult eye resulting in a red eye phenotype. However, in the w^{m4} mutants the *white* gene is expressed in some eye cells while in others is silenced, resulting in a variegated phenotype (red-white mosaic) (Girton and Johansen, 2008).

Compared with the capping and HTT domains, fewer proteins were identified to associate with TAS. Immunostaining experiments using *tel1* mutants *(stock with telomeres 10 times longer)* revealed the presence of heterochromatin marks at the TAS domain (Andreyeva et al., 2005). TAS is enriched in the heterochromatic mark H3K27me3 and targeted by the Polycomb group (PcG) of transcriptional repressor proteins, such as Brahma (Brm), Enhancer of zeste (E(Z)) and Polycomb (Pc) (Boivin et al., 2003; Andreyeva et al., 2005; Doheny et al., 2008). Additionally, ChIP experiments also identified the presence of HP1 in TAS (Frydrychova et al., 2008), although mutations in this protein do not affect TAS silencing (Cryderman et al., 1999). Recently, Antão

and colleagues identified Zf30c (a zinc finger transcription factor (Jafari et al., 2012)) and Brm (a coactivator of the trytorax group of proteins (Kal et al., 2000)) as suppressors of TPE (Antão et al., 2012), Figure 7.

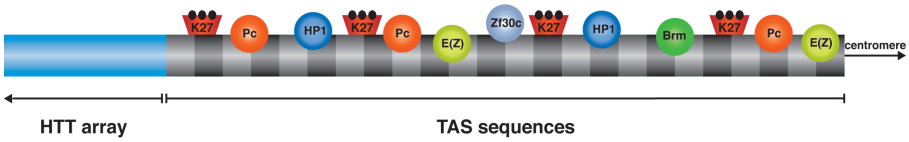


Figure 7: The subtelomeric domain. TAS sequences (dark and light grey rectangles) are associated with gene silencing and thus are rich in heterochromatin marks like H3K27me3 (red half triangle), Pc (orange circle), E(Z) (light green circle), Brm (green circle), HP1 (dark blue circle), and Zf30c (light blue circle).

Finally, TAS sequences are also responsible for the telomeric *trans*-silencing effect (TSE). TSE is a repression mechanism by which a transgene inserted in the subtelomeric chromatin (TAS) has the capacity to repress the expression of an homologous transgene inserted in euchromatic regions in the female germline in *trans* (Roche and Rio, 1998; Ronsseray et al., 2003; Mason et al., 2003). TSE displays an epigenetic transmission through meiosis and is highly sensitive to mutations affecting chromatin formation, like *Su(var)2-5* and *Su(var)3-7*, and the *rasi*RNA (repeat-associated small interfering RNA) silencing pathway, like *aub* (*aubergine*), *armi* (*armitage*) and *piwi* (*P-element induced wimpy testis*). These results suggest that in *Drosophila* the RNA silencing pathway can also depend on heterochromatin components (Josse et al., 2007; 2008; Todeschini et al., 2010)

3.5 Telomere regulation by the RNAi machinery

In addition to the epigenetic regulation, *Drosophila* telomeres can also be regulated by RNAi mechanisms. The piRNA (PIWI-interacting RNA) pathway is involved in the silencing of mobile elements in germline cells. The generation of piRNAs in germ cells is dependent on the Ping-Pong

amplification cycle. In the Ping-Pong cycle, a primary piRNA recognizes its target and recruits PIWI-proteins (Aub, Argo3 and Piwi), which will cleave the transcript, generating a secondary piRNA that will further amplify the process (Khurana and Theurkauf, 2010). Mutations in components of the piRNA pathway lead to the overexpression and mobilization of retrotransposons in the germline (Brennecke et al., 2007; Klattenhoff and Theurkauf, 2008). *Drosophila* telomere retrotransposons are no exception. Analysis of piRNA pathway mutants revealed that *HeT-A*, *TART* and *TAHRE* are also targets of the piRNA silencing (Savitsky et al., 2006; Khurana et al., 2010; Shpiz et al., 2011). The mechanisms of telomere length control by the piRNA machinery can be pre-translational or post-translational. The pre-translational control involves the piRNA-mediated spreading of heterochromatin into the HTT array. Mutations in the piRNA pathway proteins *spn-E*, *aub* and *piwi* lead to a transcriptional activation of the telomeric retrotransposons. This activation is associated with an enrichment in the histone marks H3K4me2 and H3K79me2 at the telomeric retrotransposons chromatin (Shpiz et al., 2007; 2011). Moreover, *armi* and *ago3* mutant flies show an increase in *HeT-A* copy number, which gives evidence of the involvement of the piRNA pathway in the control of telomere length (Khurana et al., 2010). Finally, the piRNA components were found to be essential for the assembly of the telomere capping proteins. *aub* and *armi* mutations lead to telomere fusions, these fusions reduce HOAP and HP1 binding to the telomeres, indicating that piRNAs are directly involved in the assembly of the telomere cap (Khurana et al., 2010). Additional evidence of piRNA control was obtained in our laboratory by finding a highly conserved sequence at the 3'UTR of *HeT-A,* HeT-A_pi1. This sequence is highly conserved across *HeT-A* copies within the *D. melanogaster* subspecies group. Moreover, a piRNA species has been found in both sense and antisense orientation corresponding directly to this sequence. The high conservation of this piRNA target suggested an important function of the HeT-A_pi1 sequence in the co-evolution of the *HeT-A* retrotransposon and the *Drosophila* genome (Petit et al., 2012).

3.6 The chromosomal proteins JIL-1 and Z4

As mentioned before, JIL-1 and Z4 were found to specifically localize at the HTT array of *Drosophila* telomeres (Andreyeva et al., 2005). Both proteins localize at the polytene chromosome interbands and have a role in maintaining the band-interband structure (Eggert et al., 2004; Deng et al., 2005).

3.6.1 JIL-1

In *Drosophila*, JIL-1 is the tandem kinase responsible for the phosphorylation of the serine 10 of histone H3 during interphase (Jin et al., 1999). Phylogenetic analyses show that JIL-1 and the human MSKs belong to the same family. Like MSKs, JIL-1 is constituted by four domains: NH_2 domain (NTD), kinase domain I (KDI), kinase domain II (KDII), and COOH-domain (CTD) (Figure 8B) (Jin et al., 1999). JIL-1 seems to have at least three different functions in the cell; it is required for maintaining the correct structure of the chromosomes, it is involved in reinforcing the active transcription of certain genes during interphase; and participates in the dosage compensation of genes in the male X chromosome.

JIL-1 localizes specifically at interband regions of larval polytene chromosomes, suggesting that it is present at the sites of actively transcribed genes (Figure 8A) (Jin et al., 1999). Reduced levels of JIL-1 lead to the condensed polytene chromosomes and lower levels of histone H3Ser10 phosphorylation (Figure 8C and 8D), indicating that the kinase activity of JIL-1 is required to maintain an open chromatin state and consequently facilitate gene expression (Wang et al., 2001). JIL-1 directly interacts with Chromator (Chro, also named Chiz) (Rath et al., 2006) and its presence at the polytene chromosomes is mediated by the Chro-Z4 complex (Gan et al., 2011). Like JIL-1, Chro and Z4 are also involved in the maintenance of the polytene chromosomes structure, suggesting that JIL-1 activity is necessary but not sufficient for maintaining chromosome morphology (Eggert et al., 2004; Rath

et al., 2006; Gan et al., 2011) . Chro was also shown to interact with histone H1 and this interaction was found to be required for its proper targeting to chromatin (Yao et al., 2011). Besides, JIL-1 was described to interact with histone H3 through its CTD (Bao et al., 2008). This interaction is necessary for a correct targeting of JIL-1 to chromatin and chromosome structure organization independent of its kinase activity.

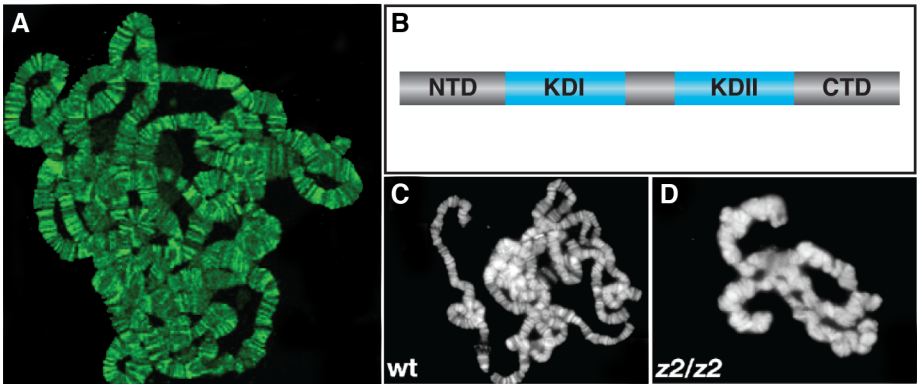


Figure 8: The JIL-1 kinase. (A) JIL-1 localizes at most interbands of third instar larvae polytene chromosomes (green, JIL-1 labeling) (Rath et al., 2006). (B) Schematic representation of the different domains of JIL-1. The JIL-1 kinase is constituted by four domains: the N-terminal domain (NTD), the kinase domains I and II (KDI and KDII), and the C-terminal domain (CTD). (C and D) Third instar larvae polytene chromosome preparations labeled with Hoechst (Deng et al., 2005). (C) Wild type polytene chromosomes show a well-defined band-interband pattern. (D) *JIL-1* mutant polytene chromosomes have a defective structure problems due to lack of euchromatin.

JIL-1 is also involved in a dynamic balance between euchromatin and heterochromatin. In *JIL-1* mutants, major heterochromatin marks like H3K9me2, HP1 and SU(VAR)3-7 spread to ectopic locations on the chromosome arms (Zhang et al., 2006; Deng et al., 2007; 2010). Several studies show that *JIL-1* and *Su(var)3-9* genetically and physically interact *in vivo* (Ebert et al., 2004; Deng et al., 2007; Boeke et al., 2010). JIL-1 was shown to strongly counteract SU(VAR)3-9 mediated heterochromatin formation (Ebert, 2004). Moreover, JIL-1 activity and localization at polytene

chromosomes is not affected in *Su(var)3-9* mutants, suggesting that JIL-1 acts upstream SU(VAR)3-9 maintaining euchromatic regions by antagonizing SU(VAR)3-9 and HP1 mediated heterochromatization (Zhang et al., 2006; Deng et al., 2010). Additionally, the JIL-1 kinase is able to phosphorylate SU(VAR)3-9 without increasing its histone methyltransferase activity, this phosphorylation may be related with a fine-tuning ability of JIL-1 to mediate heterochromatin formation and spreading (Boeke et al., 2010). Several studies of PEV show that *Su(var)2-5*, *Su(var)3-9* and *Su(var)3-7* loss of function alleles act as strong suppressors of PEV, while *JIL-1* mutants have the opposite effect, acting as enhancers of PEV. However, double mutants of these proteins and *JIL-1* show neutralized variegation effects (Deng et al., 2010; Wang et al., 2011b; 2011a; 2012). These observations led to the development of a model for a dynamic balance between euchromatin and heterochromatin. In this model the level of expression of a specific gene is determined by antagonistic functions of heterochromatic components like SU(VAR)3-9, H3K9me3, HP1 and SU(VAR)3-7 and euchromatic components like JIL-1 and H3S10ph (Lerach, 2006; Bao et al., 2008; Deng et al., 2010; Wang et al., 2011a; 2011b).

Furthermore, JIL-1 has an independent role in the regulation of dosage compensation. JIL-1 is almost 2-fold up-regulated in male X chromosomes and male flies are more susceptible to the reduction of JIL-1 levels (Jin et al., 1999; 2000). JIL-1 co-localizes with the MSL (Male Specific Lethal) complex on the male X chromosome and was shown to molecularly interact with several proteins of this complex. Moreover, the JIL-1 kinase is known to be necessary for the correct dosage compensation of *white* alleles (Deng et al., 2005; Lerach et al., 2005). Additionally, JIL-1 has a strong maternal effect and *JIL-1* null offspring can survive until pupariation using the JIL-1 maternally produced by heterozygous females (Zhang et al., 2003a).

Finally, JIL-1 has a controversial role in the process of gene transcription. Corces and co-workers defend that gene activation after heat shock is mediated by JIL-1 H3S10 phosphorylation, leading to the recruitment of 14-3-

3 and the elongator protein 3 (Elp3). These proteins will then trigger the transition of an initiated RNA polymerase II (Pol II) into the elongation mode (Ivaldi et al., 2007). By contrast, Johansen and colleagues defend that in *JIL-1* mutant alleles Pol II is still active, thus JIL-1 and H3S10 phosphorylation do not have a direct function in heat shock gene activation (Cai et al., 2008). Recently it was demonstrated that JIL-1 depletion has only mild effects on transcription, suggesting that JIL-1 is not directly required for transcription. Instead, JIL-1 contributes to a composite histone mark that may serve to reinforce the active chromatin state by preventing the association of silencing factors (Regnard et al., 2011).

In addition to Chro and the histone H3 (see above), other partners of JIL-1 were described. The first one is Lola zf5, a BTB-domain protein with a role in transcription regulation. *lola* and *JIL-1* genetically interact and function in the same pathway (Zhang et al., 2003b). Another JIL-1 partner is Lamin Dm_0, an essential protein required to maintain the nuclear envelope structure. JIL-1 interaction with Lamin Dm_0 is essential to maintain the nuclear morphology and the integrity of nurse cells during oogenesis, suggesting that JIL-1 might cooperate with Lamin Dm_0 to maintain and modify chromatin structure and nuclear architecture (Bao et al., 2005).

3.6.2 Z4

Z4, also known as Putzig, is a seven zinc-finger protein essential for fly development. Like JIL-1, Z4 localizes at most interbands of polytene chromosomes and is absent from bands and the heterochromatic chromocenter. Although Z4 localizes in most interbands, this protein does not stain transcriptional active loci like *hsp70* heat-shock puffs but is present on the edge of each puff, suggesting that Z4 is involved in the formation of band-interband boundaries. The chromosomal localization of Z4 is lost during mitosis when the protein gets diffusely distributed within the mitotic spindle region (Eggert et al., 2004).

There are evidences that Z4 is involved in chromatin compaction since *Z4* mutants enhance the expression of the *white* gene in the w^{m4} allele, acting as a suppressor of PEV. These results suggest that Z4 is involved in the establishment or maintenance of heterochromatin. Moreover, polytene chromosome squashes of *Z4* mutants show structural problems with a high degree of decompaction and a fluffy appearance (Figure 9A-C), which indicates a loss of the heterochromatic bands (Eggert et al., 2004).

Z4 immunoprecipitation experiments in Kc cells identified Chro as a major Z4 interactor. As mentioned before, Chro colocalizes with Z4 and JIL-1 in many interbands and the three proteins are required for the maintenance of the polytene chromosomes structure (Rath et al., 2004; Gortchakov et al., 2005; Gan et al., 2011).

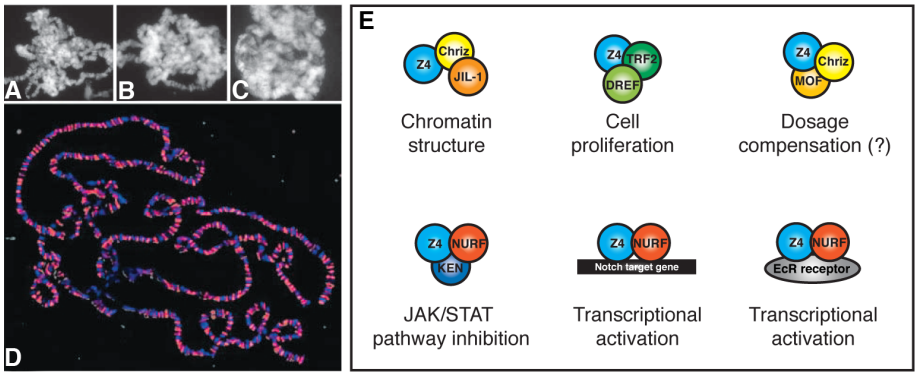


Figure 9: Z4 (or Putzig). (A-C) Z4 mutant polytene chromosomes have structural problems because they lack heterochromatin (Eggert et al., 2004). (D) Z4 localizes at most interbands of *Drosophila* polytene chromosomes (red, Z4 staining; blue, DAPI staining) (Eggert et al., 2004). (E) Schematic representation of Z4 protein complexes and their cellular roles.

Additionally, Z4 was shown to be a key regulator of several cellular pathways. Z4 is part of a macromolecular complex together with TRF2 (TATA-binding protein-Related Factor) and DREF (DNA replication-Related Element Factor). This complex has been associated with the transcriptional activation of replication-related genes that contain DREF binding sites (Hochheimer et al., 2002). Furthermore, Z4 and NURF are involved the activation of Notch target

genes and ecdysone receptor (EcR) signaling through chromatin remodeling, in these situations Z4 is involved in the formation of an active chromatin state (Kugler and Nagel, 2007; 2010). Z4 is also an important cofactor in the JAK/STAT pathway, the Z4-NURF complex acts, together with Ken, as a transcriptional repressor of the JAK/STAT target genes (Kugler et al., 2011). Finally, Z4 and Chro were shown to interact with the male-specific lethal (MSL) histone acetyltransferase MOF (males absent of first) but the role of this complex is still not completely understood (Prestel et al., 2010) (Figure 9E).

Objectives

Objectives

In *Drosophila* the genes involved in telomere maintenance reside at the telomeric retrotransposon array, making telomere function strongly dependent of telomere transcription in this organism. Therefore, the main goal of this thesis is to study the proteins that specifically affect the expression of the telomeric retrotransposons *HeT-A*, *TART* and *TAHRE*. Because the structure of the telomeric chromatin might also be relevant to understand the mechanisms of telomere regulation, we have centered our attention on proteins involved in chromatin structure regulation. To address the aim of this thesis we set the following objectives:

- Select candidate proteins that might have a role regulating the expression of the telomere retrotransposons.
- Investigate the influence of the selected proteins – JIL-1, Z4 and dSETDB1 - over the expression of the telomeric retrotransposons.
- Study the effect of the analyzed proteins in telomere length and stability.
- Characterize the chromatin modifications associated with changes in the expression of the telomeric retrotransposons in mutants of the selected proteins.
- Explain the observed effects in the context of protein complexes already described to act at the telomeres or known to interact with JIL-1, Z4 and dSetDB1.
- Understand how the observed effects are specific of the telomere domain and their secondary effects over the subtelomere domains.

Results

CHAPTER 1

The JIL-1 kinase affects telomere expression in the different telomere domains of *Drosophila*

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Submitted September 2012

ABSTRACT

While studying the regulation of telomere expression in *Drosophila*, we have observed that mutant alleles of *JIL-1* affect gene expression from the different telomere domains. JIL-1 function depends on which telomere domain, which chromosome and which promoter is embedded in the telomere chromatin.

INTRODUCTION

Drosophila telomeres are the best-studied telomerase exception to date. The transposition of three special non-LTR retrotransposons; *HeT-A*, *TART* and *TAHRE* (HTT), buffers the receding chromosome ends when needed (Frydrychova *et al*., 2008; Pardue and DeBaryshe, 2003). Therefore, in *Drosophila* the genes responsible for telomere elongation are embedded in the telomere chromatin and, to understand telomere regulation in this organism, is necessary to understand gene expression from this genomic domain.

Drosophila telomeres are organized in two different domains; the *cap* domain protecting the very end of the chromosome, and the *telomere* domain composed of the HTT array. Additionally, immediately adjacent to the HTT array is the *subtelomere* domain, composed of the Telomere Associated Sequences (TAS) (Andreyeva *et al*., 2005; Mason *et al*., 2008; Rute Silva-

Sousa, 2012). Although the subtelomere domain is not strictly part of the telomere, its closeness to the HTT array influences some of its functions.

Previous studies based on the variegation of reporter genes (PEV), suggested that TAS sequences nucleate a compacted chromatin restrictive of gene expression at the telomeres, causing telomere position effect (TPE). Later, molecular studies have confirmed the presence of chromatin marks typical of repressive chromatin (H3K27me3 and Polycomb) in this domain (Cryderman *et al.*, 1999; Boivin *et al.*, 2003; Andreyeva *et al.*, 2005; Mason *et al.*, 2008).

We aimed to better understand the regulation of gene expression in *Drosophila* telomeres. With this objective we assayed the influence of the JIL-1 kinase over the telomere and subtelomere domains of *Drosophila*. In *Drosophila*, JIL-1 is an essential gene responsible for the phosphorylation of the Ser10 of Histone H3 when cells are in interphase or as a result of a stress response (Jin *et al.*, 1999; Zhang *et al.*, 2003; Regnard *et al.*, 2011). JIL-1 localizes at the band-interband region of polytene chromosomes and has been shown to be involved in maintaining chromosome structure and protecting genomic regions from an excess of heterochromatinization (Ebert *et al.*, 2004; Deng *et al.*, 2005; Zhang *et al.*, 2006; Wang *et al.*, 2011). Additionally, JIL-1 has been found to localize among other genomic locations at the HTT array (Andreyeva *et al.*, 2005).

MATERIALS AND METHODS:

Fly stocks and crosses

Fly stocks were maintained and crosses performed at 25°C on standard *Drosophila* corn meal medium. *JIL-1^{z60}/TM6* and *JIL-1^{h9}/TM6* stocks were provided by Kristin M. Johansen. *Su(var)2-5^{05}/CyO*, *Su(Z)2$^{1.a1}$*/CyO and *Su(Z)2$^{1.b7}$*/CyO were obtained from Bloomington Stock Center. *Su(var)3-9^{17}*/TM3 and *Bmx2trx/TM6* were provided by Fernando Azorin

laboratory. Lori Walrath provided the reporter lines 39C-5, 39C-27, 118E-5, 39C-72, 39C-42 and 39C-12, and James Mason the lines EY08176, EY00453 and EY09966.

To assay for TPE modifications, males from homozygous reporter gene stocks, with a *P*-element inserted at a telomere domain, were crossed with virgin females from mutant stocks of the proteins of interest. The eye phenotype of the resulting F1 progeny with the mutation was compared with their siblings lacking the mutation. Flies were analyzed and collected 2 days after eclosion and briefly frozen. Images were obtained using Zeiss Discovery V.8 microscope with AxioCam MRE5 camera and Axiovision 4.7 software.

RNA Extraction and cDNA synthesis

Total RNA was isolated from ten whole third instar larvae and extracted using RNeasy Mini Kit (Qiagen) according to manufacturer'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 37ºC. 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 d(T) 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: HeT-A_F (CCCCGCCAGAAGGACGGA) and HeT-A_R (TGTTGCAAGTGGCGCGCA), EY0176_F (GTGGATGTCTCTTGCCGACG) and EY8176_R (GCTCTTACTCACACTGACGCTTCTC), EY09966_F (CTATAACATCGAGTACCAGCCG) and EY09966_R (GCTCTTACTCACACTGACGCTTCTC), Actin_F

(GCGCCCTTACTCTTTCACCA) and Actin_R (ATGTCACGGACGATTTCACG).

Chromatin Immunoprecipitation experiments (ChIPs)

Chromatin immunoprecipitation experiments were preformed has described in chapter 2. Chromatin was immunoprecipitated with anti-H3K27me3 (ab6002, Abcam). Three independent ChIP samples were analyzed and the amount of immunoprecipitated DNA was calculated by quantitative real-time PCR using iQ™ SYBR® Green Supermix (BioRad). Primers used for quantitative real-time PCR: HeT-A_5'UTR_F (TCGCGCTTCCTCCGGCAT) and HeT-A_5'UTR_R (GCGGTTATTACATTACGGCGG), TAS_X_F (TTGTAATTTGGTGCGGCAGC) and TAS_X_R (CAGCGTGACTGTTCGCATTC), RpL32_F (CAAGAAGTTCCTGGTGCACAA) and RpL32-R (AAACGCGGTTCTGCATGAG).

Quantitative real-time PCR

Quantitative real-time PCR was performed to determine *HeT-A* copy number and *HeT-A* expression, and in ChIP experiments. The iQ5 Multicolor Real-Time PCR Detection System was used and the iQ™ SYBR® Green Supermix (BioRad) was used to prepare the reactions. Relative levels of *HeT-A* expression were determined using the threshold cycle and normalized to actin levels (or RpL32 for ChIPs). Three independent experiments of two samples each strain were performed.

RESULTS AND DISCUSSION

In order to assess the possible role of JIL-1 regulating gene expression at the HTT array, we chose several lines that contain a mini-*white* reporter gene at telomere (HTT array) and at the subtelomere domain (TAS) from different chromosomes (Wallrath and Elgin, 1995; Cryderman *et al.*, 1999; Biessmann *et al.*, 2005).

JIL-1 is necessary for gene expression at the telomere domain (HTT)

By crossing mutant alleles of *JIL-1* with lines with a mini-*white* gene inserted at the HTT array from different chromosomes, we searched for TPE effects on the progeny eyes (Figure 1A). We did not observe a different TPE phenotype in any of the telomere lines (EY08176, EY09966, EY0453) with simple heterozygous combinations of *JIL-1* alleles (Figure 1A, 1st, 2nd and 3rd rows). Nevertheless, when we combined both alleles in a trans-heterozygous mutant (*JIL-1*^{z60}/*JIL-1*^{h9}) with the line EY08176, an enhancer effect of TPE was revealed (Figure 1A, 1st row, 4th column). A trans-heterozygous combination was difficult to obtain for the line EY0453 line since both the mini-*white* insertion and the *JIL-1* mutation are at the 3rd chromosome. On the other hand, the mini-*white* gene inserted at the HTT array in the 4th chromosome line EY09966 is heavily repressed and would not have allowed the detection of an additional enhancer effect.

