The histone deacetylase Rpd3 regulates the heterochromatin structure of Drosophila telomeres

Giosalba Burgio1,2, Francesca Cipressa3, Antonia Maria Rita Ingrassia1,2, Giovanni Cenci3,* and Davide F. V. Corona1,2,*

1Istituto Telethon Dübacco, c/o STEMBO, Viale delle Scienze, Edificio 16, 90128 Palermo, Italy
2Università degli Studi di Palermo–Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari – Sezione di Biologia Cellulare, Viale delle Scienze, Edificio 16, 90128 Palermo, Italy
3Dipartimento di Biologia di Base ed Applicata, Università dell’Aquila, 67100 Coppito, L’Aquila, Italy

*Authors for correspondence (giovanni.cenci@cc.univaq.it; dcorona@unipa.it)

Summary
Telomeres are specialized structures at the end of eukaryotic chromosomes that are required to preserve genome integrity, chromosome stability and nuclear architecture. Telomere maintenance and function are established epigenetically in several eukaryotes. However, the exact chromatin enzymatic modifications regulating telomere homeostasis are poorly understood. In Drosophila melanogaster, telomere length and stability are maintained through the retrotransposition of specialized telomeric sequences and by the specific loading of protecting capping proteins, respectively. Here, we show that the loss of the essential and evolutionarily conserved histone deacetylase Rpd3, the homolog of mammalian HDAC1, causes aberrant telomeric fusions on polytene chromosome ends. Remarkably, these telomere fusion defects are associated with a marked decrease of histone H4 acetylation, as well as an accumulation of heterochromatic epigenetic marks at telomeres, including histone H3K9 trimethylation and the heterochromatic protein HP2. Our work suggests that Drosophila telomere structure is epigenetically regulated by the histone deacetylase Rpd3.

Key words: Telomere, Rpd3, Heterochromatin, Histone acetylation, Histone H3K9 trimethylation, HP2

Introduction
Eukaryotic linear chromosomes have evolved special nucleoprotein structures at their ends, the telomeres, which counterbalance the incomplete replication of terminal DNA and protect chromosome termini from fusion events (De Lange, 2005; Ferreira et al., 2004). Telomere structure is conserved in most eukaryotes and, with few exceptions, consists of short repetitive double-stranded G-rich sequences that end in a single-stranded overhang. Telomeric DNA is elongated by telomerase, a specialized reverse transcriptase that adds short DNA repeats by reverse-transcribing the template region of its RNA component (Nugent and Lundblad, 1998). Telomere repeats bind sequence-specific factors, which in turn recruit additional telomeric proteins, forming multiprotein complexes that are crucial for chromosome-end homeostasis. A well-documented example of such terminal complexes is the six-protein complex that coats human chromosome ends allowing cells to distinguish telomeres from sites of DNA damage (Palm and de Lange, 2008). Telomeric and subtelomeric DNA from different organisms contain histone modifications that are typical of heterochromatin (Blasco, 2007). Interestingly, recent studies have highlighted some of the roles played by chromatin remodelers and covalent modifiers in the establishment of epigenetic marks essential for telomeres structure and function (Altaf et al., 2007).

The model organism Drosophila melanogaster lacks telomerase activity and fly chromosome ends do not consist of telomerase-generated simple repeats. Drosophila telomeres are elongated by transposition of three different, but related, non-long terminal repeat retrotransposons called Het-A, TART and TAHHRE (Mason et al., 2008). Like their yeast and vertebrate counterparts, fly telomeres are also protected by multiprotein complexes (Cenci et al., 2005; Ciapponi and Cenci, 2008). Interestingly, the elongation of Drosophila telomeres is uncoupled from their capping function, as chromosomes ends devoid of retrotransposons can assemble regular telomeres and can be transmitted for many generations. There is also evidence that terminally deleted Drosophila chromosomes can be properly capped regardless the sequence of terminal DNA (Cenci et al., 2005; Mason et al., 2008). Thus, Drosophila telomere protection relies on sequence-independent epigenetically determined structures.