Following, we investigated if a decrease in the expression of the HTT retroelements adjacent to the mini-*white* gene insertion was also observed in *JIL-1* mutants. Quantitative real-time PCR was used to detect expression of the transcript containing both, the telomeric element and the mini-*white* reporter. *JIL-1* heterozygous mutants show lower telomere transcription from the HTT element adjacent to the insertion in the 2nd (EY08176) and the 4th (EY09966) chromosomes (Figure 1B and D). This reduction is in agreement with a reduction of the overall expression of *HeT-A* (Figure 1C and E, and

R.S-S., E.L-P., D.P., E.C. *submitted*) suggesting a global role of JIL-1 over the HTT array.

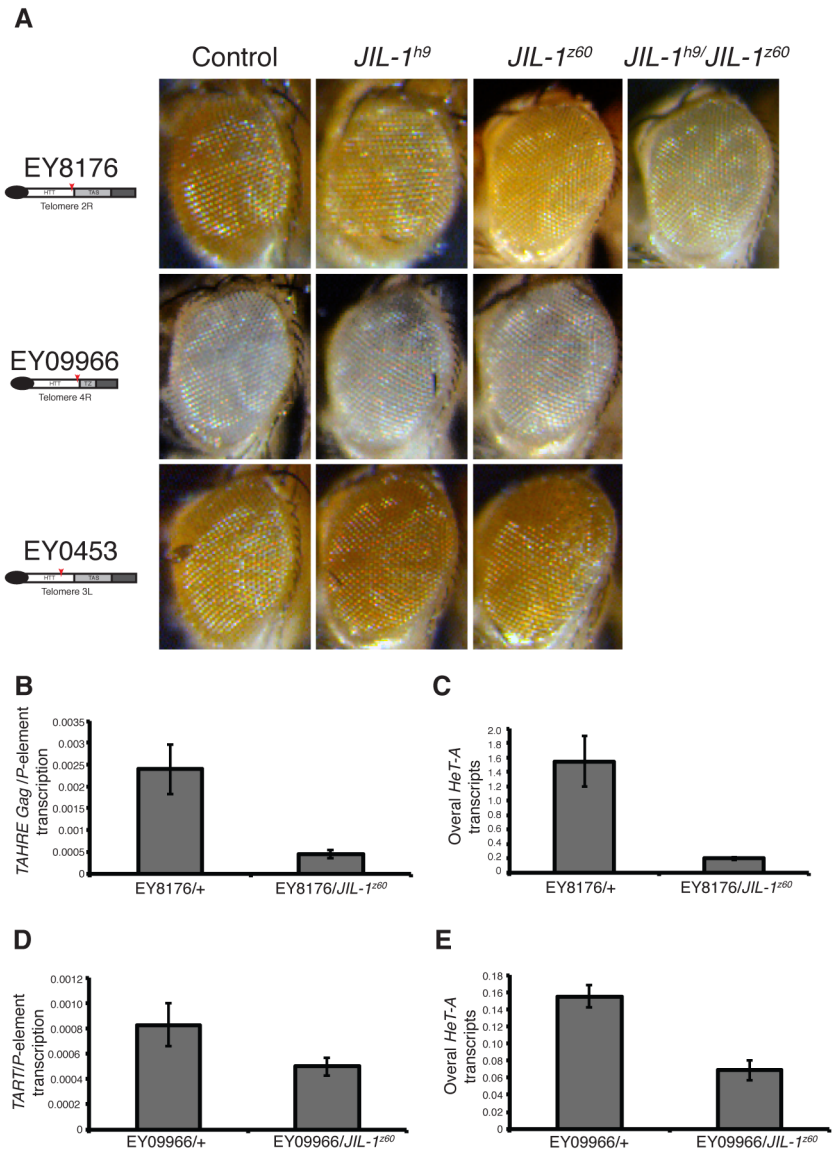

Figure1: **JIL-1 affects the expression of genes embedded in the HTT array.** (A) To assay for TPE modifications, homozygous males for the reporter gene were crossed with females from the *JIL-1* mutant alleles, *JIL-1*^{z60} and *JIL-1*^{h9}. Reporter lines with *mini-white* insertions were: EY08176 for the 2R telomere (1st row), EY00453 for the 3L telomere (2nd row) and EY009966 for the 4R telomere (3rd row). 1st column: control (descendant of males of the reporter lines crossed with *w*^{1118} females) 2nd and

3rd columns: *JIL-1* heterozygous, *JIL-1*^{z60} or *JIL-1*^{h9}, no change in the mini-*white* transcription is detected in these allelic combinations. 4th column: *JIL-1*^{z60}/*JIL-1*^{h9} trans-heterozygous allele, in this case an enhancer of effect of TPE is detected. (B-E) *JIL-1* mutations lead to a decrease in the transcription of the telomeric retrotransposon immediately downstream of the *P*-element insertion (B and D), as well as in the overall *HeT-A* transcripts (C and E).

Similarly, it has been shown by different groups that certain mutations in the *Su(var)2-5* gene have a strong suppressor effect over the expression of the telomere retrotransposons (Savitsky *et al.*, 2002; Perrini *et al.*, 2004; Frydrychova *et al.*, 2008; R.S-S., E.L-P., D.P., E.C. *submitted*). Nevertheless, when we assayed the *Su(var)2-5*^{05} mutation over the EY08176, EY09966 and EY0453 lines (Biessmann *et al.*, 2005), only a subtle change in mini-*white* expression could be detected (Figure 2).

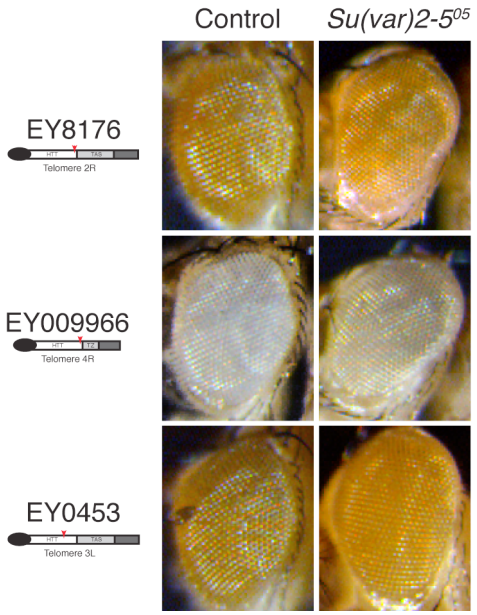

Figure 2: **HP1 effect over genes inserted at HTT array.** The mutant allele of HP1a, *Su(var)2-5*^{05}, was assayed with the same reporter lines from Figure 1. Although it has been demonstrated that *Su(var)2-5*^{05} shows a strong de-repression of *HeT-A* transcription, only a slight change in the expression of the mini-*white* reporter is detected in the telomeric lines (1st, 2nd and 3rd rows).

These results reveal that JIL-1 is needed for gene expression from the HTT array. Moreover, we have shown that, when embedded at the HTT array, the *HeT-A* and the mini-*white* promoters show different sensitivity to the presence of HP1a and JIL-1.

Our results, suggest that the use of reporter genes to study gene expression in different genomic domains will only reflect the real situation when both promoters, from the reporter and the gene under study, are similar.

JIL-1 acts as a suppressor of TPE over the TAS domain

JIL-1^{z60} and *JIL-1*^{h9} heterozygous alleles do not show any visible effect over the mini-*white* inserted at the TAS domain of chromosomes 2R (39C-27) and 2L (39C-5) (Cryderman *et al.*, 1999) (Figure 3A, 1st and 2nd columns).

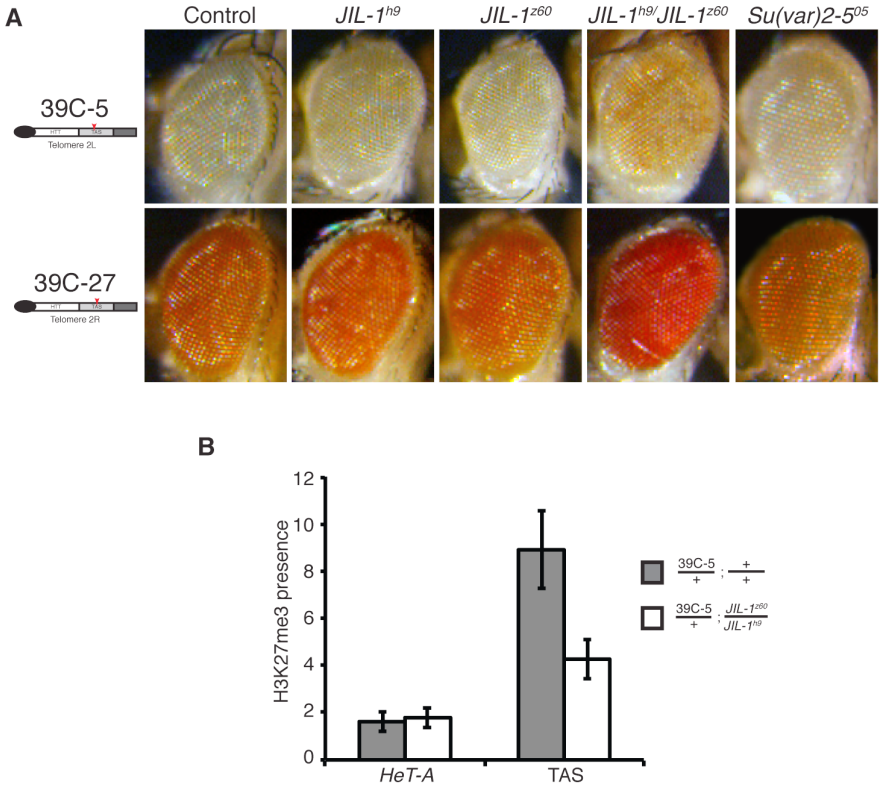

Figure 3: **JIL-1 act as suppressor of TPE over the TAS domain. (A)** TPE assays in the TAS domain were performed with the reporter lines 39C-5 (chromosome 2L, TAS-

L) and 39C-27 (chromosome 2R, TAS-R). No effect on the mini-*white* gene expression was observed when the reporter lines were crossed with the *JIL-1* heterozygous mutants *JIL-1*^{z60} and *JIL-1*^{h9}. However, when the same lines were crossed with the trans-heterozygous mutant (*JIL-1*^{z60}/*JIL-1*^{h9}) an increase in the eye color was observed revealing a suppressor effect of JIL-1 over the TAS domain. **(B)** Chromatin immunoprecipitation (ChIP) experiments reveal that JIL-1 trans-heterozygous mutants lead to a decrease in the presence of the H3K27me3 mark at the TAS domain.

Nevertheless, when the dose of JIL-1 is lowered in a trans-heterozygous combination, a significant increase in mini-*white* expression is observed, revealing a suppressor effect of JIL-1 over the TAS domain of these two telomeres (Figure 3A, 3rd column). We performed Chromatin Immunoprecipitation (ChIP) experiments in order to understand which changes in the TAS chromatin could explain the increase in mini-*white* transcription in the *JIL-1*^{z60}/*JIL-1*^{h9} combination. We developed the ChIP assay with an antibody against H3K27me3 because the TAS chromatin is enriched in this mark that is recognized by the Polycomb protein complex (Figure 3B) (Boivin *et al.*, 2003; Andreyeva *et al.*, 2005). The ChIP data reveals a significant decrease of H3K27me3 upstream of the mini-*white* insertion in the TAS domain in a *JIL-1*^{z60}/*JIL-1*^{h9} trans-heterozygous background, explaining the release of silencing observed. As suggested previously, the JIL-1 protein protects from the excessive spreading of heterochromatin to adjacent domains (Zhang *et al.*, 2006; Wang *et al.*, 2011). Our results together with previous observations (Zhang *et al.*, 2006; Wang *et al.*, 2011), suggest that JIL-1 could exert a barrier function at the HTT-TAS boundary. The JIL-1 boundary would be protecting the promoters of the HTT retrotransposons from the highly compacted chromatin of the adjacent TAS domain. The model from Figure 4, describes the local decrease of H3K27me3 inside the TAS domain and the spreading of this mark onto the adjacent domain in a *wild type* and *JIL-1* mutant scenarios. According to our model we should have detected an increase in H3K27me3 at the HTT array (Figure 3B and 4), nevertheless, it is possible that this increase is only detectable at the vicinity of the HTT-TAS border. We have obtained the same

result for both, TAS-L (2L, 3L telomeres) and TAS-R (2R, 3R and XL telomeres) (Antão *et al*., 2012), suggesting that the JIL-1 protective boundary exists in the other *Drosophila* telomeres.

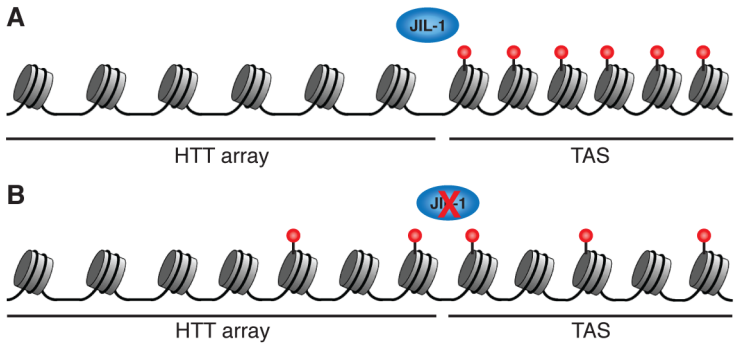

Figure 4: **Possible scenario for the TAS chromatin in a *JIL-1* mutant background. (A)** Wild type: the presence of the H3K27me3 mark, nucleates a highly repressed chromatin in the TAS domain, regulated by the Polycomb group of proteins (Supplemental Table 1). **(B)** *JIL-1* mutant background: The loss of the JIL-1 boundary, causes a spread of the H3K27me3 mark towards the flanking regions outside the TAS domain, resulting in a local decrease of the H3K27me3 mark in the TAS chromatin becoming more permissive for gene expression.

We performed different control crosses with different mutations whose effect over the subtelomeric domain had been already reported (Suppl. Table 1).

The subtelomere domain of the 4th chromosome and the TAS domains have a different chromatin

With respect to the HTT array, we have observed no difference between the 4th and the 2nd chromosomes. Interestingly, the subtelomere of the 4R telomere shows a remarkable different behavior than its equivalent in the other chromosomes, the TAS domain. The 4R telomere does not have a TAS domain flanking the HTT array, instead, a small transition zone (≈ 5Kb) composed of decayed transposable elements together with pieces of the HTT elements (*Flybase GBrowse*) lies at the subtelomere region of the 4R arm. Our experiments have revealed that the JIL-1 kinase and HP1a easily affect

the 4th chromosome subtelomeric lines 39C-72 and 118E-5 (Figure 5). The effects that these two proteins exert at the 4R subtelomere, are similar to the ones that they produce at the HTT array of the other chromosomes (Figure 1A 4th column and B, C, D and Figure 2). JIL-1 behaves as an enhancer of TPE while HP1a shows a strong supressor effect.

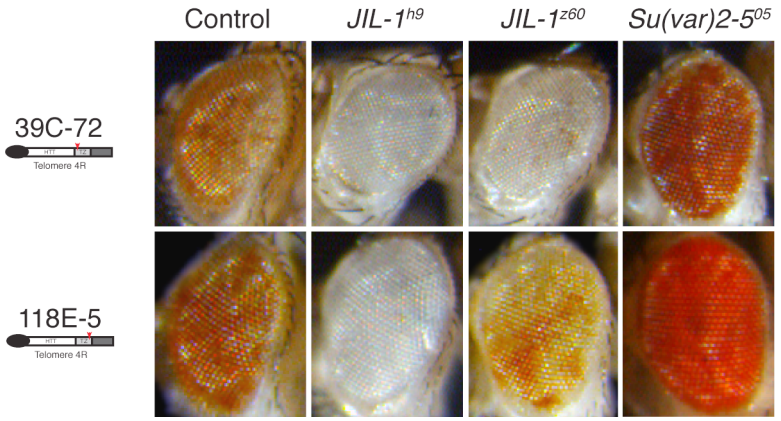

Figure 5: JIL-1 and HP1 affect the expression of genes embedded in the subtelomeric region of the fourth chromosome. TPE assays were performed with the 4th chromosome reporter lines 39C-72 (insertion near HTT array) and 118E-5 (insertion in the vicinity of first gene). A strong enhancer of TPE was observed in *JIL-1* mutants (2nd and 3rd columns). On the other hand, *Su(var)2-5* act as a strong suppressor of TPE.

Additionally, we performed control crosses with insertions in the arms of the 4th chromosome (Supplemental Figure 1). No effect in PEV was observed in *JIL*-1 mutants while in *Su(var)2-5*^{05} mutants a suppressor effect of PEV was observed. Therefore, our results suggest that the subtelomere of the 4th chromosome as well as the HTT array from the other chromosomes depends on JIL-1 for gene expression (Figure 1 and 5) and on HP1a for gene silencing (Figure 2 and 5). In summary, the results described here indicate that the chromatin at the 4R subtelomere is less compacted and more permissive to gene expression than the TAS domains from the other chromosomes, and even than the HTT array. This scenario suggests that in the 4R telomere the compaction of the chromatin is in the opposite orientation than in the rest of

the telomeres. It would be interesting to study if this reverse orientation respect to the other telomeres has any consequences for telomere function in *Drosophila*.

CONCLUSIONS

We report how, in *Drosophila*, the presence of the JIL-1 kinase at the telomere domain is needed to allow gene expression of the promoters embedded in the telomere domain. The lack of JIL-1 causes the spreading of heterochromatin towards adjacent domains. Finally, we discovered that the telomere and subtelomere domain of the 4R arm have distinct chromatin characteristics.

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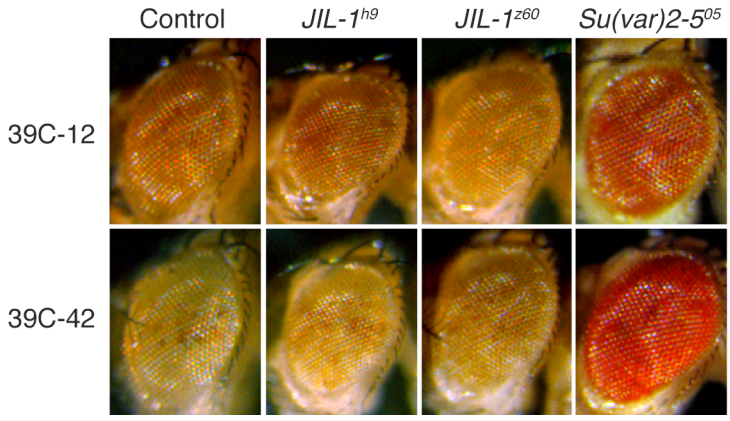
CHAPTER 1

SUPPLEMENTAL FIGURES:

Supplemental Table 1: Control crosses of *Su(var)3-9*, *polycomb* and *trythorax* mutant alleles with lines containing the reporter gene inserted in the subtelomeric domains of the 4th (39C-72 and 118E-5) and 2nd chromosomes (39C-5 and 39C-27), respectively.

Mutation	Effect HTT array	Effect TAS
Su(var)3-9^{17} [(a)]	No effect	No effect
Su(var)3-9^{06e} [(a)]	No effect	No effect
Su(Z)2^{1a1} [(b)]	No effect	Suppressor PEV
Su(Z)2^{1b7} [(b)]	No effect	Suppressor PEV
Bmx2trx [(c)]	No effect	Enhancer PEV

[(a)] *Su(var)3-9* mutant, [(b)] *polycomb* mutant; [(c)] *trythorax* mutant



Supplemental Figure 1: **Chromosome 4 control crosses.** *JIL-1* mutations have no effect on the expression of a reporter gene inserted in the arms of the 4th chromosome (39C-12 and 39C-42) (2nd and 3rd columns). A strong suppressor of PEV phenotype is observed for *Su(var)2-5^{05}* mutants.

CHAPTER 1

ADDITIONAL RESULTS:

In order to better characterize the telomeric chromatin and the proteins that affect the telomere retrotransposon expression in *Drosophila* we also tested the effects of the Z4 protein over the different telomeric domains. Z4 (also named Putzig) is a seven zinc-finger protein essential for fly development (Eggert et al., 2004). Mutant alleles of *Z4* show polytene chromosomes with severe structural problems due to lack of heterochromatin (Eggert et al., 2004). Z4 was found to specifically localize at the HTT array of *Drosophila* telomeres (Andreyeva et al., 2005), therefore we decided to investigate the role of Z4 in the regulation of the telomeric retrotransposons.

Z4 is involved in gene repression at the telomere domain (HTT)

In order to investigate the role of Z4 at the telomere domain we crossed two mutant alleles of this protein ($Z4^{7.1}$ and $Z4^{2.1}$) with reporter lines with mini-*white* insertions at the HTT array of the 2nd (EY8176), 3rd (EY0453) and 4th (EY09966) chromosomes. The $Z4^{7.1}$ hypomorph mutant allele showed an increase in the mini-*white* expression of lines EY8176 and EY0453, acting as suppressor of TPE (Figure 6 3rd column, 1st and 2nd rows). No effect of the null $Z4^{2.1}$ allele was observed for these lines. Moreover, we did not notice any observable effect of Z4 over the 4th chromosome line EY09966 (figure 6 3rd row). Similarly, when these lines were crossed with the HP1 mutant allele *Su(var)2-5*^{05}, showed only a slight increase in expression of the mini-*white* gene (Figure 2). HP1 has been identified has a strong repressor of the expression of the telomeric retrotransposons, and the increase in transcription of the telomeric retrotransposons observed in *Su(var)2-5* mutants leads to an increase in telomere length (Perrini et al., 2004; Frydrychova et al., 2008). Taking into account the effects of HP1 mutants

over the HTT retrotransposons, we expected a stronger effect of these same mutant lines over the reporter lines inserted in the HTT array (Figure 2).

Following, we checked if these reporter lines would respond to even lower levels of Z4. With this purpose, we set a cross to obtain a trans-heterozygous combination of both Z4 alleles ($Z4^{7.1}/Z4^{2.1}$), unfortunately these flies died at third instar larvae not allowing the observation of the eye phenotype. All these results suggest that Z4 can act as a weak suppressor of TPE over the genes inserted in the HTT array.

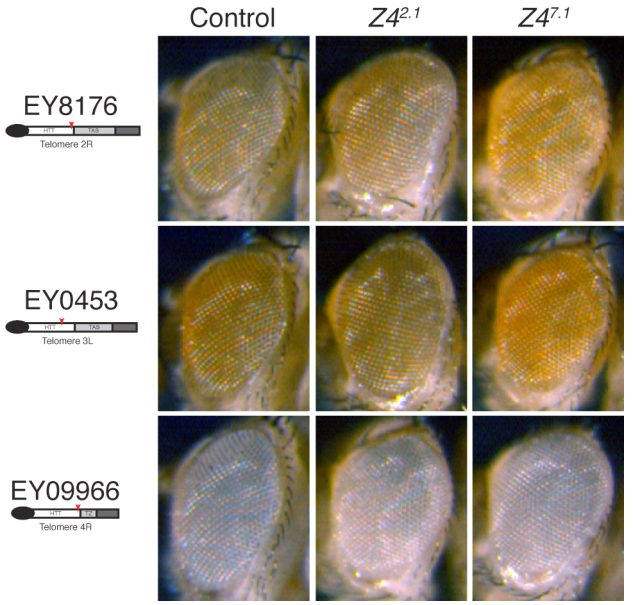


Figure 6: Z4 effect over the HTT array. Z4 mutants were crossed with reporter lines inserted at the HTT array of telomeres 2R (EY8176), 3L (EY0453) and 4R (EY09966). An increase in mini-white gene expression was observed for $Z4^{7.1}$ allele (3rd column, 1st and 2nd rows) but no effect was observed for the allele $Z4^{2.1}$ (2nd column, 1st and 2nd rows) neither for both alleles in the 4th chromosome line EY09966 (3rd row).

Z4 does not affect the expression of genes embedded at the TAS domain

To investigate whether the effect exerted by Z4 was specific of the HTT array, we crossed *Z4* mutants with reporter lines with insertions of the mini-*white* gene in the TAS domain of chromosomes 2L and 2R (39C-5 and 39C-27). We did not observe any effect of the *Z4* mutant alleles ($Z4^{7.1}$ and $Z4^{2.1}$) over the mini-*white* gene expression for the tested reporter lines (Figure 7). These results suggest that Z4 has a specific role at the telomeric but not subtelomeric domain of *Drosophila* telomeres.

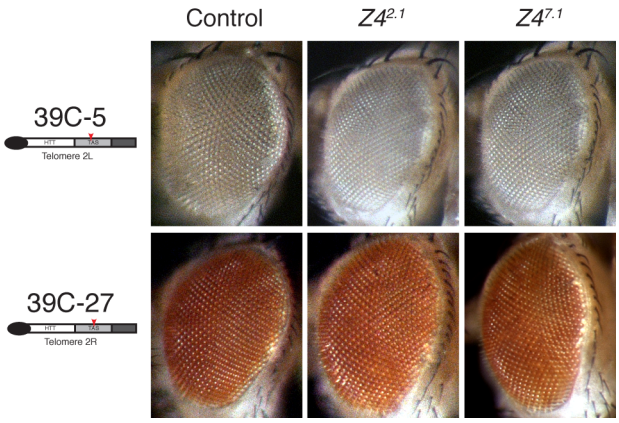


Figure 7: Z4 does not affect expression from the TAS domain. TPE assays in the TAS domain were preformed with the reporter lines 39C-5 (chromosome 2L, TAS-L) and 39C-27 (chromosome 2R, TAS-R). No effect on the mini-white gene expression was observed when the reporter lines were crossed with the *Z4* mutants, $Z4^{7.1}$ and $Z4^{2.1}$.

Z4 acts as a suppressor of TPE on the subtelomere domain of the 4th chromosome

As mentioned before, the 4R telomere of the 4th chromosome does not have a TAS domain flanking the HTT array. Instead, a much smaller region named *transition zone,* composed of pieces of HTT elements as well as other transposable elements, lies flanking the proximal end of the retrotransposon array and before the first euchromatic gene ((George et al., 2006) and

Flybase GBrowse). In order to investigate the role of Z4 in this unique region, we crossed *Z4* mutant alleles, $Z4^{7.1}$ and $Z4^{2.1}$, with lines with the reporter gene inserted at each extreme of the transition zone; line 39C-72 with a mini-*white* inserted near the HTT array and the line 118E-5 with the mini-*white* inserted towards the centromere. Like JIL-1, Z4 also affects the subtelomeric domain of the 4th chromosome (Figure 5). However, Z4 has the opposite role of JIL-1 and acts as a suppressor of TPE (Figure 8). This effect is observed by an increase in the mini-*white* gene expression and consequently an increase in eye color when comparing with the control siblings.

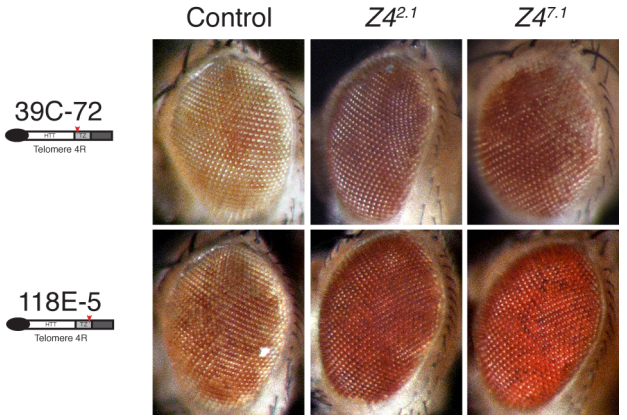


Figure 8: Z4 role at the transition zone of the 4th chromosome. Z4 mutants were crossed with the 4th chromosome lines 39C-5 and 118E-5. An increase in eye color is observed for the lines 39C-72 and 118E-5 (first and second rows), indicating that Z4 act as a suppressor of TPE over this region.

The body of results presented in this section indicate that Z4 has a role at *Drosophila* telomeres acting as a modulator of the chromatin at the HTT array, becoming a repressor of the telomeric retrotransposons. Moreover, this role seems to be specific of the HTT array since no effect of Z4 over the TAS domain was observed (Figure 7). Similarly to what we have demonstrated for the JIL-1 protein, Z4 has an effect over the subtelomere domain of the 4R chromosome, suggesting once more that this domain resembles more the HTT array of the 2nd and 3rd chromosomes than the subtelomeric domains of these same chromosomes.

CHAPTER 2

The chromosomal proteins JIL-1 and Z4/Putzig regulate the telomeric chromatin in *Drosophila melanogaster*

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In revision

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 to obtain telomere length homeostasis without compromising genomic integrity. Here we describe two chromosomal proteins, JIL-1 and Z4 (also known as Putzig), necessary for establishing a fine-tuned regulation of the transcription of the major component of *Drosophila* telomeres, the *HeT-A* retrotransposon, and 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. The decrease in *HeT-A* transcription in *JIL-1* alleles correlates with an increase in silencing chromatin marks such as H3K9me3 and HP1a. 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 the 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 similar phenotype of telomere instability 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 with other heterochromatin components characteristic of *Drosophila* telomeres, like HP1a.

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 loss of important genetic information. Moreover, a certain telomere length is required for the proper assembly of the telomere-capping complex (*shelterin* in telomerase telomeres or *terminin* in *Drosophila*) [1], [2], [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, which upon repair will be fused together and result in genomic instability [4]. Eukaryote telomeres are dynamic structures that make up their telomere length from a balanced mechanism of gains and losses, resulting in a proper telomere 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 [9], [10], [6]. The success of this mechanism is based in the targeted transposition of three different specialized non-LTR retrotransposons, *HeT-A, TART* and *TAHRE* [11], [12], [13], [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, what in the case of the telomeric transposons will translate in increased telomere length. The controlled terminal transposition of the telomeric transposons benefits both, the retrotransposons and the host genome. For this mechanism to work properly, the transcription of the telomeric retrotransposons needs to be tightly regulated, being active only when telomere elongation is needed in order to avoid excessive terminal transposition and genomic instability.

In most eukaryotes the telomeres are composed by the protective cap and the telomeric domain. Flanking the telomeric domain is the subtelomeric domain, that shows heterochromatin characteristics [8], [15], [16]. The telomere domain is composed of the telomerase repeats in telomerase organisms and of the retrotransposon array, *HeT-A*, *TART* and *TAHRE* (HTT) in the case of *Drosophila*. Andreyeva and collaborators took advantage of the *Drosophila melanogaster Telomere elongation* (*tel1*) mutant strain, which has telomeres ten times longer than wild type [17], to obtain a better resolution of the chromatin in the different telomeric domains. Their study demonstrates that the HTT array shows mixed characteristics of euchromatin and heterochromatin, and that two chromosomal proteins, JIL-1 and Z4, specifically localize in this domain [15].

Z4, also known as Putzig, is a seven-zinc finger protein known to localize at polytene chromosome interbands and necessary to maintain the band-interband structure in these special chromosomes [18]. Moreover, Z4 has been shown to be an important cofactor in at least three different pathways: the NURF and the TRF2/DREF remodeling complexes, where it acts as an activator [19], [20]; and in the JAK/STAT pathway, where Z4 acts as a co-repressor [21]. With the exception of its role as a co-repressor in the JAK/STAT pathway, where Z4 binds to the Ken protein, Z4 exerts its effects mediating chromatin changes.

In *Drosophila*, JIL-1 is the chromosomal kinase responsible for the phosphorylation of serine 10 of histone H3 during interphase [22]. Like Z4, JIL-1 also localizes at interbands in polytene chromosomes, and has a role in maintaining the band-interband structure of polytene chromosomes [23]. JIL-1 seems to have two interdependent functions; on one hand, JIL-1 is required for the structure of the chromosomes, and on the other, is involved in reinforcing active transcription of certain genes during interphase, as well as participating in the dosage compensation of genes in the male X chromosome [24], [25], [26]. Several JIL-1 interacting partners have been identified; JIL-1 interacts with Lamin Dm_0, histone H3, and the chromosomal protein Chriz, also known as Chromator, among others [27], [28], [29], [30]. A possible interaction between JIL-1 and Z4 has also been suggested [28].

Telomere function in *Drosophila* has been suggested to be epigenetically determined since the *terminin* complex that protects the telomeres assembles in a sequence independent manner [2]. Maintenance of chromatin domains is a dynamic process in which chromatin marks and proteins interchange in a complex network of interactions defining a set of characteristics that favor expression or repression of the genes embedded in such domains. Because telomere function in *Drosophila* strongly depends on transcription from the HTT array, where the genes involved in telomere elongation reside, the regulation of the chromatin structure of this domain is especially relevant to understand the regulation of telomere length in this organism. Towards this objective, we have studied the role of JIL-1 and Z4, two chromosomal proteins shown to localize at the HTT array [15], on the regulation of the telomeric chromatin and their influence upon the expression of the main telomeric component in *D. melanogaster*, the *HeT-A* retroelement [31]. We have found that JIL-1 regulates *HeT-A* transcription, being the first positive regulator of telomere expression in *Drosophila*. Moreover, we demonstrate that different *Z4* mutant alleles show telomeric fusions in metaphase chromosomes from larval neuroblasts. ChIP experiments of *Z4* and *JIL-1* mutant alleles highlight changes of other components of the telomeric

chromatin, like the HP1a protein, that can explain the transcriptional and stability effects. Importantly, we have also obtained evidences of a possible telomere targeting mechanism to recruit the JIL-1-Z4 complex to the telomeres.

MATERIALS AND METHODS

Fly stocks and crosses

Fly stocks were maintained and crosses performed at 25°C on standard *Drosophila* corn meal medium. w^{1118} strain was used as control. *JIL-1*^{z60}*/TM6B*, *JIL-1*^{h9}*/TM6B*, *JIL-1*^{z2}*/TM6B* and *JIL-1*$^{Su(var)3-1}$*/TM3SbTb* stocks were provided by Kristin M. Johansen. *Z4*$^{7.1}$*/TM3Sb* and *Z4*$^{2.1}$*/TM3Sb* came from Harald Eggert and Harald Saumweber. *pzg*^{66}*/TM6* was a kind gift of Anja Nagel. The stocks *ligIV* $^{-/-}$ and HOAP-GFP were obtained from Yikang Rong. The *woc*^{964}*/TM6* and *woc*^{B111}*/TM6* alleles were provided by Maurizio Gatti. *Su(var)2-5*^{05}*/CyO* was obtained from Bloomington 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 200µl solution A (0.1M Tris-HCl pH9.0, 0.1M EDTA and 1% SDS) and incubated at 70°C for 30 min. 28µl 8M KAc were added and the samples incubated for 30 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 13 000 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 50µl 1X TE by rotating o/n at 4°C. After genomic DNA extraction, the number of *HeT-A* copies was determined by quantitative real-time PCR using 2ng of

DNA per reaction. Primers used for real time PCR: HeT-A_3'UTR_F (CCCCGCCAGAAGGACGGA) and HeT-A_3'UTR_R (TGTTGCAAGTGGCGCGCA) for the 3'UTR region, HeT-A_Gag_F (ACAGATGCCAAGGCTTCAGG) and HeT-A_Gag_R (GCCAGCGCATTTCATGC) for the *Gag* gene, Actin_F (GCGCCCTTACTCTTTCACCA) and Actin_R (ATGTCACGGACGATTTCACG) for the actin gene.

RNA Extraction and cDNA synthesis

Total RNA was isolated from ten whole third instar larvae and extracted using RNeasy Mini Kit (Qiagen) according to manufacturer'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: HeT-A_3'UTR_F (CCCCGCCAGAAGGACGGA) and HeT-A_3'UTR_R (TGTTGCAAGTGGCGCGCA) for the 3'UTR region, HeT-A_Gag_F (ACAGATGCCAAGGCTTCAGG) and HeT-A_Gag_R (GCCAGCGCATTTCATGC) for the *Gag* gene, Actin_F (GCGCCCTTACTCTTTCACCA) and Actin_R (ATGTCACGGACGATTTCACG) for the actin gene.

Chromatin Immunoprecipitation experiments (ChIPs)

Brains and imaginal discs from third instar larvae were dissected in 1X PBS with protease inhibitors. After dissection, the brains were resuspended in 5ml buffer A (60mM KCl, 15mM NaCl, 15mM HEPES pH7.6, 0.5% Triton X-100, 0.5mM DTT, complete EDTA-free protease inhibitor cocktail from Roche) with 1.5% formaldehyde, homogenized in a Wheaton Dounce and incubated for 15 min at room temperature. Crosslinking was stopped by adding glycine to a final concentration of 0.125M and incubating 5 min at 4°C on a rotating wheel. Brains were washed 3 times with Buffer A and resuspended in lysis buffer (140mM NaCl, 15mM HEPES pH 7.6, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.5mM DTT, 0.1% sodium deoxycholate, complete EDTA-free protease inhibitor cocktail from Roche). Crosslinked nuclei were fragmented using a bioruptor sonicator (high amplitude, 15 sec ON and 45 sec OFF). For the following steps a standard protocol (Upstate) was used. Thirty brain/discs complexes were used per IP. Chromatin was immunoprecipitated with the following antibodies: anti-H3K9me3 (ab8898, Abcam), anti-H3K4me3 (ab8580, Abcam), anti-HP1 (The anti-HP1 (C1A9) antibody developed by Lori L. Wallrath was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, department of Biology, Iowa City, IA 52242), anti-JIL-1 (gift from Kristen M. Johansen), and anti-Z4 (gift from Harald Saumweber). Three independent ChIP samples were analyzed and the amount of immunoprecipitated DNA was calculated by quantitative real-time PCR using iQ™ SYBR® Green Supermix (BioRad). Primers used for real time PCR: HeT-A_5'UTR_F (TCGCGCTTCCTCCGGCAT) and HeT-A_5'UTR_R (GCGGTTATTACATTACGGCGG), RpL32_F (CAAGAAGTTCCTGGTGCACAA) and RpL32_R (AAACGCGGTTCTGCATGAG).

Quantitative real-time PCR

Quantitative real-time PCR was performed to determine *HeT-A* copy number and *HeT-A* expression, and in ChIP experiments. The iQ5 Multicolor Real-Time PCR Detection System was used and the iQ™ SYBR® Green Supermix (BioRad) was used to prepare the reactions. Relative levels of *HeT-A* expression were determined using the threshold cycle and normalized to actin levels (or RpL32 for ChIPs). Three independent experiments of two samples each strain were performed.

Chromosome cytology

Third instar larvae brains were dissected in 0.7% NaCl solution, incubated in 10mM colchicine (Roche) for 2 hours and submitted to a hypotonic shock (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 colchicine incubation and hypotonic shock were 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 larvae brains were dissected in 1X PBS with protease inhibitors and incubated in 10mM colchicine (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 (0.15M PIPES, 3mM $MgSO_4$, 1.5mM EGTA, 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 *Sart1* (non-LTR retrotransposon from *Bombyx mori*) coding sequences were amplified by PCR and cloned in the pSTBlue-1 vector (Novagen) to produce dsRNA. Single stranded RNAs were synthesized 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. 50μg of dsRNA were diluted in 1ml of supplemented Schneider medium, added drop-wise to a total of 1.5X10^7 cells, and incubated at 25°C. The same protocol was repeated 24 and 48 hours after seeding the cells. An aliquot of cells was collected at 24, 48 and 72hours after seeding. For description of cytology experiments, see next section S2 cells metaphase chromosome preparation. Gene fragments of about 550bp were amplified using the primers: HeTGag-RNAi-F (CTAGCGGCAAACAACATCG) and HeTGag-RNAi-R (GGGATTGCAGATTCTTGGC) to amplify *HeT-A* sequence with accession number: X68130 from nucleotide (nt) 2701 to nt 3255, Z4-RNAi-F (TAATTATCCAGCAGGGACAG) and Z4-RNAi-R (CAATCAGATCTGGTCTTTGTCTCCGTAAAC) to amplify the *Z4* gene acc num: CG7752 from nt 3046 to nt 3383, HOAP-RNAi-F (GCCGAGACTAAGAAGCAGAAC) and HOAP-RNAi-R (CCTGATCGTCAGGCTCTTG) amplify the *caravaggio* gene acc num: CG6219 from nt 1689 to nt 2166, and SART1-RNAi-F (CAACGGCAGCAGAATCAATG) and SART1-RNAi-R (CGTAATTTCTCCGCCAGCAA) amplify the *SART1* retrotransposon acc

num: D85594 from nt 1943 to nt 2432. All amplified regions were checked for off-site targets.

S2 cells metaphase chromosome preparation

500μl collected cells were treated with 10mM colchicine (Roche) during 2 hours in the dark. Cells were centrifuged 3min at 1500rpm and the pellet was washed with PBS. Cells were centrifuged again and the pellet resuspended with 500μl 0,5% sodium citrate. After 10 min incubation at room temperature, cells were centrifuged, resuspended in 1mL fixation solution (methanol:acetic, 3:1) and incubated for 10 min. Cells were centrifuged again, re-suspended in 50μl Fixation Solution, and 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 using the AxioVision software.

Cell Transfection

Drosophila S2 cells were seeded at $3X10^6$ 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 1X PBS and frozen at -80ºC. HeT-A Gag-GFP plasmid and an empty GFP plasmid (pPL17) were used in cell transfection [37].

Protein Co-Immunoprecipitation assays

Protein extracts from S2 cells were prepared in 1ml lysis buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 1% TritonX-100, 1mM EDTA, 1mM 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 50μl 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 500μl lysis buffer for one hour at 4°C with rotation and washed 3 times with 500μl lysis buffer. Immuno-complexes were washed 3 times with lysis buffer and eluted from the beads with 50μl 1X 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-JIL-1 [59] were used in Western Blot experiments.

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 *tel1* stock, a stock that offers a better resolution due to the presence of telomeres ten times longer than wild type flies, to perform immunocytochemistry experiments [17]. 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, 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 HP1a (Figure 1A and B) [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 (Supplemental Figure 1A and B). 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 (Supplemental Figure 2 A and B, Figure 1 A and B). 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 3kb along the full length *HeT-A* transcript (*see materials and methods for primer details*).

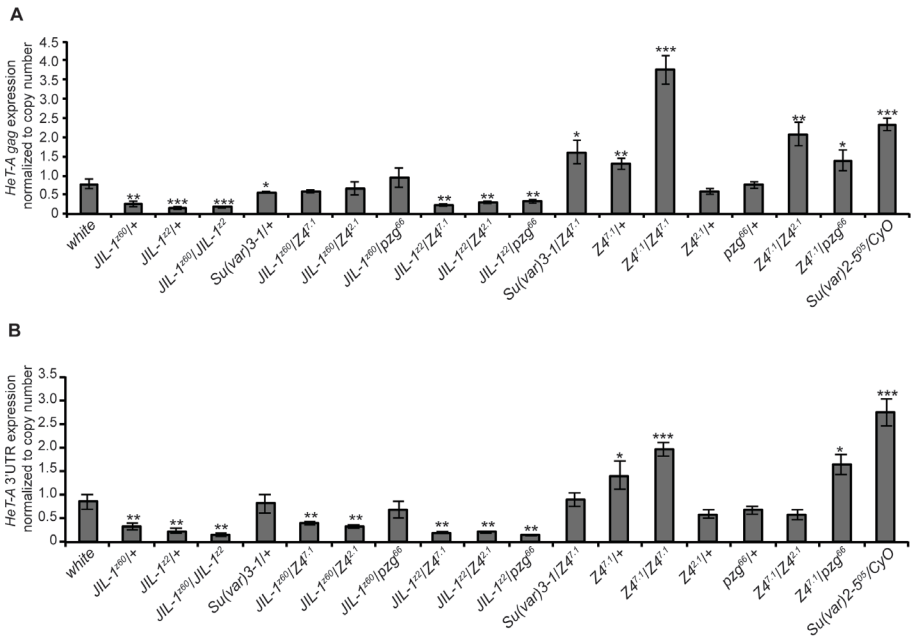


Figure 1. *HeT-A* expression normalized to copy number in different mutants. *HeT-A Gag* **(A)** and *HeT-A* 3'UTR **(B)** transcripts decrease in *JIL-1* mutants (*JIL-1^{z60}*/+, *JIL-1^{z2}*/+ and *JIL-1^{z60}*/*JIL-1^{z2}*) and in *Z4* mutants when combined with *JIL-1^{z2}* null allele (*JIL-1^{z2}*/*Z4$^{7.1}$*, *JIL-1^{z2}*/*Z4$^{2.1}$* and *JIL-1^{z2}*/*pzg^{66}*). *Z4$^{7.1}$* allele also affects *HeT-A Gag* transcripts but in this case an increase in the expression is observed. The *Su(var)2-5^{05}* 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 w^{1118}.

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, $JIL\text{-}1^{z60}$ that contains a molecular lesion, which results in low levels of functional protein [22], and also a null allele $JIL\text{-}1^{z2}$ [25]. In addition, we analyzed the $JIL\text{-}1^{Su(var)3\text{-}1}$ allele, which has been suggested to be a gain-of-function allele [36]. We obtained a significant reduction of *HeT-A* transcription with both sets of primers (3'UTR and *gag* gene) compared to the control strain (w^{1118}) for all the *JIL-1* alleles with the exception of the $JIL\text{-}1^{Su(var)3\text{-}1}$ allele (Figure 1 A and B, right 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 [30]. The *JIL-1* alleles show telomere length comparable to wild type (Supplemental Figure 1A and B). The three *JIL-1* alleles tested are all 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 w^{1118} strain in order to minimize the contribution of the genetic background in these measures.

Similarly, we analyzed three different *Z4* mutant alleles, $Z4^{7.1}$, $Z4^{2.1}$ *and* pzg^{66}. The $Z4^{7.1}$ allele is a hypomorph allele that lacks the promoter region of the *Z4* gene and is lethal at the pupal stage [18]. The $Z4^{2.1}$ and the pzg^{66} 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 $Z4^{7.1}$ (Figure 1A and B). The results are similar for both sets of primers indicating that the increase affects most *HeT-A* copies. The increased *HeT-A* transcription in the $Z4^{7.1}$ allele is consistent with a major

level of *HeT-A* transcripts in all the allelic combinations where this allele is present (Figure 1A and B). In addition, the number of copies of the *HeT-A* element in this allele was substantially increased indicating longer telomeres in this stock (Supplemental Figure 1A and B). Note that the level of *HeT-A* transcription in the $Z4^{7.1}/Z4^{7.1}$ homozygous is close to the *HeT-A* transcription in the $Su(var)2\text{-}5^{05}$ mutation of HP1a. Intriguingly, the $Z4^{2.1}$ and pzg^{66} alleles did not show a different level of *HeT-A* transcription compared to our control strain (w^{1118}) for neither the 3'UTR 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 (w^{1118}). Only when the $Z4^{7.1}$ allele is combined with the $Z4^{2.1}$ or the pzg^{66} alleles the levels of *HeT-A* transcription increase above the ones in the w^{1118} strain.

We also investigated if the low level of *HeT-A* transcription in *JIL-1* mutants could be rescued in a *Z4* mutant background. We obtained larvae of a total of seven allelic combinations between *JIL-1* and *Z4* and proceeded to test for the level of *HeT-A* transcription in these backgrounds (Figure 1A and B, supplemental Figure 2A and B). Although for the $Z4^{2.1}$ and pzg^{66} alleles we had not detected levels of *HeT-A* transcription significantly above the ones of w^{1118}, we found that the combinations $JIL\text{-}1^{z60}/Z4^{7.1}$ and $JIL\text{-}1^{z60}/Z4^{2.1}$ for the *gag* gene, and the $JIL\text{-}1^{z60}/pzg^{66}$ for both the 3'UTR region and the *gag* gene recover *HeT-A* transcription to w^{1118} levels (Figure 1A and B). Accordingly with the single mutation results, in the combination $JIL\text{-}1^{Su(var)3\text{-}1}/Z4^{7.1}$ we obtained levels of *HeT-A gag* transcription above the ones in w^{1118}.

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, as well as of HP1a, a protein already known to localize at the HTT array and to affect *HeT-A* transcription (Figure 1A and C) [33], [15], [16].

To analyze the relative chromatin changes in the homozygous mutant allele *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 the trimethylation of lysine 9 and 4 of histone H3 (H3K9me3 and H3K4me3), the most characteristic histone modifications indicative of repressed and active chromatin, respectively. 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 *Z4*$^{7.1}$*/Z4*$^{7.1}$ allele results from a double change, a substantial decrease in H3K9me3 and a simultaneous increase in H3K4me3, indicative of active transcription.