The remarkable functional similarity between mammalian and fly telomere-protecting mechanisms makes Drosophila an excellent model organism to study telomere biology (Cenci et al., 2005). In order to identify factors responsible for the deposition of epigenetic marks required for the establishment and maintenance of telomere structure and function, we conducted a small biased pilot genetic screen scoring for telomere morphology defects on polytene chromosomes. We found that loss of the essential and evolutionarily conserved histone deacetylase Rpd3, the homolog of mammalian HDAC1, causes aberrant telomeric fusions on polytene chromosome ends. Surprisingly, these Rpd3-knockdown-induced telomere fusion defects are associated with a marked decrease of histone H4 acetylation, as well as an accumulation of heterochromatic epigenetic marks at telomeres, including histone H3K9 trimethylation and the overloading of the heterochromatic protein HP2. Our work suggests that telomere chromatin structure is regulated by the histone deacetylase Rpd3, providing one of the first insights into the epigenetic determination of telomere organization in higher eukaryotes.
Results

Loss of the histone deacetylase Rpd3 causes polytene chromosome telomeric fusions

Polytene chromosomes represent a special structural organization of *Drosophila* salivary glands, consisting of polyploid interphase nuclei, which originate by repeated rounds of DNA replication without cell division. *Drosophila* polytene chromosomes have proven to be an invaluable cytogenetic tool to examine chromosome structure and, at the same time, to rapidly assess the genome-wide distribution of chromatin-binding proteins. Indeed, several studies have revealed that the genome-wide distribution of countless structural and regulatory proteins on polytene chromosomes reflects cellular and interphase chromosome physiology occurring in other developmental stages or tissues (Johansen et al., 1999; Stephens et al., 2004). Therefore, we conducted a biased genetic screen on a small group of genes encoding known chromatin remodelers and members of the histone deacetylase family, by searching for telomere morphology defects on polytene chromosomes. As canonical mutations in these genes are embryonic or early-larval lethal, thereby preventing their role in polytene chromatin structure being addressed, we decided to use salivary-gland-specific RNA interference (RNAi), with a classic GAL4:UAS driving system (Steitz et al., 1998), to knockdown gene expression in the corresponding VDRC (*Vienna Drosophila RNAi* Center) lines. To drive UAS-controlled induction of the RNAi in salivary glands we used *eyeless* (*ey*) promoter-mediated GAL4 expression (*ey*GAL4); in addition to the eye imaginal disks, the *eyeless* promoter has been shown to also efficiently drive the expression of GAL4 in salivary glands (Corona et al., 2007).

RNAi-mediated knockdown for some of the genes analyzed led to modest alterations in polytene chromosome structure (data not shown). However, loss of *Sin3A* or *Rpd3* histone deacetylase function on polytene chromosomes (supplementary material Fig. S1A–F), using multiple VDRC lines with no predicted off-target sites, resulted in the formation of apparently elongated chromosome ends that also underwent frequent telomeric fusions (Fig. 1A–E and supplementary material Fig. S1G–J). Interestingly, eye-specific RNAi-mediated knockdown of *Sin3A* or *Rpd3* caused developmental and differentiation defects in adult eyes (supplementary material Fig. S2A–C) confirming a role for the *Sin3A*–*Rpd3* histone deacetylase complex in cell division and proliferation (Ahringer, 2000). However, silencing of *Sin3A* or *Rpd3* in larval neuroblasts, using a *daGAL4* driver (Corona et al., 2007), did not cause telomeric fusions on mitotic chromosomes (supplementary material Fig. S2D–F). Indeed, previous experiments have suggested that the establishment of telomere function in polytene and mitotic chromosomes are similar, although they probably rely on different mechanisms of maintenance and protection (Cenci et al., 2005; Rashkova et al., 2002), thereby explaining why chromosome-end fusions specifically occur only after RNAi-mediated silencing of *Sin3A* or *Rpd3* on interphase polytene chromosomes.