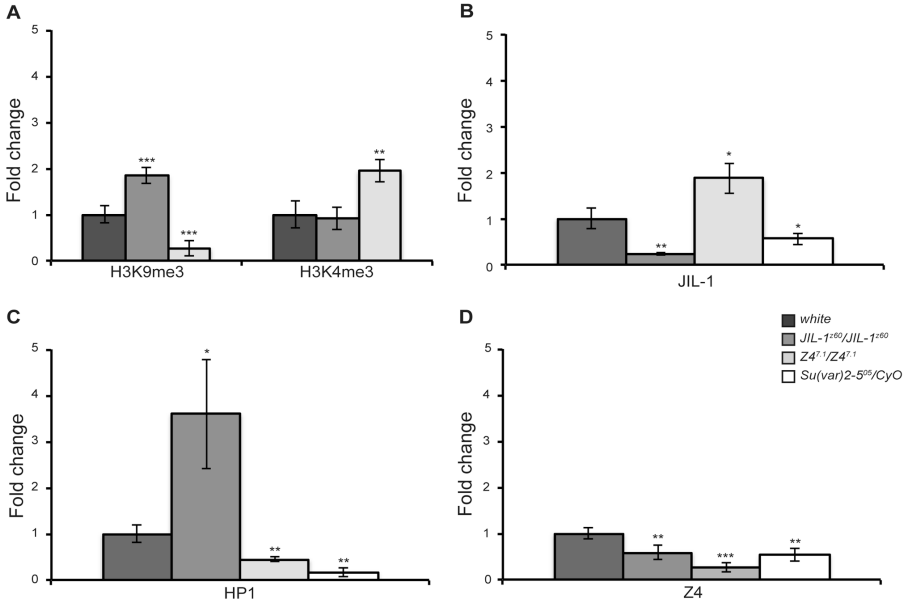


Figure 2. Chromatin changes at *HeT-A* promoter. Chromatin Immunoprecipitation (ChIP) analyses were performed at the *HeT-A* promoter of *JIL-1, Z4* and *Su(var)2-5* mutants. Specific antibodies were used against H3K4me3 and H3K9me3 **(A),** JIL-1

(B), HP1 **(C)** and Z4 **(D).** In **(A)** significant changes are observed at H3K9me3 in both *JIL-1* and *Z4* mutants, while H3K4me3 only varies in *Z4* mutants. In **(B),** *Z4* mutants show an increase in JIL-1 protein at *HeT-A* promoter. In **(C)** HP1 occupancy increases in *JIL-1* and decreases in *Z4* mutants. **(D)** Both *JIL-1* and *Su(var)2-5* mutants show a decrease in Z4 levels at *HeT-A* promoter. Graphs represent ChIP quantitation using real-time PCR. Each sample was normalized to 10% input DNA, to RpL32 locus and to the *white* control. Error bars represent standard deviations of three independent experiments. Asterisks indicate statistically significant differences (one asterisk, $P < 0.05$ to 0.01; two asterisks, $P < 0.01$ to 0.001; three asterisks, $P < 0.001$) between mutant and w^{1118} strains.

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. $Z4^{7.1}/Z4^{7.1}$ mutant flies show an increase in JIL-1, in accordance with a higher expression of *HeT-A* in this mutant allele. In contrast, $Su(var)2\text{-}5^{05}/CyO$ flies, heterozygous mutant for HP1a, show a moderate decrease of JIL-1 occupancy suggesting a subtle dependence between these two proteins. Interestingly, $JIL\text{-}1^{z60}/JIL\text{-}1^{z60}$ shows a substantial increase in the presence of HP1a, which could be in part responsible for the silencing of *HeT-A* expression in this same allele (Figure 2C). On the other hand, the $Z4^{7.1}/Z4^{7.1}$ allele shows HP1a levels comparable to $Su(var)2\text{-}5^{05}/CyO$ mutants 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 relating 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 $Z4^{2.1}$ and pzg^{66} alleles for the *HeT-A* promoter since in these alleles we did not find a significant difference in *HeT-A* transcription.

JIL-1 interacts with Z4

Because JIL-1 and Z4 localize similarly in polytene chromosomes and in the HTT array [22], [18], [23], [15], and both of them had been found to directly interact with Chromator [27], [28], we questioned if the two proteins could be

interacting. In Figure 3A we show how JIL-1 and Z4 endogenous proteins are able to co-immunoprecipitate each other in Schneider S2 cells. The input lane of JIL-1 shows a very faint signal due to the fact that a considerable amount of protein is not extracted and remains in the pellet. Nevertheless, the interaction with Z4 allows the complex to be clearly detectable in the IP with the Z4 antibody. In the case of Z4, no substantial amount of the protein was detected in the pellet (*not shown*) and the Z4 signal is present in both IPs as well as in the input lane. Although the co-immunoprecipitation experiments do not demonstrate a direct interaction, they 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.

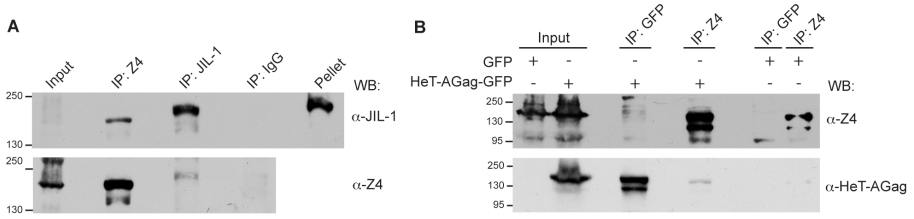


Figure 3. Z4 interacts with JIL-1 and *HeT-A* GAG. (A) Z4 and JIL-1 immunoprecipitation was performed in S2 cells using anti-JIL-1 and anti-Z4 antibodies. Negative control experiments were performed by immunoprecipitating with unspecific IgGs. **(B)** Z4 and *HeT-A* GAG immunoprecipitation was done by transfecting S2 cells with HeT-A Gag-GFP and immunoprecipitating with anti-GFP and anti-Z4. Control experiments were performed by transfecting an empty GFP vector (pPL17). Presence of the recombinant protein is indicated on the top of the panel (+ and - symbols). Antibodies used for immunoprecipitation are indicated on the top. All extracts were fractionated by SDS-PAGE, western blotted, and developed with specific antibodies (indicated on the right of each figure). Molecular markers (kDa) are indicated on the left.

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 *HeT-A* element [37], [38]. Therefore, we tested if HeT-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 HeT-A Gag fused to GFP and the endogenous Z4 protein. Figure 3B shows that the HeT-A Gag-GFP protein co-immunoprecipitated with Z4, and that conversely Z4 co-immunoprecipitated with HeT-A Gag-GFP. We did not detect a JIL-1-HeT-A Gag interaction (*data not shown*).

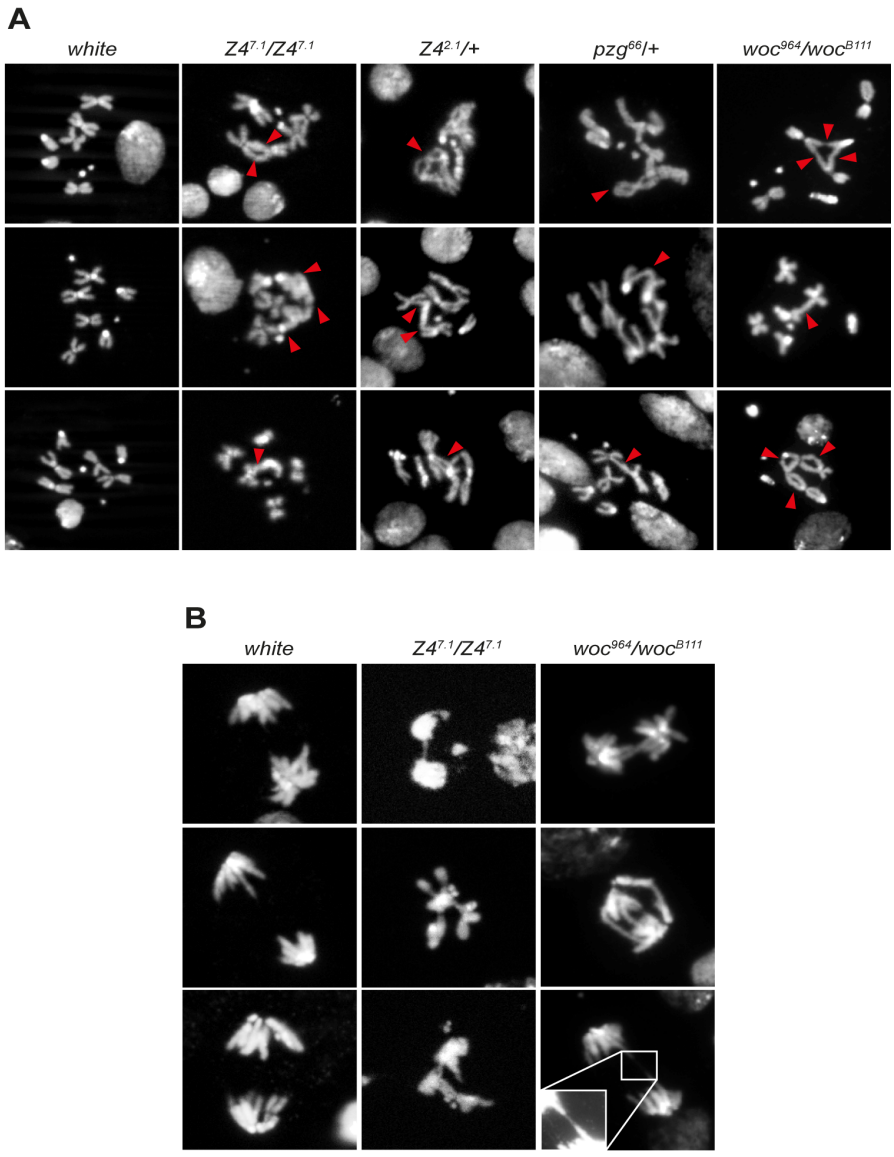
Z4 and *HeT-A gag* mutants show telomere instability in mitotic cells revealed by the appearance of telomeric fusions

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 larvae 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 (intra-chromosomal) and different chromosomes (inter-chromosomal) in all the *Z4* mutant alleles present in this study $Z4^{7.1}$, $Z4^{2.1}$ and pzg^{66} (Figure 4A, 2^{ond}, 3rd and 4th column). Similarly, TFs were observed in neuroblasts from the positive control, woc^{964}/woc^{B111} 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 $Z4^{7.1}/Z4^{7.1}$ third instar larvae and compared them again with a positive (woc^{964}/woc^{B111}) and a negative control (w^{1118}). Figure 4B (2nd column) shows

different anaphases of the *Z4*$^{7.1}$/*Z4*$^{7.1}$ 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*^{B111} larval brains (3rd column Figure 4B). No abnormal anaphases were observed for the *w*^{1118} neuroblast preparations (1st column Figure 4B).



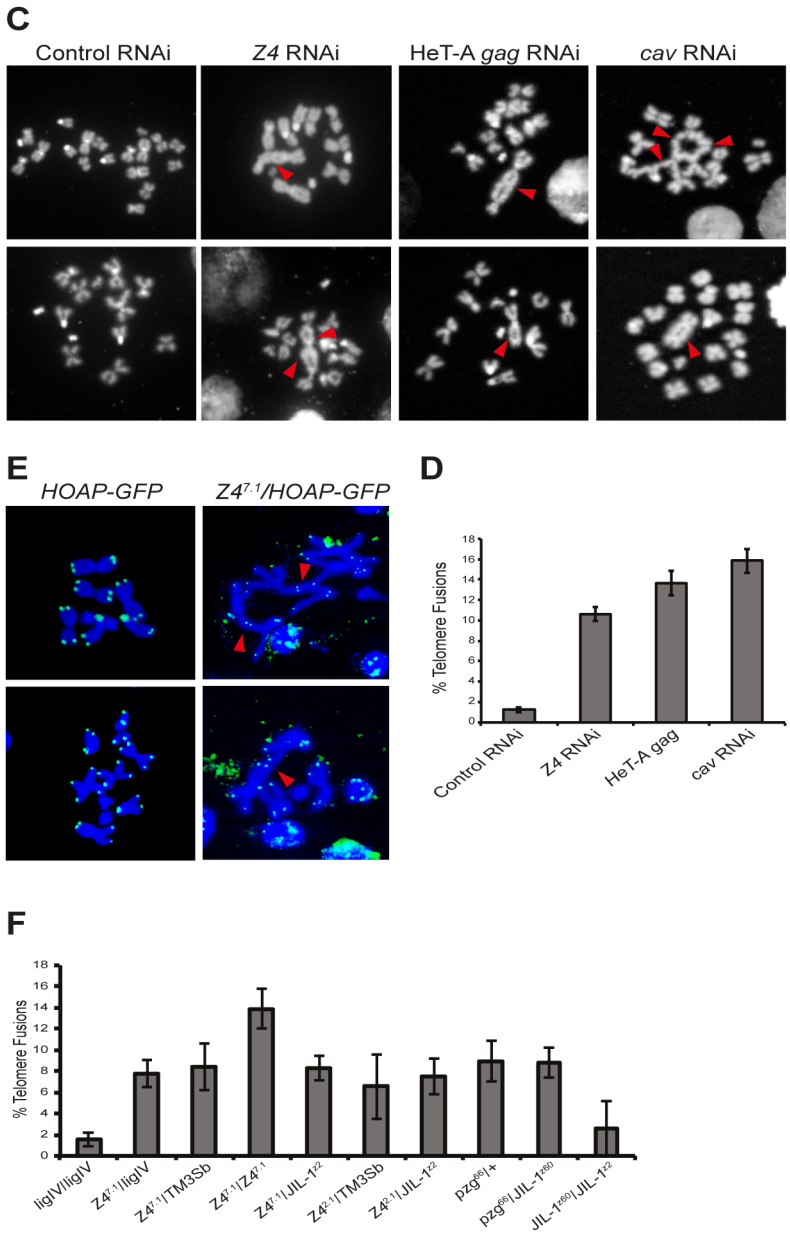


Figure 4. Telomere fusions in *Z4* and *HeT-A* mutants. (A) Mitotic chromosome preparations of third instar larvae. $Z4^{7.1}/Z4^{7.1}$, $Z4^{2.1}$/+ and pzg^{66}/+ alleles show telomere fusions. woc^{964}/woc^{B111} mutants were used as a positive control and w^{1118} as negative control. Red arrowheads indicate telomere fusions. **(B)** Anaphase preparations from third instar larvae neuroblasts of the $Z4^{7.1}/Z4^{7.1}$, woc^{964}/woc^{B111} alleles and w^{1118}. Defective anaphases due to telomere fusions visible in $Z4^{7.1}/Z4^{7.1}$ and woc^{964}/woc^{B111} (positive control). Normal anaphases in w^{1118} (negative control). **(C)** Mitotic S2 cells stained with DAPI after *Z4*, *HeT-A gag*

gene, *cav* (HOAP), and unspecific (*Sart1*) RNAi treatment. Telomere fusions are observed in *Z4* and *HeT-A Gag* RNAi mutants; RNAi for the *cav* and the *Sart1* non-LTR retrotransposon from *Bombyx mori* were used as positive and negative control respectively. Red arrowheads indicate telomere fusions. **(D)** Percentage of telomere fusions found in mitotic S2 cells after RNAi treatment. **(E)** Mitotic chromosome preparations of HOAP-GFP/HOAP-GFP and $Z4^{7.1}$/HOAP-GFP third instar larvae neuroblasts. Chromosomes stained with DAPI (blue) and HOAP-GFP fusion protein (green). HOAP is present in *Z4* telomere fusions (red arrowheads). **(E)** Percentage of telomere fusions found in $Z4^{7.1}$/*ligIV* and *Z4*/*JIL-1* double mutants. *ligIV* and *JIL-1* mutants do not affect the number of telomere fusions in *Z4* mutants. For each mutant a minimum of 100 metaphases/anaphases of three different preparations were analyzed.

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 *caravaggio* 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 M&M 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 proceeded 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 pathway [41]. Thus, we investigated if the loading of one of the major capping components, the HOAP protein [40], was perturbed in *Z4* mutants by crossing them with flies with an endogenous HOAP-GFP protein. In Figure 4D (2nd column), HOAP signals can be distinguished in TFs from metaphase chromosomes of the $Z4^{7.1}$/*HOAP-GFP* allele suggesting that at least part of the capping complex is still able to recognize and be loaded at the telomere (see arrowheads for HOAP signals over different TFs, Figure 4D). No TFs are seen in the HOAP-GFP metaphase chromosomes (Figure 4D 1st column). In order to rule out a possible contribution of the non-homologous end joining repair complex, we analyzed the contribution of the Ligase IV enzyme in the observed TFs in the *Z4* mutant alleles [41], by combining the *Z4* mutation ($Z4^{7.1}$) with a mutation for the gene encoding the Ligase IV enzyme ($ligIV^{-/-}$). Therefore, in case that the TFs observed in the *Z4* mutants were caused by this mechanism, we should have seen a decrease in the number of TFs when the two mutations are combined. The *ligase IV* allele that we assayed, ($ligIV^{-/-}$) does not show a TF phenotype compared with our control strain, (w^{1118}) (Figure 4F). As shown in Figure 4E, the TF number detected in $Z4^{7.1}$/*ligIV* double mutant was not statistically different from the $Z4^{7.1}$/*TM3Sb* allele. The results from these experiments strongly suggest that Z4 controls telomere stability independently of the DNA repair machinery and that Z4 is not directly involved in the loading of the telomere-capping complex.

We also investigated if the mutant alleles for *JIL-1* might also have a problem of telomere stability. We examined metaphase chromosomes of third instar larva neuroblasts of the $JIL\text{-}1^{z60}$, $JIL\text{-}1^{z2}$ and $JIL\text{-}1^{Su(var)3\text{-}1}$mutant alleles and did not find any significant telomere fusion (TFs) phenotype compared to w^{1118} (*data not shown*). We also assessed the possibility of TFs in the trans-

heterozygous combination *JIL-1*^{z60}/*JIL-1*^{z2} and found no significant differences from the *w*^{1118} strain (Figure 4F).

Following, we decided to test if *JIL-1* mutants could rescue the TF phenotype of *Z4* mutants. Because in a *JIL-1* mutant background some heterochromatin marks increase their presence in the *HeT-A* promoter, (H3K9me3 and HP1a, Figure 2 A and C and Figure 5) 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*$^{7.1}$/*JIL-1*^{z2}, *pzg*^{66}/*JIL-1*^{z60}, *z4*$^{2.1}$/*JIL-1*^{z2}) 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*.

DISCUSSION

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

Much effort has been put forward to study the negative regulation of the telomeric retrotransposons [42] (reviewed in [6]). Because the telomeric transposons have been able to maintain their personality as transposable elements while fulfilling a cellular role [9], it is important to understand their negative regulation [43], [44], [45]. Nevertheless, *HeT-A* is a retrotransposon entitled with the essential function of telomere elongation, and therefore a fine tuned regulation capable of achieving both, telomere replication and avoid putative harmful transpositions and consequently genomic instability should be in place. 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, JIL-1 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 is expected. Interestingly, the mammalian *JIL-1* orthologues 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.

Interestingly, a work by Phalke and co-workers [52] suggests that JIL-1 has a role in retrotransposon silencing in general but has no effect on telomere transcription. This result is in opposition with the results shown here. A possible explanation for this discordance is that the mutant allele of *JIL-1* assayed by Phalke and co-workers, the $JIL\text{-}1^{Su(var)3\text{-}1}$ allele, corresponds to a C-terminal deletion of the JIL-1 protein that causes the protein to miss-localize and phosphorylate ectopic positions [30,36]. The ectopic phosphorylation caused by the $JIL\text{-}1^{Su(var)3\text{-}1}$ allele activates 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 *Invader4* retrotransposon. In our study, we have assayed the $JIL\text{-}1^{Su(var)3\text{-}1}$ allele and we have obtained the same result than for the wild type stock likely because of the ectopic

phosphorylation in this allele (Figure 1A and B). In addition to the *JIL-1*$^{Su(var)3\text{-}1}$, we present data from two more alleles (Figure 1), *JIL-1*^{z60} *and JIL-1*^{z2}, that correspond to loss of function alleles and, in both cases, a substantial decrease in *HeT-A* transcription was detected (Figure 1A and B). 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 gene like in the work of Phalke and co-workers [52]. Our data demonstrates that JIL-1 is necessary to maintain active transcription from *Drosophila* telomeres, particularly of the telomeric retrotransposon *HeT-A*.

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 analyzed (*JIL-1*^{z60} and *JIL-1*^{z2}) have been maintained as heterozygous. Therefore, it is possible that one copy of *JIL-1* is enough to promote enough *HeT-A* transcription to elongate the telomeres when needed.

Z4 is necessary to guarantee telomere stability in *Drosophila*

Although in the case of the hypomorph mutation *Z4*$^{7.1}$ we have observed an increase in *HeT-A* transcription and *HeT-A* copy number significantly above the control strain (*w*^{1118}), the null alleles *Z4*$^{2.1}$ and *pzg*^{66} do not show an up-regulation of *HeT-A* transcription or an increase in copy number (Figure 1A and B, Supplemental Figure 1 and 2). Although we have crossed all the stocks to the *w*^{1118} strain to minimize the effects of the genetic background, it could still have a certain influence when comparing the *pzg*^{66} allele with the *Z4*$^{7.1}$. Nevertheless the *Z4*$^{7.1}$ and *Z4*$^{2.1}$ alleles come from the same genetic background [18]. A possible explanation could rely on the fact that the *Z4*$^{7.1}$ 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 the control level, 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 $Z4^{7.1}$ allele is enough to recruit JIL-1 to the *HeT-A* promoter. In the pzg^{66} and the $Z4^{2.1}$ 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.

On the other hand we have detected a phenotype of telomere instability in the three *Z4* mutant alleles $Z4^{7.1}$, $Z4^{2.1}$ and pzg^{66} (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 *woc* or *caravaggio* (HOAP), show multiple and more numerous TFs in larval neuroblasts (Figure 4A, C and [39], [40]) than the ones that we have observed in the *Z4* 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* 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).

The results from 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 the capping function and the repression of gene expression [33], [53]. In the HP1a $Su(var)2\text{-}5^{05}$ allele, known to have increased levels of *HeT-A* transcription and problems of telomere stability, we have observed a pronounced decrease in Z4 and JIL-1 (Figure 2B and D). In the $Z4^{7.1}$ allele

the decrease in Z4 protein is accompanied by a similar decrease in H3K9me3 and HP1a (Figure 2A, C and D). Finally, in the *JIL-1*^{z60} allele the increase in silencing marks like H3K9me3 and HP1a is also accompanied by a decrease in Z4 (Figure 2). Indeed, the pronounced interdependence of HP1a and Z4 presence points toward the loss of HP1a and H3K9me3 to a possible cause for telomere instability in the *Z4* mutant alleles. Interestingly, in the *Su(var)2-5*^{04}*/Su(var)2-5*^{05} 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, together with JIL-1 and HP1a, is an important component of the telomere chromatin in *Drosophila*. Reduced levels of Z4 cause significant changes in the chromatin of the HTT array, which are the source of the observed telomere instability in 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, demonstrating 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 recruitment mechanism 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 required for the targeting of *HeT-A* and *TART* transposition intermediates [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 [27], [19], [21]. Because it has been demonstrated that Z4 is able to associate with a variety of proteins in these complexes, it is especially relevant to describe a mechanism for its specific targeting to telomeres through one of the telomeric retrotransposon proteins.

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

Integrating information from previous literature and the results exposed in this study, we propose a possible model to describe the state of the chromatin at the HTT array in each of three mutant scenarios; *JIL-1*, *Z4* and *Su(var)2-5*, as well as in *wild type* (Figure 5). From the following premises: 1) HP1a 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, HP1a and Su(var)3-7 [54], [55], [49], [50]. 4) JIL-1 has been found to co-localize with Z4 at the band-interband transition in polytene chromosomes and also to co-purify with Z4 in different protein complexes [18], [27], [28]. Moreover, we have been able to detect a biochemical interaction between these two proteins (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. 5) Finally, the ChIP analyses in this study suggest a certain dependence of Z4 on HP1a or onto similar chromatin requirements for the loading of both

proteins at the HTT array (Figure 2A, C and D). In summary, the chromatin at the *HeT-A* promoter could be described as follows:

In a *wild type* situation (Figure 5A), the *HeT-A* promoter contains intermediate levels of HP1a, JIL-1 and Z4. HP1a 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.

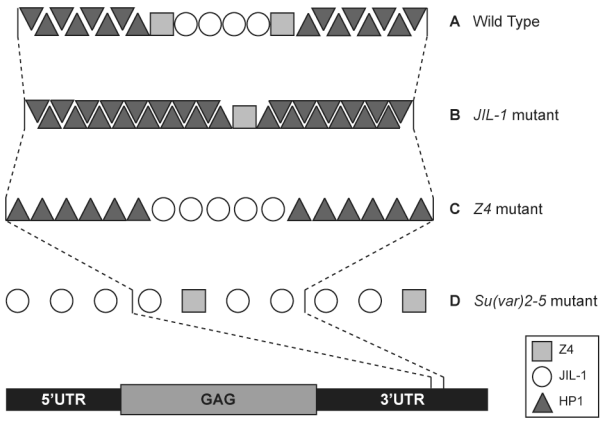


Figure 5. Model of chromatin environment at the *HeT-A* promoter. (A) Wild type: Z4 defines a boundary at *HeT-A* promoter, protecting it from the action of HP1a and other heterochromatin markers. JIL-1 guarantees a certain level of euchromatin inside the *HeT-A* promoter in order to allow gene expression. **(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 (HP1a 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 the spreading of JIL-1 and Z4 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.

In *JIL-1* mutants, (Figure 5B), the lack of JIL-1 disturbs the Z4 boundary and causes a slight decrease in the Z4 presence. This result is in agreement with a Z4-JIL-1 partial interaction (Figure 3A and [28]). The decrease in JIL-1 presence and the disturbance of the boundary causes a spreading of HP1a

into the *HeT-A* promoter, leading to the repression of transcription from the HTT array (Figure 1 and 2).

In *Z4* mutants (Figure 5C), the disappearance of the boundary together with the significant decrease in H3K9me3 causes a decrease in HP1a binding and an increase in the presence of JIL-1 if some Z4 protein is still present (Figure 2A and B). The significant decrease in HP1a availability in this region would trigger the telomere instability in a *Z4* mutant scenario (Figure 4).

Finally, in a *Su(var)2-5* mutant background (Figure 5D), the lack of HP1a 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 a permissive chromatin environment and releasing *HeT-A* silencing (Figure 1 and 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 related with the ones presented here. For example, other chromatin regulatory components that have been related with *Drosophila* telomeres are the deacetylase Rpd3, with a regulatory role on chromatin structure [56], the histone methyltransferase SETDB1 and the DNA methylase Dnmt2 [52], [57], which by acting in the same epigenetic pathway repress *HeT-A* transcription [52]. Future in depth studies on additional chromatin components will make possible to complete 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.

CONCLUSIONS

The results shown here demonstrate the role of JIL-1 as the first positive regulator of telomere 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. On the other hand, we 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 like HP1a. Moreover we have shown that JIL-1 and Z4 biochemically interact, justifying their presence in the same protein complex. Importantly, we describe the interaction of Z4 with HeT-A Gag, providing evidences for a targeting mechanism of the Z4-JIL-1 complex to the telomeres independent of its role at other genomic locations.

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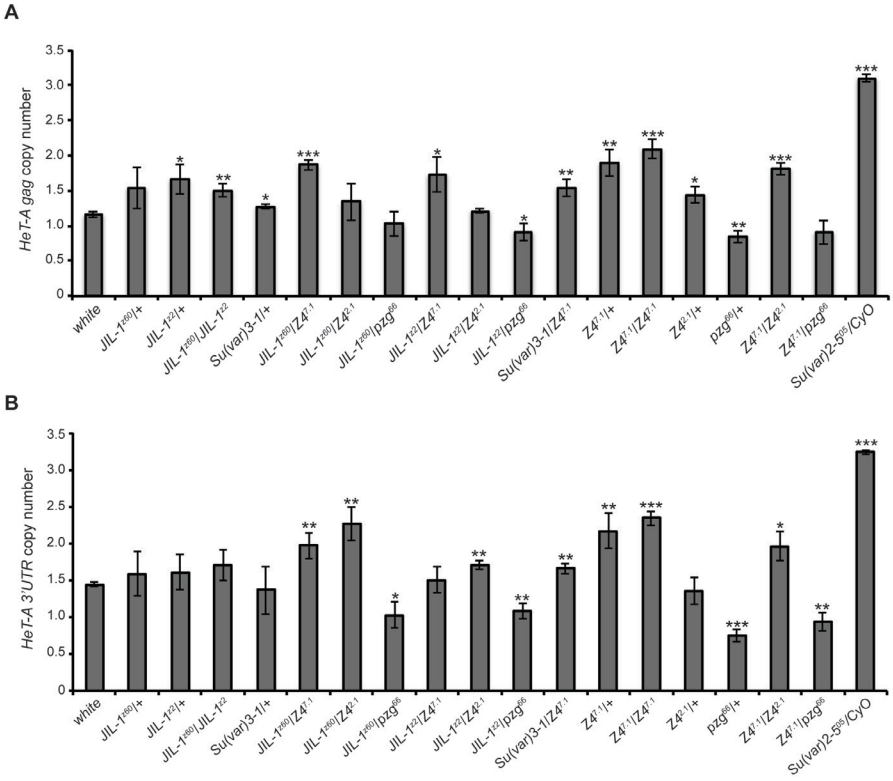
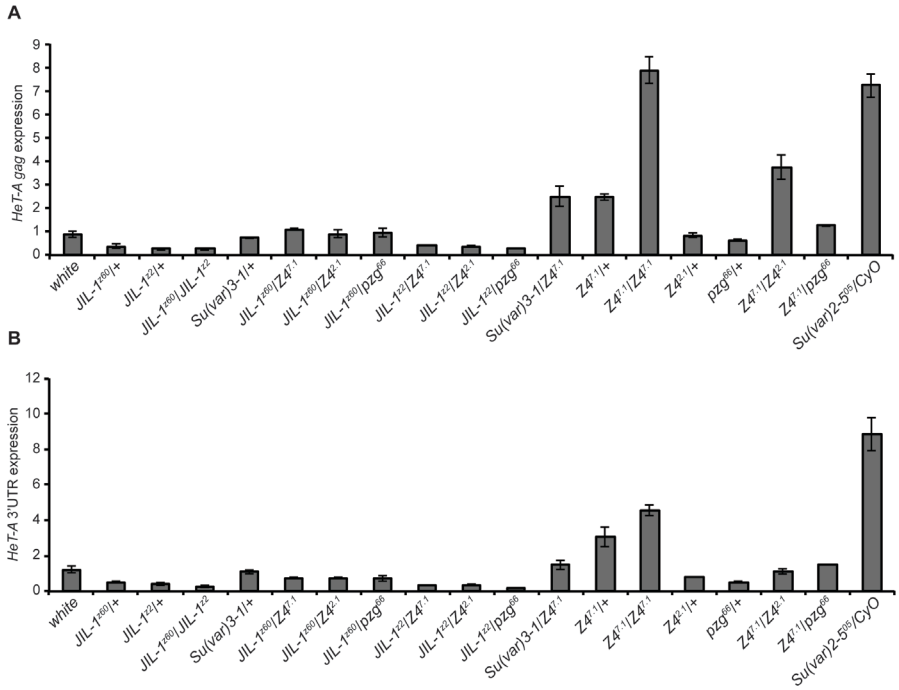
CHAPTER 2

SUPPLEMENTAL FIGURES:



Supplemental Figure 1: *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. $Z4^{7.1}$ and $Su(var)2\text{-}5^{05}$ 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 w^{1118}.



Supplemental Figure 2: *HeT-A* expression in *JIL-1* and *Z4* mutants. Absolut 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.

CHAPTER 2

ADDITIONAL RESULTS:

JIL-1 interacts with Z4 through its C-terminal domain

In order to identify which domain of the JIL-1 kinase interacts with Z4 we generated JIL-1 and Z4 fusion proteins. For JIL-1 three GFP fusion proteins containing the full-length (FL) and different deletions of the protein were generated, while for Z4 one FLAG fusion protein with the full length protein was generated. The fusion proteins were transfected in S2 cells and collected 48 hours after transfection. First, we checked if both JIL-1^{FL}GFP and Z4FLAG recombinant proteins co-immunoprecipitated, like the respective endogenous proteins (Figure 6A and Figure 3). JIL-1 is not very soluble and we had several problems extracting the protein, however in figure 6A its is possible to observe that Z4FLAG immunoprecipitates with JIL-1^{FL}GFP. The opposite, JIL-1^{FL}GFP immunoprecipitated with Z4FLAG, is not detectable probably due to the low amounts of JIL-1^{FL}GFP in solution. To determine which domain of JIL-1 interacts with Z4 we did two constructs, one lacking the CTD of JIL-1 (JIL-1$^{\Delta CTD}$GFP) and other containing only of the CTD (JIL-1^{CTD}GFP) (see Introduction Figure 8B). JIL-1$^{\Delta CTD}$GFP failed to immunoprecipitate with Z4FLAG (Figure 6B). But JIL-1^{CTD}GFP was able to immunoprecipitate Z4FLAG, Z4FLAG also immunoprecipitated JIL-1^{CTD}GFP but with low efficiency (Figure 6C). These results suggest that JIL-1 and Z4 interact through the CTD of JIL-1.

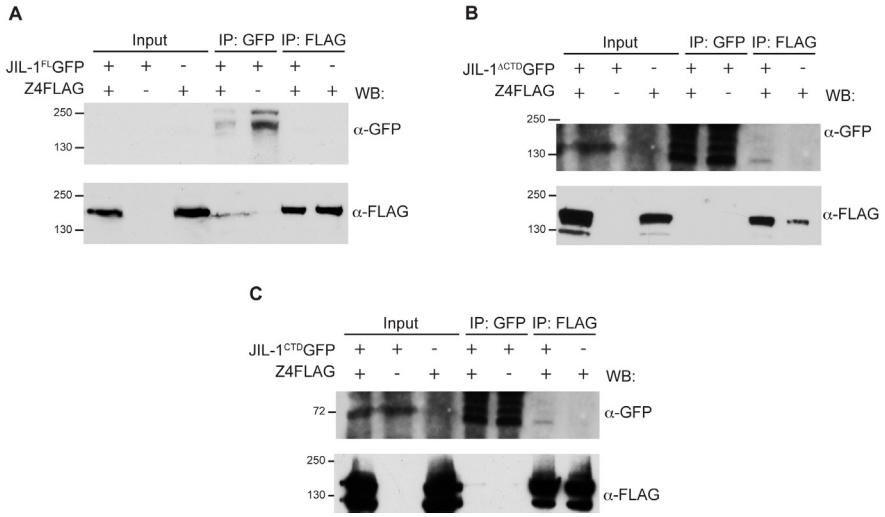
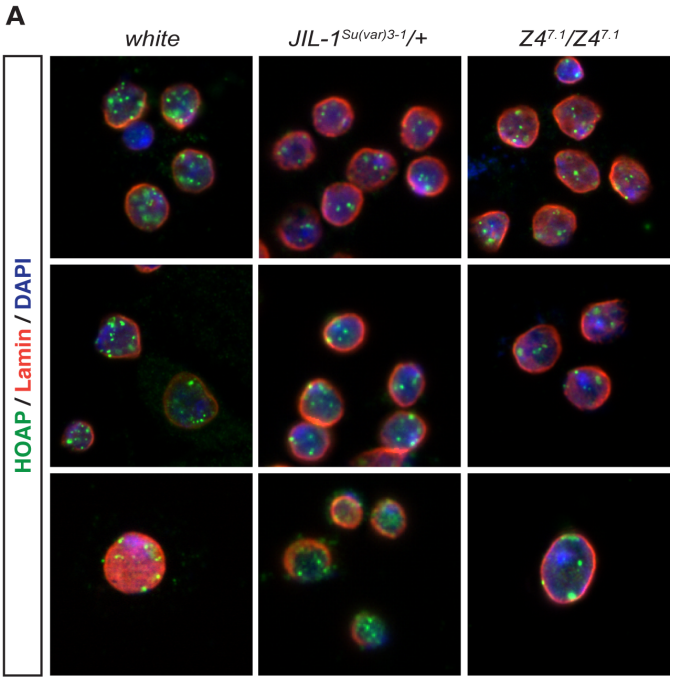


Figure 6: Mapping of the JIL-1 interaction domain with Z4. (A) Immunoprecipitation (IP) of JIL-1^{FL}GFP with Z4FLAG. (B) The truncated JIL-1, JIL-1$_{\Delta}^{CTD}$GFP, failed to immunoprecipitate with Z4FLAG. (C) JIL-1^{CTD}GFP and Z4FLAG immunoprecipitation. S2 cells were transfected with the different combinations of recombinant proteins and immunoprecipitated with anti-GFP and anti-FLAG. Control experiments were performed by transfecting the empty GFP and FLAG vectors. Presence of the recombinant protein is indicated on the top of the panel (+ and - symbols). Antibodies used for immunoprecipitation are indicated on the top. All extracts were fractionated by SDS-PAGE, western blotted, and developed with specific antibodies (indicated on the right of each figure). Molecular markers (kDa) are indicated on the left.

Do JIL-1 and Z4 regulate telomere localization in the nucleus?

Yeast and mammalian telomeres require an appropriate lamin structure for a proper localization in the nucleus (Cowan et al., 2001; Gonzalez-Suarez et al., 2009). Moreover, loss of the lamin structure affects the distribution of the telomeres leading to telomere shortening, defects in telomere chromatin and increased genomic instability (Cowan et al., 2001; Gonzalez-Suarez et al., 2009). JIL-1 directly interacts with Lamin Dm_0 through its CTD and this interaction is required to maintain an appropriate nuclear morphology (Bao et al., 2005). Therefore, we decided to investigate if *Drosophila* telomeres depend on an interaction with Lamin for a correct localization, and if this interaction is mediated by JIL-1 and/or Z4.

To investigate this hypothesis we looked for telomere delocalization in *JIL-1* and *Z4* mutants. With this purpose we prepared *JIL-1* and *Z4* mutant third instar larvae neuroblasts and co-stained with anti-Lamin (to visualize the nuclear membrane) and anti-HOAP or anti-HeT-A Gag (to visualize the telomeres). In this experiment we used the *JIL-1*$^{Su(var)3-1}$ gain-of-function mutant because this allele carries a molecular lesion that deletes the CTD domain involved in the JIL-1 interaction with Lamin Dm_0 (Ebert et al., 2004; Bao et al., 2005). Due to the abundance of the telomeric signals (up to 16 spots in the nucleus) and to the irregular pattern of telomere distribution through the nucleus it was very difficult to evaluate if *JIL-1* and *Z4* mutants display abnormal telomere localization (Figure 7). However, we did not observe any significant difference in the telomere signals, suggesting that in the case that JIL-1 and Z4 have a role in telomere positioning, this role is subtle in larval neuroblasts under our working conditions.



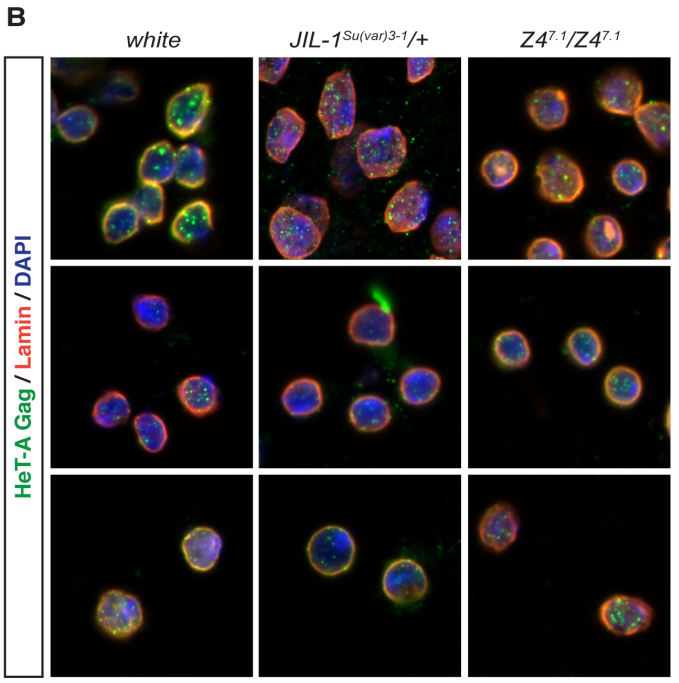


Figure 7: Immunolocalization experiments of HOAP and HeT-A GAG in *JIL-1* and *Z4* mutants. *white*, *JIL-1*$^{Su(var)3-1}$/+, and *Z4*$^{7.1}$/*Z4*$^{7.1}$ third instar larvae neuroblast preparations. Cell nucleus was stained with DAPI (blue), nuclear membrane was stained with anti-lamin (red) and telomeres were marked with (A) anti-HOAP (green) and (B) anti-HeT-A Gag (green).

A possible alternative to solve the technical problem encountered in this experiment would be to use a line that carries a 256-copy lacO array located at a single telomere (unpublished results from the laboratory of Dr. Yikang Rong). lacO is the binding site for the *E. coli* DNA binding protein LacI (Jacob and Monod, 1961). This line can be crossed with a line expressing a LacI-GFP fusion protein, which would allow the visualization of the marked telomere. Upon binding of multiple LacI-GFP molecules to the lacO array at the telomeres, the localization of this telomere could be monitored by the GFP signal. Introduce this LacI-lacO system into *JIL-1* or *Z4* mutant backgrounds would be very useful to address this question.

Additionally, we performed protein immunoprecipitation experiments to check if HeT-A Gag (experiment preformed by Elisenda López-Panadès) and Z4 interacted with Lamin. In both situations the immunoprecipitations were negative, indicating that HeT-A Gag and Z4 do not mediate the interaction between the telomeres and Lamin. At the moment the possible role of Lamin at *Drosophila* telomeres remains uncertain and more experiments need to be performed in order to characterize the importance of this protein in the regulation of telomere length and stability.

Chromator does not effect telomere expression

JIL-1 and Z4 interact with Chromator (Chro) and the three proteins are necessary for a correct morphology of polytene chromosomes (Eggert et al., 2004; Rath et al., 2004; Gan et al., 2011; Wang et al., 2012). Polytene chromosomes of *Chro* mutants have problems in band-interband segregation resulting in compacted structures (Rath et al., 2006). It was recently suggested that the Chro-Z4 complex is involved in the recruitment of JIL-1 to chromosomes, leading to the phosphorylation of nearby nucleosomes (Gan et al., 2011). Since we found that JIL-1 and Z4 affect the expression of the telomeric retrotransposons, we decided to investigate whether Chro was also involved in telomere regulation.

To investigate the role of Chro at *Drosophila* telomeres we analyzed the levels of mRNA of the *HeT-A* element in the $Chro^{KG03258}$ mutant. The *Chro* mutant consists in a lethal *P*-element insertion that disrupts the *Chro* gene (Rath et al., 2004). To determine the level of *HeT-A* expression in $Chro^{KG03258}$ mutants we used whole third instar larvae to extract total mRNA (Figure 8A). Since the number of *HeT-A* copies varies among stocks, we analyzed the number of *HeT-A* copies in *Chro* mutants (Figure 8B) and used this value to normalize the expression results (Figure 8C). In this experiment we used ry^{506} flies as control to minimize the possible genetic background effects, since the $Chro^{KG03258}$ allele is in a ry^{506} background. No changes were observed in the

number of *HeT-A* copies in the *Chro*^{KG03258} allele (Figure 8A), neither in the levels of *HeT-A* expression (Figure 8B and C). These results reveal that Chro is not involved in the regulation of the telomere retrotransposons in *Drosophila*.

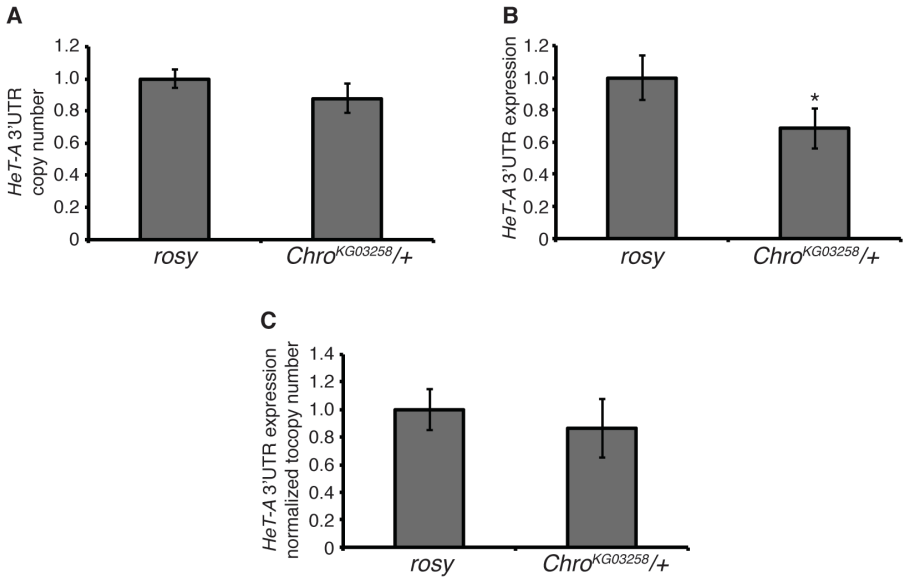


Figure 8: *HeT-A* expression and copy number in the *Chro*^{KG03258} mutant. (A) The number of *HeT-A* copies does not change between control and *Chro*^{KG03258} mutant flies (B) *HeT-A* transcripts slightly decrease in the *Chro*^{KG03258} alleles. (C) *HeT-A* transcript levels were normalized to actin transcripts and to the respective *HeT-A* copy number. *Chro*^{KG03258} mutants have the same level of expression as the control flies. Error bars represent standard deviations of three independent experiments. Asterisks indicate statistically significant differences (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 three independent experiments.

Since Chro was described to directly interact with Z4 (Gortchakov et al., 2005) and our results demonstrate that *Z4* mutants have problems in telomere stability, we decided to check if *Chro* mutants had problems in telomere protection. To investigate the possible role of Chro in telomere stability, we checked metaphase chromosome preparations from third instar larvae neuroblasts of *Chro* mutants. No TFs were observed in the *Chro*^{KG03258} allele (results not shown) indicating that Chro is not involved in telomere stability in *Drosophila*. These results strongly indicate that, despite its

interaction with JIL-1 and Z4, Chro is not involved in the recruitment of these proteins to the telomeric domains; neither is involved in the telomere retrotransposons regulation nor in telomere stability. A screen of candidate chromodomain proteins that act as suppressor of TPE also showed that Chro does not have effect on TPE (Doheny et al., 2008), which, in accordance with the results presented here and other published studies, suggests that the overlapping function of these three proteins is not complete.

CHAPTER 3

The Z4 partners DREF, TRF2 and KEN are involved in the regulation of *Drosophila* telomeres

INTRODUCTION

Z4 is a seven zinc-finger protein known to localize at polytene chromosome interbands and necessary to maintain the band-interband structure in these chromosomes (Eggert, 2004). A study using the *Drosophila tel1* mutant (mutant with telomeres ten times longer) identified Z4 as a component of the telomeric domain (Andreyeva, 2005). These findings lead us to investigate the role of Z4 at *Drosophila* telomeres. In summary, we found that *Z4* mutants specifically affect the expression of genes embedded at the telomeric domain but not at the TAS domain (Chapter 1). Additionally, we demonstrated that Z4 is necessary to guarantee telomere stability and that it specifically interacts with HeT-A Gag and JIL-1 (Chapter 2). Furthermore, other studies have identified Z4 as a component of different protein complexes with different cellular roles (see Introduction, Figure 9):

1. Transcriptional activation of replication-related genes: Z4 was identified as part of a complex with DREF and TRF2 (Hochheimer et al., 2002). The DREF/TRF2 complex is involved in core promoter selection, where DREF functions as a transcriptional activator of replication-related genes (Hochheimer et al., 2002). The DREF homo-dimer binds specifically to the DRE sequence (5'-TATCGATA) in the promoter of many cell proliferation-related genes, making DREF required for the cellular shift from the resting state into the proliferating state. As part of the DREF/TRF2 complex, Z4 is required for the expression of cell cycle and replication related genes (Kugler and Nagel, 2007). Depletion of Z4 strongly affects DNA replication and the

number of cells entering mitosis is strongly reduced. Moreover, low levels of Z4 lead to a strong decrease in the expression of cell cycle genes such as *dE2F1*, *cyclin A* or *cyclin D* (Kugler and Nagel, 2007). In addition to Z4, several proteins were identified to belong to the DREF/TRF2 complex. Among them there are the NURF (Nucleosome Remodeling Factor) subunits ISWI, NURF55 and NURF38 (Hochheimer et al., 2002). NURF triggers nucleosome sliding changing the dynamic properties of the chromatin, which can result in chromatin activation or repression (Kwon et al., 2008). The role of the NURF complex in this context has been speculated to be involved in potentiating gene activation by changing the chromatin structure (Hochheimer et al., 2002).

2. Notch target gene activation signaling: Z4, through its interaction with NURF, binds to Notch target genes leading to its activation (Kugler and Nagel, 2007). In *Z4* mutants the typical open chromatin structure of Notch target genes is no longer detectable, which strongly suggests that Z4 functions at the level of chromatin activation. The role of Z4 and NURF in the Notch pathway is independent of DREF since only Z4, and not DREF, is detected at the promoters of different Notch target genes (Kugler and Nagel, 2010).

3. Metamorphosis: Z4 and NURF-301 directly bind to the ecdysone receptor (EcR) in the presence of ecdysone, suggesting that they act as direct effectors of the nuclear receptor activity (Badenhorst et al., 2005; Kugler et al., 2011). In *Z4* and *Nurf301* mutants most ecdysone target genes are downregulated and consequently developmental delay and early larval lethality are observed (Kugler et al., 2011).

5. Innate immune response: Immunoprecipitation experiments detected Z4 in a complex with KEN and NURF (Kugler et al., 2011). KEN and NURF function as repressors of the JAK/STAT pathway and both are found in gene promoters with Ken binding sites, suggesting that NURF is recruited by Ken to repress STAT sequences (Kwon et al., 2008). In *Z4* mutant flies the

expression of defense response genes is elevated, leading to the formation of melanotic tumors (Kugler et al., 2011). Z4 and NURF are thus involved in the transcriptional repression of the JAK/STAT pathway genes (Kugler et al., 2011; Kwon et al., 2008).

Since we found that Z4 was involved in the regulation of telomere stability and that *Z4* mutants lead to chromatin changes at the *HeT-A* promoter, we decided to investigate if any of the described Z4 complexes was involved in the remodeling of the telomeric chromatin, regulating this way the expression of the telomeric retrotransposons *HeT-A*, *TART* and *TAHRE*.

MATERIALS AND METHODS

Fly stocks and crosses

Fly stocks were maintained and crosses preformed at 25ºC on standard *Drosophila* corn meal medium. w^{1118} and ry^{506} were used as control, depending on the genetic background of each strain. *w* ; Trf^{260071}/*FM7c*, ry^{506}; $Dref^{KG09294}$/*CyO* and ry^{506} ; Ken^{1}/*CyO* were obtained from Bloomington Stock Center. *w* ; Trf^{260071}/*FM7c* balancer was changed to *FM7c-GFP* to allow the selection of hemizygous males to perform the experiments.

Sequence alignments

The sequence alignments were carried out using the ClustalW software. For the accession number of the *HeT-A* sequences used in the alignments see (Piñeyro et al., 2011) and for the accession number of *TART* and *TAHRE* sequences see (George et al., 2006).

Genomic DNA extraction, RNA extraction and quantitative real-time PCR

Genomic DNA extraction, RNA extraction, cDNA synthesis and quantitative real-time experiments were performed has described in chapter 2. Primers used for *TART* amplification: TART_5'UTR_F: GATAATAGCCGATAAGCCCGCCA and TART_5'UTR_R: AAGACACAGCGGTTGATCGATATG. Primers used for HeT-A amplification: HeT-A_3'UTR_F (CCCCGCCAGAAGGACGGA) and HeT-A_3'UTR_R (TGTTGCAAGTGGCGCGCA).

RESULTS

TART promoter contains DREF binding sequence

To investigate if the DREF/TRF2 complex had a role controlling the expression of the telomeric retrotransposons, we started by searching if the DREF binding sequence (5'-TATCGATA) was present in any of the telomeric retrotransposons. The presence of this sequence indicates that the DREF/TRF2 complex is able to bind to the telomeric array, and thus regulate the expression of the telomeric retrotransposons. We did not find any DREF binding sequence in the retrotransposons *HeT-A* and *TAHRE*. However, we were able to identify two DREF binding sequences in *TART*; one in the 5'UTR around 170bp downstream the transcription start site and another around 600bp upstream the end of the 3'UTR. To check if this sequence was conserved between the different *TART* subfamilies we did a nucleotide sequence alignment using the ClustalW software (Figure 1). The sequence alignment revealed that the DREF binding site at the 5'UTR was highly conserved among all *TART* subfamílies (Figure 1A) while the 3'UTR sequence was only present in the *TART A* subfamily (Figure 1B). The DREF binding site at the *TART* 5'UTR is located at the *TART* promoter that drives sense RNA transcription while the 3'UTR binding site is probably present at

the *TART* anti-sense promoter. These data strongly suggest that DREF, and consequently TRF2, bind at *TART* promoter controlling its expression.

Figure 1: DREF binding sites. Nucleotide sequence alignment of *TART A*, *B* and *C* subfamilies using the ClustalW software. (A) *TART* 5'UTR alignment; DREF binding site is 100% conserved in all *TART* subfamilies. (B) *TART* 3'UTR alignment; DREF binding site is only conserved in the *TART A* subfamily.

HeT-A, *TART* and *TAHRE* contain KEN binding sites

Similarly, we searched for KEN binding sites (5'-GAGAAAK, K=G/T) (Arbouzova and Zeidler, 2006) in *HeT-A*, *TART* and *TAHRE*, and found that this sequence was present in the three telomeric retrotransposons. *HeT-A* has a KEN binding site at the 5'UTR, but this sequence is only conserved in three of the analyzed sequences (Figure 2A) For this analysis we used the complete *HeT-A* sequences available in the databases (for more information see (Piñeyro et al., 2011)). *TART* has two KEN binding motifs inside the Gag coding sequence (ORF1); one is present in the three *TART* subfamilies while the other is only present in the *TART A* subfamily (Figure 2B and C). Finally, we found six copies of the KEN binding site in *TAHRE*, three at the 5'UTR, one at the RT coding sequence (ORF 2) and two at the 3'UTR. In the case of *TAHRE* we could not perform a sequence multi-alignment analysis since only one full sequence is available in databases. These results strongly suggest

that KEN is able to bind at the HTT array and that it might be involved in the regulation of the telomeric retrotransposons.

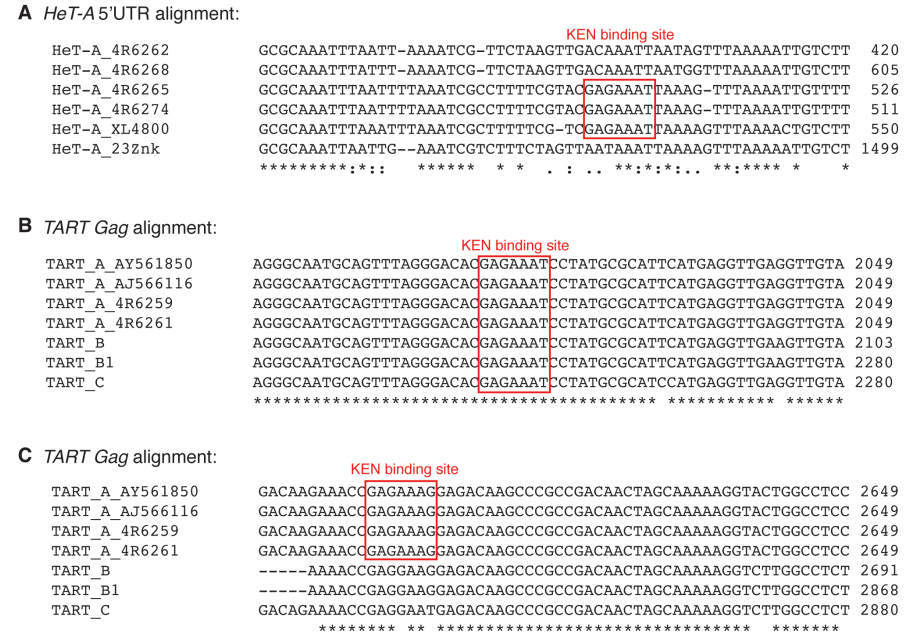


Figure 2: KEN binding sites. (A) *HeT-A* 5'UTR alignment; KEN binding site is conserved in three of the six *HeT-A* sequences analyzed. (B) *TART Gag* alignment; KEN binding site is conserved in the three *TART* subfamilies. (C) *TART Gag* alignment; the second KEN binding site is only conserved in the *TART A* subfamily. Nucleotide sequence alignment was performed using the ClustalW software.

DREF, TRF2, KEN and Z4 differently affect the telomeric retrotransposons *HeT-A* and *TART*

After finding DREF and KEN binding sites in the telomeric retrotransposons nucleotide sequence we decided to investigate if mutations in these proteins affect the expression of the telomeric retrotransposons *HeT-A* and *TART*. In our analysis we used $Dref^{KG0994}$, Trf^{260071} and Ken^{1} mutants; all of them consisting in *P*-element insertions that disrupt the coding sequence. We also included two different *Z4* mutant alleles, a *Z4* hypomorph ($Z4^{7.1}$) and a null mutant (pzg^{66}) in order to investigate if the role of DREF, TRF2 and KEN on *Drosophila* telomeres could be mediated by Z4. For all mutants we analyzed

the *HeT-A* and *TART* mRNA levels by quantitative real-time PCR. As in all the other expression studies of this thesis, in order to obtain the real level of expression of each *HeT-A* and *TART* element we first measured the levels of *HeT-A* and *TART* copies in each stock and used this value to normalize the mRNA data.

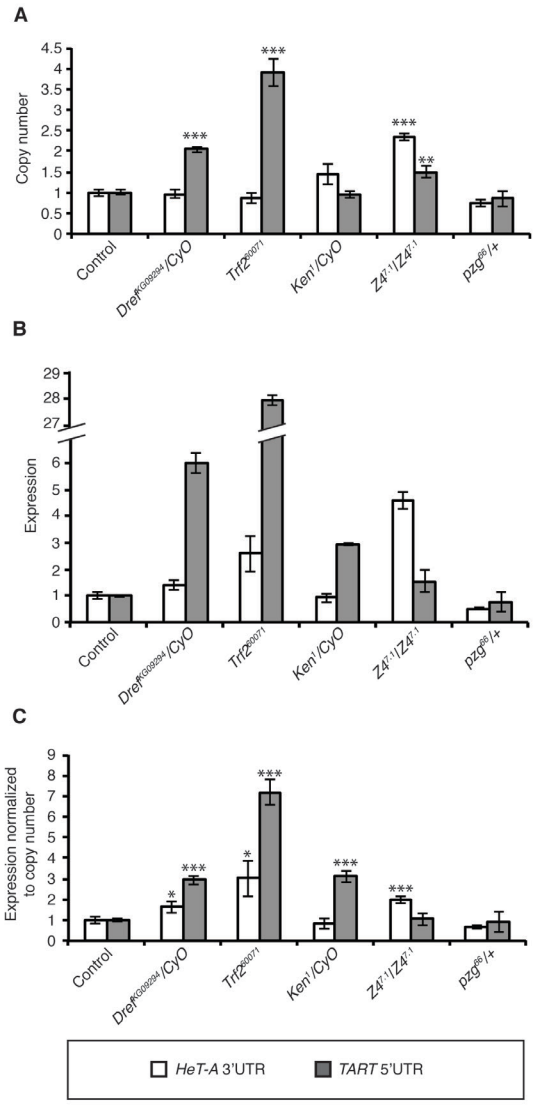


Figure 3: *HeT-A* and *TART* expression and copy number in *Dref*, *Trf2*, *Ken* and *Z4* mutants. (A) *Dref*^{KG09294} and *Trf*^{260071} mutants have more *TART* copies than control flies but no difference in *HeT-A* copies is observed. *Ken*^{1} and *pzg*^{66}/+ mutants

do not affect *HeT-A* and *TART* copy number. $Z4^{7.1}/Z4^{7.1}$ mutants have more *HeT-A* and *TART* copies. (B) and (C) *HeT-A* transcripts increase in $Dref^{KG09294}$, Trf^{260071} and $Z4^{7.1}/Z4^{7.1}$ mutants and no effect is observed in *Ken* mutants. *TART* transcripts increase in $Dref^{KG09294}$, Trf^{260071} and Ken^{1} mutants and do not change in *Z4* mutants. *HeT-A* is represented in white bars and *TART* in grey bars. Error bars represent standard deviations of three independent experiments. Asterisks indicate statistically significant differences (one asterisk, $P < 0.05$ to 0.01; two asterisks, $P < 0.01$ to 0.001; three asterisks, $P < 0.001$) in *HeT-A* and *TART* expression and copy number of each mutant compared to the respective control.

To determine the number of *HeT-A* and *TART* copies in each stock we extracted genomic DNA from third instar larvae without salivary glands. For *TART* amplification we designed a pair of primers at the 5'UTR flanking the DREF binding sequence. *Dref*, *Trf2* and *Ken* mutants did not show differences in *HeT-A* copy number (Figure 3A), but in the $Z4^{7.1}/Z4^{7.1}$ hypomorph mutant an increase in *HeT-A* copy number was observed (discussed in Chapter 2). However, the number of *TART* copies increased in $Dref^{KG0994}$, Trf^{260071} and Ken^{1} but not in *Z4* mutants (Figure 3A). Following, we analyzed the mRNA levels of *HeT-A* and *TART* in the same mutants. To obtain the expression data we extracted mRNA from whole third instar larvae and analyzed them by quantitative real-time PCR. An increase in *HeT-A* and *TART* expression was observed for $Dref^{KG0994}$ and Trf^{260071} mutants, with the increase in *TART* expression being more accentuated than in *HeT-A* (Figure 3B and C). Ken^{1} mutants show an increase in *TART* expression and no effect in *HeT-A* expression (Figure 3B and C). Finally, the $Z4^{7.1}/Z4^{7.1}$ hypomorph mutant shows an increase in *HeT-A* expression while the null mutant pzg^{66}/+ does not affect *HeT-A* or *TART* expression (Figure 3B and C and Chapter 2). These results indicate that DREF, TRF2 and Ken modulate the expression of the telomeric retrotransposons *HeT-A* and *TART*.

DISCUSSION

DREF and TRF2 control the expression of *HeT-A* and *TART*

DREF and TRF2 have been described by their role as activators of transcription related genes (Hochheimer et al., 2002; Kugler and Nagel, 2007; Hirose et al., 2001; Kim et al., 2007; Yoshioka et al., 2012; Fujiwara et al., 2012). TRF2 affinity purification experiments allowed the identification of several proteins that form a complex with DREF and TRF2, among them are Z4 and the NURF complex (Hochheimer et al., 2002). Since we had previously found that Z4 was involved in telomere stability (Chapter 2), we decided to investigate whether the role of Z4 at *Drosophila* telomeres was performed through the DREF/TRF2/Z4 complex. We started by identifying two DREF binding sites (5'-TATCGATA) in the *TART* element of *D. melanogaster*, one highly conserved at the 5'UTR and another at the 3'UTR only conserved in the *TART A* subfamily (Figure 1). The fact that one of the DREF binding sites is strongly conserved among all *TART* subfamilies and the other conserved in all the different copies of the *TART A* subfamily here analyzed, strongly suggests an important role of DREF in the control of *TART* transcription. Interestingly the *TART A* subfamily in *D. melanogaster* is the one with higher levels of expression. Based on the DREF/TRF2 role on the activation of gene transcription, we were expecting that DREF and TRF2 would be involved in the control of active *TART* expression when cells proliferate and DNA replication is needed. However, when we analyzed *HeT-A* and *TART* expression in *Dref* and *Trf2* mutants an increase of the transcription of these retrotransposons was observed, indicating that DREF and TRF2 were involved in the repression of the telomeric retrotransposons.

Since the results presented in this chapter were the very last obtained during my thesis, we did not have time to completely characterize the role of the DREF/TRF2 at *Drosophila* telomeres. However, some literature research allowed us to formulate two possible hypotheses to explain the obtained results. Both hypotheses are compatible with the obtained results but

additional experiments should be performed in order to discern between the two or to prove that both of them are true.

Our first hypothesis is based on the fact that BEAF (boundary element-associated factor) competes with DREF for DNA binding because both have overlapping binding sites (BEAF binds to 5'-CGATA motifs) (Hart et al., 1999). Boundary elements have the ability to insulate a transgene from its chromosomal context by blocking enhancer-promoter interactions and heterochromatin spreading (Barkess and West, 2012). In *D. melanogaster*, BEAF associates preferentially with active transcribed genes (Vogelmann et al., 2011). A study from Emberly and co-workers described the role of DREF and BEAF at the promoters of cell-cycle genes (Emberly et al., 2008). In this study the authors demonstrated that in DREF depleted cells, the expression of DREF regulated genes is upregulated up to 4 to 5-fold. This increase in gene transcription is due to the binding of BEAF to the different loci that DREF is also able to bind. The presence of BEAF at DREF regulated genes leads to a decrease in the deposition of the heterochromatic mark H3K9me3, de-repressing those genes from the surrounding heterochromatin (Hart et al., 1997; Cuvier et al., 2002). Our results show an increase in *HeT-A* and *TART* transcription in *Dref* mutants. A possible explanation could be the binding of BEAF to the DREF binding sites at the *TART* promoter, protecting it from the surrounding heterochromatin environment. The levels of *HeT-A* expression also increase in this situation because the influence of the BEAF boundary spreads through the HTT array due to the random disposition of the telomeric retrotransposons. To test this hypothesis we should measure the levels of *HeT-A* and *TART* expression in *Beaf* mutants as well as in *Beaf*/*Dref* double mutants. Additionally, ChIP experiments should be performed to confirm the presence of DREF and BEAF at the *TART* promoter and to quantify the levels of H3K9me3 in *Dref* and *Beaf* mutants.

Our second hypothesis is related with the role of TRF2. In accordance with what happens with *Dref* mutants, *Trf2* mutants also show an increase in *HeT-A* and *TART* transcription. TRF2 has been associated with chromatin

organization and protein synthesis, and its mammalian ortholog TLF (TBP-like factor) was found to be required for the formation of the chromocenter heterochromatin (Martianov et al., 2002). Moreover, *Drosophila Trf2* mutants show defects in meiotic chromosome condensation (Kopytova et al., 2006). In this context, TRF2, together with the PcG protein CRAMPED, was found to be required for the expression of the histone *H1* gene (Isogai et al., 2007; Gibert and Karch, 2011). Linker histone H1 is an essential chromatin component that binds to internucleosomal DNA and mediates DNA packaging by the folding of nucleosomes into a higher-order structure (Kasinsky et al., 2001; Bassett et al., 2009). Recent studies show that histone H1 is involved in transcription regulation near or within heterochromatin (Lu et al., 2009). The assembly of histone H1 at *Drosophila* chromosomes is dependent on the NURF subunit ISWI (involved in ATP dependent nucleosome sliding). Loss of ISWI leads to decondensation of mitotic and polytene chromosomes and change in the spacing of nucleosomes due to the loss of H1 (Bouazoune and Brehm, 2006; Siriaco et al., 2009). ISWI forms a complex with TRF2 and DREF, thus the depletion of TRF2 can lead to the loss of H1 at *Drosophila* chromosomes by two different pathways: 1) TRF2 is required for the expression of the histone *H1* gene, thus mutations in *Trf2* lead to a global decrease on the levels of histone H1 available (Isogai et al., 2007; Gibert and Karch, 2011); 2) The loss of TRF2 probably reduces the level of ISWI at gene promoters and this will lead to a relaxation of the chromatin at those promoters due to the loss of H1. These findings support the fact that *Trf2* mutants show an increase in *HeT-A* and *TART* expression. In this situation, ISWI is not recruited to the *TART* promoter and the nucleosome spacing increases, leading to a decompaction of the neighboring chromatin due to the absence of histone H1. The increase in expression observed in *Trf2* mutants is higher for *TART* than for *HeT-A* because TRF2 directly binds to the *TART* promoter, the increase observed in *HeT-A* expression is probably an indirect effect of the relaxation of the HTT chromatin. In agreement, it was recently reported that the linker histone H1 is

required for the silencing of retrotransposons in *Drosophila* (Vujatovic et al., 2012). Moreover, the histone H1.1 is involved in telomere silencing in *S. Cerevisiae* (Jedrusik and Schulze, 2003). Therefore, all of the above findings strongly support our hypothesis on the role of the linker histone H1 controlling the telomere retrotransposons expression in *Drosophila*. In order to confirm this hypothesis ChIP experiments should be performed to evaluate the amount of ISWI and H1 present at *TART* promoter in wild type and *Trf2* mutant flies. Additionally, an increase in *HeT-A* and *TART* expression should be observed in *Iswi* and *H1* mutants. However, it is important to note that several experiments still need to be performed in order to identify the role of the DREF/TRF2 complex at *Drosophila* telomeres since the results presented in this chapter are preliminary and are not enough to support our hypotheses.

KEN is a repressor of *TART*

The JAK/STAT signaling pathway is responsible for the activation of the immune response genes (Arbouzova and Zeidler, 2006; Hombría and Sotillos, 2006). KEN is a well-defined repressor of the JAK/STAT pathway and it competes with STAT for binding to target genes (Hombría and Sotillos, 2006). Recently, NURF301 and Z4 were also identified to be recruited by Ken and repress the JAK/STAT pathway (Kwon et al., 2008; Kugler et al., 2011). When we analyzed the effect of *Ken* mutations in *Drosophila* telomeres, an increase in *TART* expression was observed but no effects on *HeT-A* expression were detected (Figure 3C). Accordingly, we found that the KEN binding sequences are not conserved in all the analyzed *HeT-A* sequences (Figure 2A) suggesting that KEN might bind only to a portion of the *HeT-A* copies. A possible explanation could be that KEN might be involved in the recruitment of the chromatin remodeling complex NURF to *TART* sequences. Therefore, in a *Ken* mutant background, the NURF complex is not recruited to the telomeres and that leads to a relaxation of the chromatin. The fact that no effect on *HeT-A* expression is observed in *Ken* mutants suggests that the

degree of chromatin relaxation is weaker than the one observed in *Dref* and *Trf2* mutants. However, as mentioned before these are preliminary results and we have not ruled out any possibility. More experiments need to be performed in order to better characterize the role of the JAK/STAT pathway in the regulation of the telomeric retrotransposons.

In conclusion, we have identified three new proteins involved in the regulation of the expression of the telomeric retrotransposons, DREF, TRF2 and Ken. Although we were not able to completely characterize the role of these proteins at *Drosophila* telomeres, our results offer some clues on other chromatin related proteins that might be involved in telomere regulation.

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CHAPTER 4

The role of dSETDB1 and POF at *Drosophila* telomeres

INTRODUCTION

Eukaryotic chromosomes are composed of two types of chromatin, euchromatin and heterochromatin. Each type of chromatin is regulated by specific epigenetic marks such as DNA methylation and histone modifications (Grewal and Jia, 2007). Heterochromatin consists of highly compacted DNA and is present at transcriptionally silent regions. In *Drosophila*, the most heterochromatic regions are present around the centromeres and telomeres, and throughout the 4th chromosome (Riddle et al., 2009). The most common epigenetic mark for heterochromatin formation is the histone H3 lysine 9 methylation (H3K9me). The methylation of H3K9 allows the recruitment of *chromodomain* proteins like HP1, which will promote chromatin condensation and gene repression (Bannister et al., 2001; Schotta et al., 2002).

In *Drosophila*, the methylation of H3K9 is accomplished by the histone methyltransferases SU(VAR)3-9, dSETDB1 and dG9a, orthologous to the human SUV39H1, SETDB1 and EHMT2/G9a, respectively (Seum et al., 2007; Schotta et al., 2002). SU(VAR)3-9 and dG9a have overlapping functions in heterochromatin formation and gene silencing (Mis et al., 2006). SU(VAR)3-9 is mainly associated with H3K9 methylation at the chromocenter, although this protein was also detected binding to the 4th chromosome and the telomeres (Ebert et al., 2004). However, in *Su(var)3-9* mutants H3K9 methylation and HP1 presence are diminished in the chromocenter but not in the 4th chromosome nor at the telomeres (Schotta et al., 2002; Perrini et al., 2004). Moreover, *Su(var)3-9* mutations do not affect the silencing of the telomeric retrotransposon *HeT-A* (Perrini et al., 2004).

These findings indicate that another methyltransferase should be responsible for the H3K9me at the telomeres and at the 4th chromosome. dSETDB1 is the methyltransferase responsible for H3K9me at the 4th chromosome (Seum et al., 2007; Tzeng et al., 2007). This histone methyltransferase is an essential protein for fly development (Stabell et al., 2006) and is constituted by two *tudor* motifs, a methyl-CpG-binding domain, and a PreSET/SET domain (Hung and Shen, 2003). Additionally, this protein is responsible for H3K9me3 in germline cells and required for egg chamber formation during oogenesis (Clough et al., 2007; Yoon et al., 2008).

dSETDB1 is also responsible for the binding of HP1 and POF (Painting Of Four) to the 4th chromosome (Tzeng et al., 2007). POF is a 4th chromosome-specific RNA binding protein essential for the correct transcription of the genes from this chromosome (Larsson et al., 2001; Johansson et al., 2007a). POF and HP1 bind interdependently to the 4th chromosome and dSETDB1 directly interacts with POF *in vivo* (Johansson et al., 2007a; Tzeng et al., 2007). Tzeng and colleagues suggested a model for heterochromatin formation and gene regulation specific of the 4th chromosome. In this model dSETDB1 binds to the 4th chromosome and generates H3K9me3, leading to the recruitment of HP1. At the same time, dSETDB1 represses *Su(var)2-5* expression, limiting the amount of HP1 available. dSETDB1 and POF physically interact, facilitating their recruitment and/or stabilizing their binding to the 4th chromosome. The recruitment of HP1 (transcription repressor) and POF (transcription activator) by dSETDB1 allows a global regulation of gene expression from the 4th chromosome (Johansson et al., 2007b; Tzeng et al., 2007). Moreover, dSETDB1 together with Dnmt2 is involved in the silencing of *Rt1b{}* and the *HeT-A* retrotransposon by DNA methylation (Phalke et al., 2009; Gou et al., 2010).

At the beginning of this thesis the methyltransferase responsible for H3K9 methylation at *Drosophila* telomeres was still unknown, on the view of the observations described above, we decided to investigate whether dSETDB1 was responsible for this role. Moreover, dSETDB1 and HP1 interact with POF

in the 4th chromosome, so we wondered if POF could also play a role in the control of telomere retrotransposons expression from this chromosome. In this chapter we will present our results on the role of dSETDB1 and POF at *Drosophila* telomeres.

MATERIALS AND METHODS

Fly stocks

Fly stocks were maintained at 25ºC on standard *Drosophila* corn meal medium. *egg*^{1473}*/CyO* was obtained from Tulle Hazelrigg and *Pof*^{D31A}*/Cyo* was provided by Jan Larsson. *w*^{1118} was used as control. For TPE assays see materials and methods section from Chapter 1.

For further materials and methods see *Genomic DNA Extraction*, *RNA Extraction and cDNA synthesis*, *Quantitative Real Time PCR* and *Chromosome cytology* sections in Chapter 2.

RESULTS

In *Drosophila*, telomere length control means control of the retrotransposons *HeT-A*, *TART* and *TAHRE*. To date several proteins were described to control the expression of the major component of *Drosophila* telomeres, the *HeT-A* retrotransposon. Several studies state that in *Drosophila* the telomeric domain is enriched in H3K9me3 (Cowell et al., 2002; Andreyeva, 2005) and HP1 (Perrini et al., 2004; Frydrychova et al., 2008). Perrini and co-workers investigated whether the H3K27 methyltransferase E(Z) and the H3K9 methyltransferase SU(VAR)3-9 could be involved in the maintenance of those heterochromatin marks at *Drosophila* telomeres. Their study revealed that

neither E(Z) nor SU(VAR)3-9 were involved in the silencing of the telomeric retrotransposons, and suggested that H3K9me3 at telomeres should depend on another histone methyltransferases (Perrini et al., 2004).

dSETDB1 and POF control telomeric gene expression from the 4th chromosome

Because dSETDB1 was identified as the methyltransferase responsible for H3K9me3 at the 4th chromosome and to recruit POF to this chromosome (Seum et al., 2007; Tzeng et al., 2007) we decided to start by investigating whether *dSETDB1* and *Pof* mutants affected the expression of reporter genes inserted in the 4th chromosome telomeres. In our analyses, we used a *dSETDB1* mutant (*egg*^{1473}) that contains an internal deletion that removes the entire SET domain (the catalytic domain of histone methyltransferases (Rea et al., 2000)) of the protein (Clough et al., 2007). A hypomorph mutant of *Pof* (*Pof*^{D31A}) that lacks the promoter region of the *Pof* gene was also used (Larsson et al., 2004). In order to investigate the possible role of dSETDB1 and POF regulating gene expression at the HTT array, we crossed mutant alleles of *dSETDB1* (*egg*^{1473}) and *Pof* (*Pof*^{D31A}) with lines with a reporter gene inserted at the HTT array (EY09966) and at the subtelomeric transition zone of the 4th chromosome (39C-72 and 118E-5). The subtelomeric domain of the 4th chromosome is much smaller (~5.4kb) than the one of other chromosomes and is not composed of TAS sequences. Instead, following the HTT array and before the first gene, there is a transition zone composed of truncated fragments of different transposable elements mixed with small fragments of *HeT-A* and *TART* (George et al., 2006). We observed a suppressor of TPE phenotype for both *dSETBD1* and *Pof* mutants at the transition zone of the 4th chromosome (39C-72 and 118E-5 lines) but no effect was observed at the HTT array (EY09966) (Figure 1). Our data from TPE suggest that dSETDB1 is more effective controlling expression from the subtelomeric transition zone than from the telomeric domain. However, these

results are in agreement with the results obtained with the *Su(var)2-5*^{05} mutant allele where only a slight release of silencing was observed when this mutant was crossed with the same reporter line (see Chapter 1, Figure 2). Similarly, *Pof* mutants showed an increase in eye color, indicating that POF acts as a suppressor of TPE. Because in the 4th chromosome, dSETDB1 and HP1 were described to be responsible for gene repression and POF to activate gene expression, we were expecting to observe an increase in TPE in *Pof* mutants and a release in dSETDB1 and HP1 mutants.

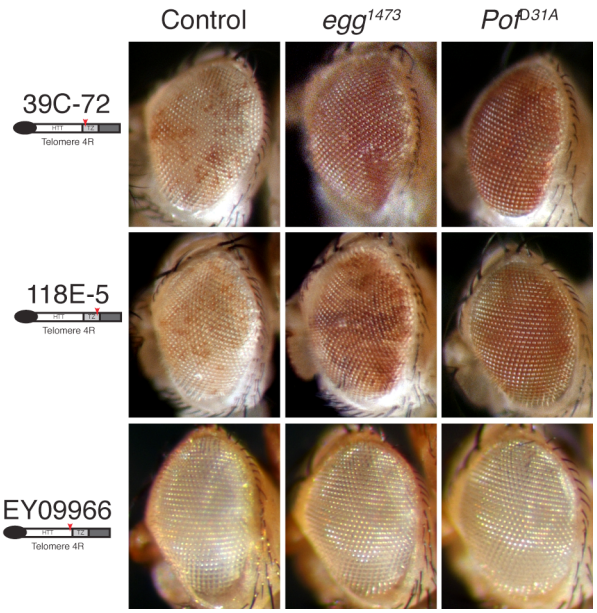

Figure 1: dSETDB1 and POF affect the expression of genes in the transition zone of the 4th chromosome but not in HTT array. TPE assays were performed with the reporter lines 39C-72 and 118E-5, with the mini-*white* gene insertions at the telomeric transition zone of the 4th chromosome, and EY09966, with mini-*white* gene insertion at the HTT array of the 4th chromosome. A suppressor of TPE was observed when the *dSETDB1* and *Pof* mutants were crossed with the transition zone reporter lines (1st and 2nd rows) but no effect was observed when the same mutants were crossed with a reporter line inserted at the HTT array (3rd row).

dSETDB1 and POF are required for *HeT-A* repression

To further investigate the role of dSETDB1 and POF controlling the expression from the telomeric domain we analyzed the *HeT-A* mRNA levels

in these mutants by quantitative real-time PCR. Since different *Su(var)2-5* mutant alleles were already described to have an effect in *HeT-A* expression and the recruitment of HP1 is known to be driven by H3K9 methylation (Savitsky et al., 2002; Perrini et al., 2004; Tzeng et al., 2007), we decided to include this mutant in our analyses. In *Drosophila,* the number of *HeT-A* copies varies among stocks; therefore we determined the number of *HeT-A* copies for each of the analyzed stocks. For each mutant the levels of mRNA expression were normalized to the respective number of copies.

To determine the level of *HeT-A* transcription we extracted mRNA from whole third instar larvae since the larval brains and discs are actively dividing at this stage and thus *HeT-A* expression is active (George and Pardue, 2003). A significant increase in *HeT-A* transcription was observed for egg^{1473} when compared to the control strain (w^{1118}) (Figure 2B and C). No differences in *HeT-A* copy number were observed between control and *dSETDB1* mutant flies (Figure 2A). Similarly, we analyzed the Pof^{D31A} hypomorph allele and an increase in *HeT-A* transcription was observed (Figure 2B and C). Moreover, the number of *HeT-A* copies was slightly increased in these mutants indicating longer telomeres in this stock (Figure 2A). Our results reveal that both dSETDB1 and POF are involved in the control of telomere retrotransposon expression in *Drosophila*.

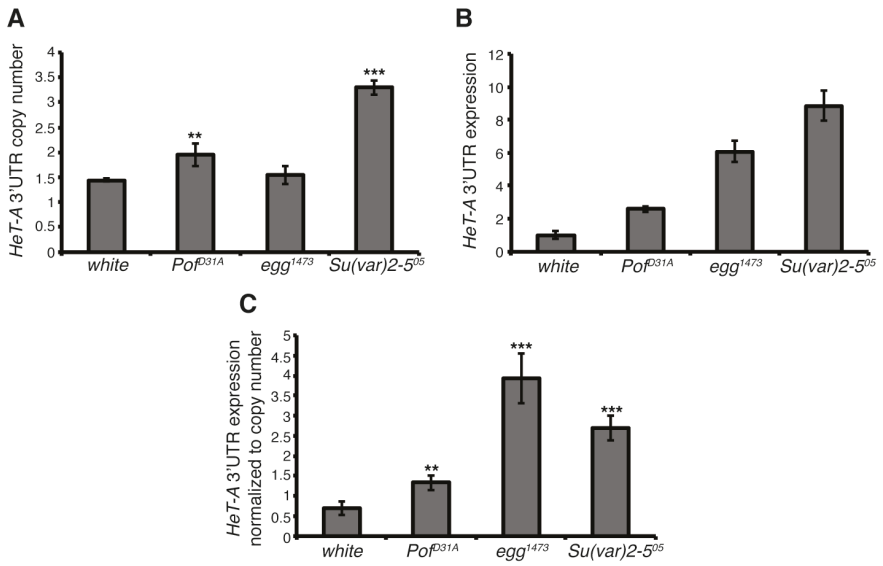

Figure 2: *HeT-A* expression and copy number in *dSETDB1*, *Pof* and *Su(var)2-5^{05}* mutants. (A) *Pof* (*Pof^{D31A}*) and *Su(var)2-5^{05}* mutants have more *HeT-A* copies than control flies, while in *dSETDB1* (*egg^{1473}*) mutants no difference is observed. (B) and (C) *HeT-A* transcripts increase in *dSETDB1*, *Pof* and *Su(var)2-5^{05}* mutants. (C) *HeT-A* transcript levels were normalized to actin transcripts and to the respective number of *HeT-A* copies. Error bars represent standard deviations of three independent experiments. Asterisks indicate statistically significant differences (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 white.

dSETDB1 and POF are not involved in telomere stability

Drosophila telomeres are capped by a complex of proteins that assembles at the end of the chromosomes and protects the chromosome of being recognized as a double strand break (Raffa et al., 2011; Silva-Sousa et al., 2012). HP1 is one of the capping proteins and its depletion causes a high percentage of telomere fusions (Perrini et al., 2004). To test whether dSETDB1 and POF were involved in the telomere capping stabilization or in the loading of HP1 in the 4th chromosome telomeres, we did neuroblast preparations of third instar larvae and analyzed their metaphase chromosomes. No effect on telomere stability was observed in mutant alleles

of *dSETD1* (*egg*^{1473}) or *Pof* (*Pof*^{D31A}) (Figure 3). These results suggest that neither dSETDB1 nor POF are involved in telomere capping in *Drosophila*.

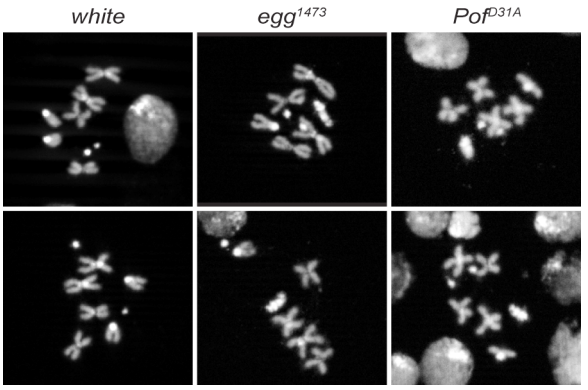

Figure 3: dSETDB1 and POF do not prevent telomere fusions. Third instar larvae neuroblast metaphases of *white* (*w*^{1118}) (1st column), *egg*^{1473} (2nd column) and *Pof*^{D31A} (3rd column). No telomere fusions are observed.

DISCUSSION

Role of dSETDB1 and POF at the 4th chromosome telomeres

In the telomeres of the 4th chromosome, dSETDB1 is responsible for the methylation of H3K9 and recruitment of HP1 to the HTT array, leading to the silencing of the telomeric retrotransposons. Moreover, dSETDB1 and POF binding and/or stabilization at the 4th chromosome is interdependent. When dSETDB1 is removed the levels of H3K9me3 decrease and HP1 is not efficiently recruited to the HTT array, leading to a relaxation of the heterochromatin at the promoters of the transposable elements and consequently an increase in transcription, a rule that applies to all telomeres (Figure 2C and (Gou et al., 2010)). At the moment we are unable to describe the role of POF in the regulation of the telomeric retrotransposons from the 4th chromosome. It could be that POF is able to bind to the 4th chromosome telomere even in the absence of dSETDB1, this way the increase in transcription observed in *dSETDB1* mutants is not only due to the reduction of H3K9me3 levels but also due to the effect of POF as transcription

activator. On the other hand, the depletion of dSETDB1 can lead to the destabilization of POF binding and the increase in transcription observed is only due to the reduction of heterochromatin marks. To address this question it would be necessary to investigate the levels of *HeT-A* transcription in a *dSETDB1*/*Pof* double mutant. If the levels of *HeT-A* transcription were similar in a *dSETDB1* single mutant than in a *dSETDB1*/*Pof* double mutant, POF would not be responsible for the increase in transcription. However, if the levels of *HeT-A* transcription decrease in a double mutant compared to a *dSETDB1* single mutant, POF would be responsible for the increase in *HeT-A* transcription together with the decrease of H3K9me3 and HP1 binding.

Because POF was described as a 4th chromosome transcription activator, we were expecting to observe a decrease in *HeT-A* expression in *Pof* mutants. Interestingly, *Pof* mutants show an increase in *HeT-A* transcription. These findings suggest that dSETDB1 binding to the 4th chromosome telomeres might be in part dependent of POF. Therefore, in *Pof* mutants dSETDB1 binding to the 4th chromosome telomeres is less efficient and the amount of H3K9me3 decreases, leading to a decrease in the binding of HP1 and a relaxation of the chromatin is produced as a consequence. A good strategy to address this question would be to preform ChIP experiments to quantify the amount of dSETDB1 and HP1 bound to the *HeT-A* promoter in a *Pof* mutant background.

dSETDB1 **and** ***Pof*** **mutations do not affect the stability of telomere capping**

HP1 is a protein involved in telomere protection in *Drosophila* and its depletion leads to telomere fusions (Perrini et al., 2004). Since the recruitment of HP1 to the HTT array is dependent on H3K9me3 by dSETDB1 and to the 4th chromosome is dependent of dSETDB1 and POF (Johansson et al., 2007a; Tzeng et al., 2007) we decided to investigate if *dSETDB1* and *Pof* mutations also lead to telomere fusions. Metaphase chromosome

preparations of *dSETDB1* and *Pof* mutants show that these proteins are not involved in maintaining the stability of *Drosophila* telomeres (Figure 3). Moreover, the fact that the number of copies of the *HeT-A* retrotransposon does not increase in *dSETDB1* and *Pof* mutants as it does in *Su(var)2-5* mutant alleles (Figure 2A) already pointed to a normal telomere protection and not the increased telomere accessibility shown by *Su(var)2-5* mutants.

General role of dSETDB1 at *Drosophila* telomeres

The role of dSETDB1 at *Drosophila* telomeres is not exclusive of the 4th chromosome. There are evidences that dSETDB1 controls the silencing of the telomeric retrotransposons in general, including the *HeT-A* telomeric retrotransposon (Phalke et al., 2009; Gou et al., 2010). dSETDB1 controls the transposition of the telomeric transposons by two interdependent pathways: 1) dSETDB1 is responsible for the methylation of the H3K9 at the promoters of the HTT array. The presence of H3K9me3 leads to the recruitment of HP1 and results in the compaction of the chromatin and consequently telomere silencing (Gou et al., 2010). 2) dSETDB1 directly interacts with the Dnmt2 methyltransferase leading to DNA methylation and additionally silencing of the telomeric retrotransposons (Phalke et al., 2009). Like *dSETDB1* mutants, *Dnmt2* mutants also lead to an increase in the expression of the telomeric retrotransposon *HeT-A*. Moreover, mutants of both proteins, as well as double mutants, lead to the loss of DNA methylation at *HeT-A* sequences. These findings suggest that dSETDB1 mediates DNA methylation (by Dnmt2) leading to the silencing of *HeT-A* (Phalke et al., 2009; Gou et al., 2010).

In conclusion, the histone methyltransferase dSETDB1 is responsible for the silencing of the telomeric retrotransposons through the interaction with Dnmt2, HP1 and POF, being the last one exclusive of the 4th chromosome.

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Discussion

Discussion

Telomeres are essential for protecting the genome from losing important genetic information and instability. The study of the epigenetic characteristics of telomeres in several organisms has recently demonstrated how much the telomere regulation is driven by the chromatin environment at the telomeric and subtelomeric domains (Schoeftner and Blasco, 2009). *Drosophila* telomeres are no exception and, despite their very different DNA composition, they share some epigenetic layers of regulation with other eukaryote telomeres. However, *Drosophila* telomere regulation reaches one more step in complexity because the genes involved in the maintenance of telomere length are embedded in the telomeric chromatin, making necessary the action of chromatin remodeling complexes in order to release and re-establish the appropriate chromatin modifications. The release of gene repression is necessary when the telomeres need to be elongated. However, the re-establishment of the repressive state should be tightly controlled because the telomeric retrotransposons maintain their personality as transposable elements and their uncontrolled expression could result in excessive transposition and, as a consequence, abnormal telomere elongation and genomic instability.

The presence of a highly compacted chromatin structure at the telomeres was discovered several years ago because of the TPE (telomere position effect) phenomena to which transgenes inserted close to the telomeres were subjected (Sandell and Zakian, 1992) (see Introduction, box 5). The study of TPE revealed that different heterochromatic proteins, nucleosome remodeling complexes and histone modifying enzymes were involved in the regulation of the telomeric chromatin (Ottaviani et al., 2008). Currently it is known that the

telomere function is fine-tuned by modifications on the telomeric chromatin (Blasco, 2007). Epigenetic regulation of telomeres refers to a dynamic process that involves a complex network of DNA modifications, nucleosome remodeling and histone modifications, together with the establishment of an equilibrium of the presence/absence of different heterochromatic proteins at a given developmental stage, tissue or time frame.

1. Positive regulation of *Drosophila* telomeres

In *Drosophila*, the enzymes responsible for the release of gene silencing or the establishment of activation marks at the HTT array are still poorly characterized. Nevertheless, the regulation of *Drosophila* telomeres should contemplate activation of the telomeric retrotransposons because the expression of these elements is vital to maintain telomere length through end transposition. Therefore, the fact that gene activation marks like H3K4me3 and JIL-1 are present at the HTT array of *Drosophila* telomeres is not surprising (Andreyeva et al., 2005).

To date, the enzyme responsible for the trimethylation of H3K4 at *Drosophila* telomeres remains unidentified. In *Drosophila* there are three COMPASS-like complexes responsible for the methylation of histone H3K4: dSet1, Trythorax (Trx) and Trythorax-related (Trr) (Mohan et al., 2011). TPE analysis of *Trx* mutants showed that Trx is not involved in the regulation of genes inserted in the HTT array (Results, Chapter 1 (supplementary table 1) and (Boivin et al., 2003)). The fact that dSet1 was recently identified as the enzyme responsible for the majority of H3K4me3 (Ardehali et al., 2011; Mohan et al., 2011; Hallson et al., 2012) makes it a good candidate for the methylation of H3K4 at *Drosophila* telomeres. dSet1 trimethylates H3K4 at promoter-proximal nucleosomes, leading to chromatin changes. These chromatin changes positively modulate the release of Pol II and produce gene translation, contributing in this way to optimal mRNA levels (Ardehali et al., 2011). Mutations in the *dSet1* gene lead to a reduction of the transcription levels and

accumulation of Pol II at gene promoters (Ardehali et al., 2011). Moreover, Set1 was described to be involved in the regulation of the H3K4me3 levels in yeast telomeres (Schoeftner and Blasco, 2009). These findings strongly suggest an involvement of dSet1 in the regulation of the telomeric retrotransposons expression. It is possible that, whenever the telomeres need to be elongated, dSet1 methylates H3K4 at the promoters of the telomeric retrotransposons, leading to the release of Pol II. At this point, the JIL-1 kinase might bind to the telomeric retrotransposons and phosphorylate H3 at serine 10, reinforcing the active chromatin state and leading to their expression (Ivaldi et al., 2007; Cai et al., 2008). Future experiments using *dSet1* mutants should be performed in order to characterize the role of the dSet1 protein methylating H3K4 at *Drosophila* telomeres. The levels of JIL-1 at *HeT-A* promoters in *dSet1* mutants should also be investigated. We believe that in addition to JIL-1 and dSet1 other chromatin factors are involved in the regulation of the telomere chromatin in *Drosophila* (*see below*).

The role of the JIL-1 kinase activating the telomeric retrotransposons expression is double. On one hand, JIL-1 acts by reinforcing the open chromatin state by phosphorylating H3S10 and, on the other hand, JIL-1 avoids the heterochromatinization of the HTT array by counteracting the spreading of heterochromatin marks, such as H3K9me3 and HP1a (Zhang et al., 2006; Wang et al., 2011a; 2011b). We have demonstrated that JIL-1 is essential to achieve wild type levels of *HeT-A* transcription, being the first positive regulator of telomere transcription described in *Drosophila* (Results, Chapter 2). These results are in agreement with epigenetic changes at the *HeT-A* promoter that reveal an open chromatin state. In a *JIL-1* mutant background, the presence of the heterochromatin marks HeK9me3 and HP1 is increased, indicating that the JIL-1 presence is necessary to avoid excessive heterochromatinization at the *HeT-A* promoter. Finally, we found that the presence of JIL-1 at the telomeres is essential to avoid the spreading of the heterochromatic marks from the subtelomeric TAS domain (Results,

Chapter 1). Our results, together with previous observations (Zhang et al., 2006; Wang et al., 2011b), suggest that JIL-1 exerts a barrier function that in the case of the telomeres is concentrated at the HTT-TAS boundary.

A recent study shows how the genome-wide distribution of JIL-1 correlates with the deposition of the euchromatic marks H3K16Ac and H3K36me3, suggesting that the recruitment of JIL-1 might occur downstream of these two histone marks (Regnard et al., 2011). It is thus likely that these two histone modifications are involved in the activation of the transcription of the telomere retrotransposons. Furthermore, other chromatin marks that might activate the expression of the telomeric retrotransposons are the acetylation of histone H3K12 and the methylation of the histones H3K79 and H4K20. Nevertheless, the presence of H4K12Ac was not found at the HTT array (Andreyeva et al., 2005) and there are evidences that the depletion of SU(VAR)2-4, the methylase of H4K20, does not affect the expression of genes inserted in the HTT array (Phalke et al., 2009). In summary, some of the histone modifications important for telomere expression in *Drosophila* will be common to other telomeres or transposable elements, while others will be specific of these particular retrotransposon telomeres. Therefore, a wider and more detailed picture on the specific histone modifications that drive the activation of *HeT-A*, *TART* and *TAHRE*, should be drawn in the future.

2. Balance between euchromatin and heterochromatin at *Drosophila* telomeres

Based on our results, we proposed a model where JIL-1 functions at the telomeres guaranteeing a certain level of *HeT-A* expression. In this model, another chromosomal protein, Z4, is needed to set an equilibrium between the activation effect of JIL-1 and the repressive effect of HP1a (Results, Chapter 2). Z4 has been described to be involved in the maintenance of chromosome structure by establishing the proper boundaries to euchromatic domains (Eggert et al., 2004). Moreover, Z4 and JIL-1 have been proposed

to act coordinately in chromosome structure preserving the heterochromatin-euchromatin boundaries (Gan et al., 2011). We have shown that *Z4* mutations specifically affect the expression of genes inserted in the HTT array but not in the TAS domain (Results, Chapter 1) and that the presence of H3K4me3 and JIL-1 at *HeT-A* promoter increases in some *Z4* mutant alleles, suggesting that JIL-1 acts upstream Z4 (Chapter 2, Results section). In fact, we have been able to detect a biochemical interaction between JIL-1 and Z4, demonstrating that these two proteins can be components of the same protein complex. 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 (Andreyeva et al., 2005; Gan et al., 2011). All these observations are in agreement with the role of Z4 maintaining the boundaries between the activation effect of JIL-1 and the repression effect of HP1a. Finally, we have found that Z4 directly binds the Gag protein of the *HeT-A* element. The Z4-HeT-AGag interaction offers a specific mechanism for recruiting the Z4-JIL-1 complex at the HTT array. In summary, these results point towards a coordinated action of Z4, JIL-1 and HP1 in the regulation of the chromatin structure at the HTT array.

Equally important, Z4 is necessary to guarantee telomere stability, probably by maintaining the appropriate chromatin characteristics necessary for the loading of the capping components, like HP1a (Results, Chapter 2). ChIP analyses of *Z4* mutations ($Z4^{7.1}$ allele) show a decrease in the level of H3K9me3 and HP1a at the *HeT-A* promoter. The loss of H3K9me3, which serves as target for HP1a and helps to its spreading from the HTT array towards the cap domain, in these mutants could result in telomere instability.

3 Negative regulation of *Drosophila* telomeres

3.1 Telomere silencing by chromatin remodeling complexes

In addition to its role establishing euchromatin boundaries, Z4 has been described as part of several protein complexes with different cellular roles.

Among them there are the transcription activator DREF/TRF2 complex (Hochheimer et al., 2002; Kugler and Nagel, 2007), the NURF remodeling complex (Kugler and Nagel, 2010; Kugler et al., 2011) and the KEN/NURF complex, involved in the repression of the JAK/STAT pathway (Kugler et al., 2011). We identified two DREF binding sites in the *TART* promoters (sense and antisense), one of them highly conserved among all *TART* subfamilies and the other one conserved in the *TART A* subfamily. After analyzing the effect of *Dref* and *Trf2* mutations on the expression of the telomeric retrotransposons *HeT-A* and *TART*, we found that this complex is involved in the repression of the telomere retrotransposons (Results, Chapter 3). From our results and the published literature we hypothesized that the role of the DREF/TRF2 complex at *Drosophila* telomeres could involve two different strategies. First, based on the fact that BEAF competes with DREF for the DNA binding sequence, it is possible that DREF and BEAF compete for binding at *TART* promoter. When cells are actively dividing and telomeres need to be elongated BEAF binds to *TART* promoter acting as a boundary element and avoiding the spreading of the surrounding heterochromatin. In this situation the methylation of H3K9 by dSETDB1 is limited and kept outside the HTT array. Additionally, the presence or absence of TRF2 also helps the modulation of the telomeric chromatin. When no telomere elongation is needed, DREF binds to the *TART* promoter recruiting TRF2 and the NURF complex. The presence of TRF2 and the ISWI subunit of the NURF complex will facilitate the assembly of the linker histone H1 leading to a compaction of the chromatin. Interestingly, Elisenda López-Panadès found the protein TRAL (Trailer Hitch) in protein complexes pulled down with TART RT and HeT-A Gag proteins (see Annex 1). TRAL is a protein mainly associated with RNA processing found to be a component of a RNA-protein complex required for efficient membrane trafficking (Wilhelm et al., 2005). Moreover, this protein was also identified as a member of the DREF/TRF2 complex (p67 in (Hochheimer et al., 2002)) and to be essential for the transposon silencing through the piRNA pathway (Liu et al., 2011). All

together, these findings further support the idea that the DREF/TRF2 complex is important for the regulation of *Drosophila* telomeres, opening new lines of research to better characterize the chromatin regulation of *Drosophila* telomeres. Finally, we found KEN binding sites in *HeT-A*, *TART* and *TAHRE*, suggesting that KEN will provide another layer of chromatin repression by recruiting the NURF complex to the HTT array.

A recent study showed that the loss of the deacetylase Rpd3 produces changes in the telomeric chromatin of polytene chromosomes, like the loss of deacetylation of histone H4, increase in histone H3 acetylation and increase in H3K9me3 presence (Burgio et al., 2011). These changes are enough to produce telomere fusions in polytene chromosomes without increase in telomere transcription, giving evidences on the necessity of certain epigenetic marks for a correct capping assembly. Moreover, *Rpd3* mutants show a substantial increase in the presence of HP2 at the telomeres. HP2 is a heterochromatic protein related with chromosome structure and gene silencing (Shaffer et al., 2006). Several HP2 partners were described; among them are HP1a, the NURF remodeling complex and the nucleosome chaperone NAP-1 (Stephens et al., 2005; 2006). *Nap-1* mutations act as suppressor of PEV, suggesting that NAP-1 functions to help the assembly of closed chromatin structure, as HP2 does (Stephens et al., 2006). It was suggested that HP2 may cooperate with NURF and NAP-1 in chromatin remodeling (Stephens et al., 2006). Moreover, NAP-1 was shown to form a complex with Rpd3 and Lid (H3K4 demethylase), therefore coupling deacetylation with demethylation (Moshkin et al., 2009). In this context, NAP-1 facilitates H3K4 demethylation activity by LID and H3 deacetylation by Rpd3 and *Nap-1* depletion causes a dramatic loss of histones at regulatory elements (Moshkin et al., 2009). Interestingly, we have isolated NAP-1 as part of a protein complex with HeT-A Gag (see Annex 1) and experiments evaluating the possible telomere phenotypes in *Nap-1* mutants are ongoing by Elisenda López-Panadès as part of her PhD thesis.

Accordingly, our results together with the literature concerning Z4 suggest that the presence of the NURF complex is needed to regulate the expression of the telomeric retrotransposons (Kugler and Nagel, 2007; 2010). Because the NURF complex is a common component of both protein complexes, the one interacting with Z4 and the other with NAP-1, we suggest that NURF could act as a coordinator of these two complexes, which would have been independently recruited to the HTT by their interaction with the HeT-A Gag protein (Results, Chapter 2 and Annex 1). This scenario points towards a coordinated activity of several protein complexes to regulate the telomeric chromatin in *Drosophila*, the telomere targeting of these complexes would be directed by the interaction with HeT-A Gag (Z4-HeT-A Gag and Nap-1-HeT-A Gag).

Additionally, NAP-1 was also described to be in the same protein complex as PROD (Moshkin et al., 2009). PROD was described to bind at the 3'UTR of the *HeT-A* element near the poly(A) tail (Török et al., 2006). Mutations in the *prod* gene lead to increased levels of *HeT-A* mRNA in ovaries, suggesting that PROD functions as a repressor of *HeT-A* transcription. The fact that PROD interacts with NAP-1 and NAP-1 interacts with HeT-A GAG strongly suggests that PROD is targeted to the telomeres as part of a complex with HeT-A Gag and NAP-1. Adding all these results, we suggest that NAP-1 could be involved in the repression of the telomeric retrotransposons in at least two different pathways: First, by targeting the NURF complex to the HTT array and leading to chromatin remodeling. As a nonexclusive hypothesis, the targeting of NURF to the HTT array could also be mediated by Z4 (see above). The fact that NAP-1 and Z4 might be involved in the recruitment of the NURF complex to the telomeres suggests cooperation between both proteins. Second, NAP-1 is probably involved in the recruitment of Rpd3, HP2, LID and PROD to the telomeres. Rpd3, HP2 and LID will reinforce the histone modifications at the promoters of the telomeric retrotransposons leading to their repression.

3.2 Telomere silencing by DNA and histone modifications

In yeast and vertebrates, the telomeres are methylated at the subtelomeric repeats. Hypomethylation of the subtelomeric repeats results in an increase in the recombination rate with fatal consequences for genome stability (Schoeftner and Blasco, 2009). DNA methylation is also present in *Drosophila* and *Arabidopsis*, in these species is the telomeric domain and not the TAS sequences that are subject to DNA methylation (Schoeftner and Blasco, 2009; Phalke et al., 2009; Vrbsky et al., 2010; Gou et al., 2010).

In *Drosophila*, the enzyme responsible for telomeric DNA methylation is Dnmt2 (Phalke et al., 2009; Gou et al., 2010). Mutations in the *Dnmt2* gene lead to an increase in *HeT-A* expression, with no increase in telomere length nor problems with telomere stability (Phalke et al., 2009; Gou et al., 2010). The DNA methylation at *Drosophila* telomeres is dependent of dSETDB1 (Gou et al., 2010). dSETDB1 is probably responsible for the recruitment of Dnmt2 to the telomeres since in *dSETDB1* mutants no DNA methylation is observed at the telomeres (Gou et al., 2010). However, the fact that the disruption of the methylation at the telomeric repeats does not result in a telomere phenotype suggests that other layers of telomere regulation exist.

In addition to its role mediating telomeric DNA methylation, dSETDB1 directly regulates the expression of the telomeric retrotransposons through the methylation of H3K9. The increase in di- and tri-methylation of H3K9 by dSETDB1, leads to HP1a recruitment. The presence of HP1a will recruit more dSETDB1 molecules, coordinately increasing the levels of H3K9me3 available and HP1a as a consequence. This process will create a positive feedback that will lead to the formation of a repressed chromatin state and silencing of the telomeric retrotransposons.

Finally, the dSETDB1/POF/HP1 complex seems to have a role controlling the expression of the telomeric retrotransposons from the 4^{th} chromosome. In this situation, the binding of dSETDB1 to the 4^{th} chromosome telomeres seems to be dependent of POF (Results, Chapter 4). These findings suggest

that the telomeres of the 4th chromosome have an additional layer of regulation when compared to the other chromosomes.

4. Model for chromatin regulation of *Drosophila* telomeres

In order to integrate all the information described above we have drawn a schematic representation of the possible chromatin marks present HTT array (Figure 1) and a protein network of all the proteins mentioned during this discussion (Figure 2).

Whenever the telomeric retrotransposons need to be elongated, the presence of gene activation marks at HTT promoters is increased. It is very likely that dSet1 methylates H3K4 leading to a release of Pol II. At this point, JIL-1 will be recruited and phosphorylate H3S10, reinforcing this way the active chromatin state. The presence of BEAF at *TART* promoters will avoid the spreading of the silent mark H3K9me3. Additionally, we hypothesize that the telomeric chromatin could also be enriched in the gene expression marks H3K16Ac and H3K76me3 (Figure 1 and 2).

On the other hand, when telomere elongation is not necessary and the telomeric retrotransposons need to be silenced, a heterochromatic environment is defined at the HTT array. In this situation, the presence of SETDB1 will lead to an increase in H3K9me3 and HP1 recruitment. Additionally, SETDB1 will also lead to Dnmt2 recruitment and the consequent DNA methylation. At the same time HeT-A Gag will target Z4 and NAP-1 to the telomeric sequences. Z4 and NAP-1 will recruit the DREF/TRF2, KEN, and NURF complexes leading to major chromatin remodeling. Finally, NAP-1 will also recruit PROD, LID and Rpd3, which will add another layer of silencing marks (Figure 1 and 2).

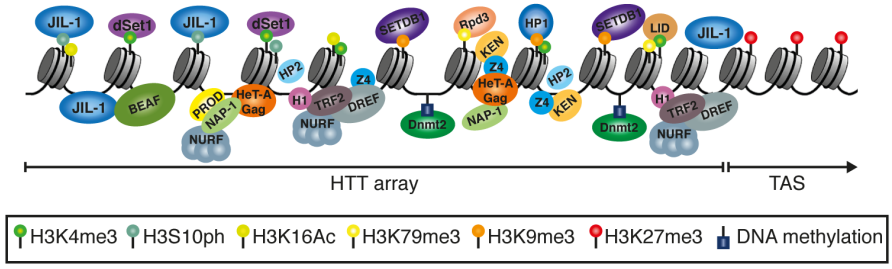

Figure 1: Model of the chromatin marks present at the HTT array. In *Drosophila*, the telomeric domain needs to be tightly regulated because the genes involved in the maintenance of telomere length are embedded in the telomeric chromatin. Therefore, the coordinated action of chromatin remodeling complexes, histone modification enzymes and chromatic proteins is necessary in order to release and re-establish the appropriate chromatin modifications. For a detailed description see the text.

In summary, the results obtained during this thesis help to further characterize the chromatin environment at the HTT array and TAS domains, leading to a better understanding of the chromatin changes necessary for the silencing and the activation of the expression of the telomeric retrotransposons. Additionally, we have found new telomeric components involved in guaranteeing telomere stability and obtained evidence of the specific targeting mechanism of different regulatory complexes to the telomeres and their possible interplay. We have demonstrated that different protein complexes, which act coordinately and are specifically targeted at the HTT array, contribute to the multilayer regulation of *Drosophila* telomeres. Finally, we have also found evidences that the 4R subtelomere is composed of a slightly different chromatin, which could differently affect the HTT array in this chromosome. In the end, my thesis has opened new lines of research that will help to complete and detail even more the description of the chromatin at *Drosophila* telomeres, the first retrotransposon telomeres to be studied in detail.

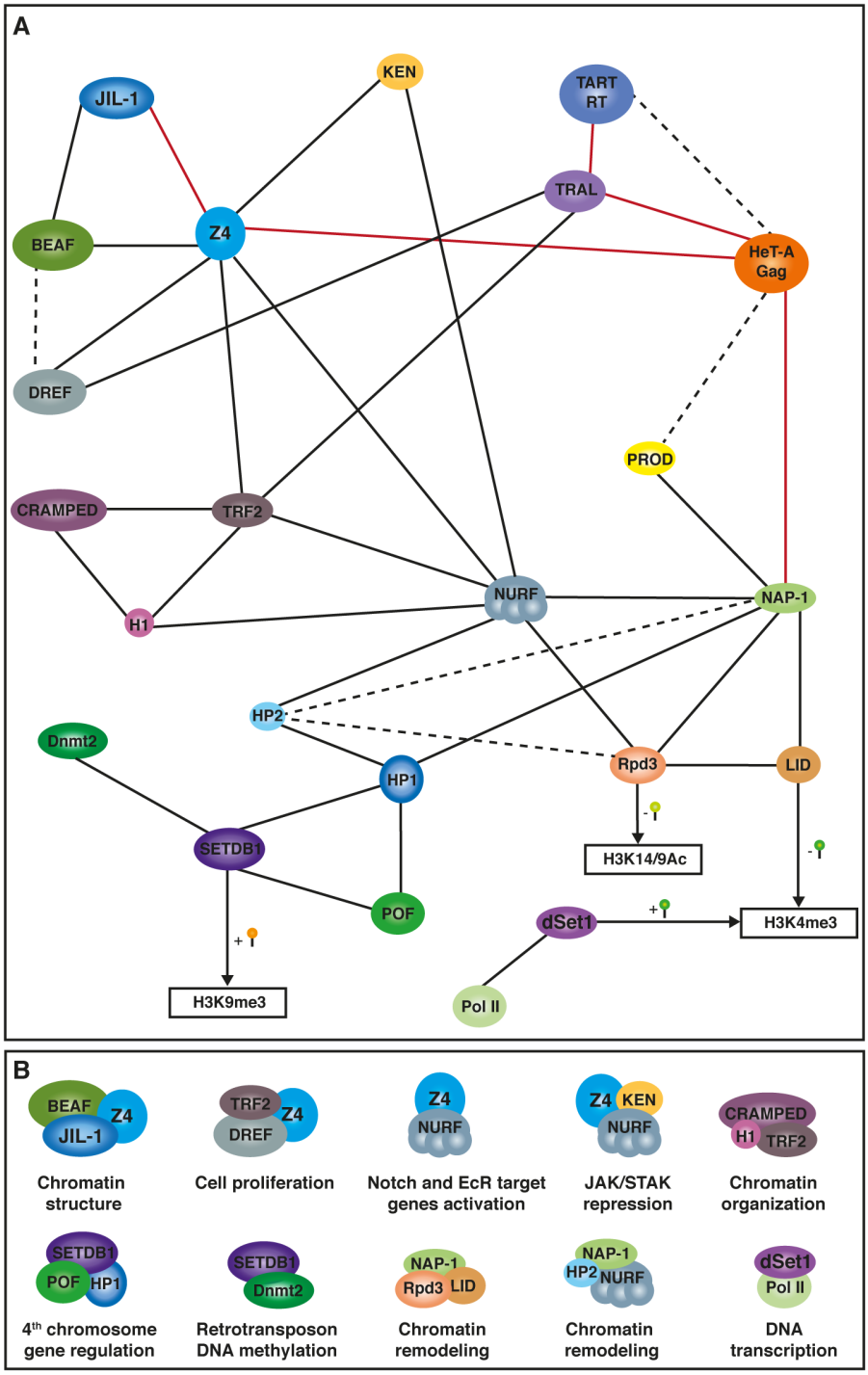

Figure 2: Protein interaction network. (A) Schematic representation of the protein interactions described to date. Red lines represent the interactions found in our laboratory; black lines represent protein interactions and dashed lines represent other

type of interactions. (B) Schematic representation of the different protein complexes and their cellular roles.

Conclusions

Conclusions

From the results obtained during this thesis, the following can be concluded:

1. The JIL-1 kinase is necessary for the expression of genes embedded in the telomeric domain. The lack of JIL-1 leads to the spreading of the heterochromatin from the TAS domain towards the HTT array, indicating that JIL-1 exerts a barrier function at the HTT-TAS boundary.
2. The chromatin at the subtelomere domain of the 4th chromosome is less compacted and more permissive to gene expression than the TAS domain of other chromosomes, and even than the HTT array.
3. The JIL-1 kinase is important to achieve wild type levels of *HeT-A* transcription, being the first positive regulator of telomere expression characterized in *Drosophila*.
4. JIL-1 and Z4 biochemically interact suggesting that both proteins can be part of the same protein complex.
5. Z4 does not directly participate in the capping of the telomeres, however its presence is necessary to guarantee telomere stability. Z4 depletion leads to major chromatin changes at the HTT array that will result in a secondary loss of necessary chromatin and capping components, like HP1a.
6. JIL-1, Z4 and HP1a are involved in fine-tuning the chromatin structure at the HTT array. The interplay of at least these three chromosomal proteins is fundamental to obtain the correct chromatin environment at the HTT array. The disturbance of any of these proteins leads to

changes in the telomeric chromatin that will result in the loss of the telomere homeostasis and/or instability.

7. Z4 specifically interacts with HeT-A Gag and both show the same telomere instability phenotype. The further interaction of JIL-1-Z4 strongly suggests that this complex is targeted to the telomeres through the interaction of Z4 with HeT-A Gag.

8. The binding sites of the Z4 partners DREF/TRF2 and KEN are present at the telomeric retrotransposons. DREF, TRF2 and KEN are involved in the repression of the telomeric retrotransposons by still uncharacterized mechanisms.

9. dSETDB1 is the enzyme responsible for the methylation of histone H3K9 and consequent HP1 recruitment at *Drosophila* telomeres. In the 4th chromosome, POF provides another layer of telomere regulation together with dSETDB1 and HP1.

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Annexes

ANNEX 1

Identification of new telomere components

INTRODUCTION

Although the telomere retrotransposons were discovered almost two decades ago, it is still unknown if they require cellular components in order to perform their telomeric role and complete their life cycle. It was therefore interesting to isolate possible cellular partners involved in the targeting of the telomere retrotransposons. To address this question we decided to purify possible protein complexes that might be interacting with the telomeric proteins (proteins produced by the telomeric retrotransposons, *HeT-A* and *TART*). Because the expression and the protein production of the telomere retrotransposons are low in most tissues and developmental stages, we decided to use the TAP-tag system to purify the protein complexes. With this system is possible to obtain stable transfected S2 cells by selection with Hygromicin B. Moreover, the TAP vector contains a cooper inducible metallothionein promoter that allows expression of the fusion proteins by induction, avoiding in this way toxicity problems. In the TAP vector the protein of interest is fused to the TAP tag at its N-terminal or C-terminal end, allowing the performance of a sequential and high affinity purification protocol that enhances the chance to purify specific interactions (Veraksa et al., 2005).

MATERIALS AND METHODS

Cloning vectors for inducible expression of TAP-tagged proteins

HeT-A Gag, *TART RT* and *TART Pol* were amplified from *Drosophila* genomic DNA using primers that included *Bam*HI restriction enzyme sites. The amplified sequences were cloned in a TOPO® vector (Invitrogen). The sequences were cut from TOPO with *Bam*HI and ligated in the pMK33-CTAP and pMK33-NTAP vectors (Veraksa et al., 2005) previously cut with the same restriction enzyme. The orientation of the inserts was checked with suitable restriction enzymes. Primers used to clone HeT-AGag-CTAP: HeT-AGag_BamHI_F GGATCCATGTCCATGTCCGACAACCT and HeT-AGag_BamHI_R GGATCCTCTATTAGCTAAGCTTGTTG. HeT-AGag-NTAP: HeT-AGag_BamHI_F GGATCCATGTCCATGTCCGACAACCT and HeT-AGag_BamHI_R GGATCCTCTATTAGCTAAGCTTGTTG. TARTPol-CTAP: TARTPol_BamHI_F GGATCCATGACTCGGGCGTGCAACAG and TARTPol_BamHI_R(nonstop) GGATCCGTCACCTTCGTAGTCGGGTAG. TARTPol-NTAP: TARTPol_BamHI_F GGATCCATGACTCGGGCGTGCAACAG and TARTPol_BamHI_R GGATCCTTAGTCACCTTCGTAGTCGG. TARTRT-CTAP: TARTRT_BamHI_F GGATCCATGGACGCCTGGAAACATGCC and TARTPol_BamHI_R(nonstop) GGATCCGTCACCTTCGTAGTCGGGTAG. TARTRT-NTAP: TARTRT_BamHI_F GGATCCATGGACGCCTGGAAACATGCC and TARTPol_BamHI_R GGATCCTTAGTCACCTTCGTAGTCGG.

Generation of stable S2 cell lines

Drosophila S2 cells were grown in Schneider's medium (Sigma) in the presence of 10% fetal calf serum at 25ºC. Cells were transfected with 2μg of pMK33-based constructs (HeT-AGag-CTAP, HeT-AGag-NTAP, TARTPol-CTAP, TARTPol-NTAP, TARTRT-CTAP, TARTRT-NTAP and empty vector CTAP) in 25cm^2 flasks using Effectene transfection reagent (Qiagen). After

three days of incubation, cells were subjected to selection in the presence of 300μg/ml of Hygromycin B (Sigma). The cell medium was changed every week without disturbing the selected cells. Stable cells lines were established after approximately one month.

Verification of construct functionality

The functionality of the TAP-tagged constructs was checked by protein size on Western Blot and cellular localization by immunostaining. To verify the size of the fusion protein, stable cell lines were induced overnight with different concentrations of $CuSO_4$. After 24 hours of incubation, cells were harvested and re-suspended in protein loading buffer. Proteins were separated by SDS-PAGE electrophoresis and blotted to a nitrocellulose membrane. Anti-TAP (OpenBiosystems) was used to develop western blots at a concentration of 1:500.

To verify the cellular localization of the fusion proteins, the induced cells were placed on concavalin-treated coverslips, fixed with 4% paraformaldehyde in PBT for 10 min and washed in PBT. Cells were incubated overnight with the primary antibody (anti-TAP or anti-HeT-A Gag), washed with PTB and incubated for 1 hour with the respective secondary antibody. Finally, cells were washed with PBT and PSB and mounted with mowiol/DAPI medium. Preparations were analyzed on a Zeiss Axio Imager.Z2 fluorescence microscope using the Axio Vision Software.

Tandem Affinity Purification

HeT-AGag-CTAP and TARTRT-CTAP stable lines were induced with a final concentration of 100μM and 200μM $CuSO_4$ respectively, and the control line CTAP was induced with both amounts of $CuSO_4$. Cells were induced for 24 hours at 25ºC. After induction cells were pelleted at 500 X *g*, washed twice with cold PBS and stored at -80ºC until approximately 8 grams of pellet was

collected. The protocol used to purify the protein complexes was performed as described (Veraksa et al., 2005). Some minor modifications were preformed; due to insolubility problems, HeT-AGag-CTAP was extracted using Buffer A (5mM Tris-HCl pH 7.4, 1.5mM KCl, 2.5mM $MgCl_2$, 1% (w/v) deoxycholic acid, 1% (v/v) TritonX-100, 2X Protease Inhibitor Cocktail (Sigma)).

RESULTS

Verification of construct functionality

In order to check the construct functionality we induced HeT-AGag-CTAP stable line with different amounts of $CuSO_4$ and did immunostaining experiments using anti-CTAP and anti-HeT-AGag antibodies (Figure 1). Immunostaining analysis demonstrated that the tagged HeT-A Gag protein was efficiently expressed. Moreover, increasing concentrations of $CuSO_4$ lead to an increase in the expression of the HeT-AGag-CTAP fusion protein.

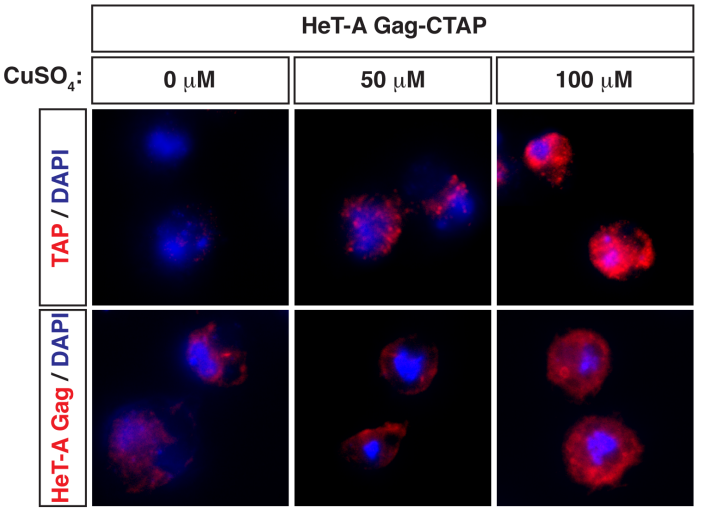

Figure 1: HeT-AGag-CTAP expression. HeT-AGag-CTAP stable line induced with different amounts of $CuSO_4$ indicated on the top. Non-induced cells were used as a control, left column. Nuclear staining in blue (DAPI), TAP (first row) and HeT-A Gag (second row) staining in red.

Additionally, the amount of cooper sulphate ($CuSO_4$) necessary to express the fusion proteins was selected by western blot (Figure 2). All stable lines express the correspondent bait protein with the expected molecular size, a 20kDa shift is observed for all the proteins due to the expression of the TAP tag. For each protein the optimal level of induction was selected: for HeT-AGag-CTAP 100μM $CuSO_4$ (final concentration) was selected to induce the cells for purification, TARTRT-CTAP showed optimal induction at 200μM $CuSO_4$ and TART Pol-CTAP at 500μM $CuSO_4$. The control line CTAP (empty vector) was always induced at the level of the bait proteins.

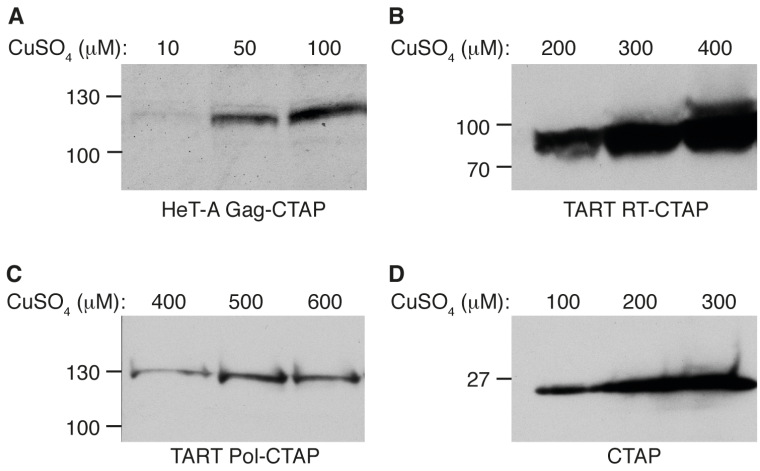

Figure 2: Expression assays of the different cell lines. The different stable cell lines established (A) CTAP, (B) HeT-AGag-CTAP, (C) TARTRT-CTAP and (D) TARTPol-CTAP were induced with different amounts of $CuSO_4$ (indicated on the top panel) and the level of protein expression was analyzed by western blot and developed with anti-TAP. Molecular markers are indicated on the left.

The following experiments of this project were performed by Elisenda López-Panadès as part of her PhD thesis and will not be presented in this annex. Nevertheless, some of the identified proteins in the purified complexes are discussed in the discussion section due to their relevance with the results presented in this thesis.

Silva-Sousa, R., López-Panadès, E., and Casacuberta, E. Drosophila telomeres: an example of co-evolution with transposable elements. Genome Dyn. 2012; 7: 46-67.