In order to quantify the chromosome-end fusions in *Sin3A* and *Rpd3* RNAi lines, we developed a scoring system to measure the penetrance of telomeric attachments (supplementary material Fig. S3). On the basis of this scoring system, the observed telomeric fusions upon *Sin3A* and *Rpd3* RNAi were not due to independent mutations present in the driving (*eyGAL4*) or UAS–RNAi (UAS-*Sin3A*-RNAi; UAS-*Rpd3*-RNAi) lines (Fig. 1F), nor are they indirectly caused by the activation of the RNAi machinery in salivary glands because the overexpression of *Dicer2* (*eyGAL4; UAS-Dicer2*) does not cause an increase in telomere fusions when compared with wild-type *Df(1)yw* chromosomes (Fig. 1F) (Dietzl et al., 2007). Our scoring system also revealed that knockdown of the histone deacetylase catalytic subunit encoded by *Rpd3* yielded a higher frequency of telomere fusions when compared with knockdown of *Sin3A* (Fig. 1F). Therefore, to address further the role of the *Sin3A–Rpd3* deacetylase complex in telomere
homeostasis, we decided to focus our telomere fusion characterization only on the \textit{Rpd3}-encoded catalytic subunit.

Abnormal telomere elongation and chromosome-end fusions have been observed in \textit{Drosophila} polytene chromosomes as consequence of mutations in the HP1-encoding \textit{Su(var)205} gene and the Telomere elongation (Tel) gene (Perrini et al., 2004; Siriaco et al., 2002). Telomeres of polytene chromosomes are characterized by the presence of three distinct chromatin domains: the telomere-associated sequences (TASs), the terminal tract of the HTT retrotransposons (HeT-A, TART and TAHRE) and the cap, the most-terminal region that is associated with capping proteins that bind independently of the DNA sequence (Andreyeva et al., 2005; Cenci et al., 2005). In both \textit{Su(var)205} and \textit{Tel} mutants, elongated telomeres result from an increase in \textit{HeT-A} and TART elements in the HTT domain. It has been hypothesized that the presence of many tandemly arranged copies of telomeric retrotransposons on long polytene telomeres can increase the likelihood of recombination between telomeric sequences leading to telomere–telomere attachments (Cenci et al., 2005; Perrini et al., 2004; Siriaco et al., 2002).

To ascertain whether the apparent telomere elongation and fusions upon \textit{Rpd3} RNAi were caused by an increase in the amount of telomeric retrotransposable elements at chromosome ends, we conducted classic fluorescent in situ hybridization (FISH) analysis using \textit{HeT-A}, TART and TAHRE sequences as probes. This analysis revealed that the levels of \textit{HeT-A} (supplementary material Fig. S4A–C), TART (supplementary material Fig. S4D–F) and TAHRE (supplementary material Fig. S4G–I) sequences were very similar in wild-type telomeres and \textit{Rpd3} RNAi fused telomeres. These results were also confirmed by dot-blot analysis conducted on genomic DNA from \textit{Rpd3} RNAi and \textit{Tel} mutant larval salivary glands. As expected, the dot-blot analysis revealed a significant increase of telomeric genomic DNA \textit{HeT-A} sequences in \textit{Tel} mutants but not upon \textit{Rpd3} RNAi, when compared with wild type (Fig. 2A). Furthermore, because \textit{Rpd3} plays a key role in transcription repression (De Rubertis et al., 1996; Pile and Wassarman, 2000; Rundlett et al., 1996), we checked whether \textit{Rpd3} knockdown would affect transcription of telomeric retrotransposons. RT-PCR on RNA samples extracted from salivary glands of wild-type and \textit{Rpd3} RNAi larvae did not show any significant difference in transcription of \textit{HeT-A} sequences (Fig. 2B). Collectively, our data indicate that, unlike for \textit{Tel} and \textit{Su(var)205}, upon \textit{Rpd3} RNAi telomeres are not longer than wild-type, suggesting that the ‘apparently elongated telomeres’ \textit{Rpd3} RNAi phenotype is due to changes in chromatin condensation.

**Loss of \textit{Rpd3} affects histone acetylation at telomeres**

To assess whether the apparent elongation and fusion phenotypes of the telomere tip upon \textit{Rpd3} RNAi were linked to chromatin condensation changes underlying telomeric histone acetylation defects, we performed immunofluorescence analysis on wild-type and \textit{Rpd3} RNAi chromosomes using antibodies directed against acetylated histones H3 and H4 (AcH3 and AcH4; specifically recognizing K9 and K14 histone H3 acetylations and K5, K8, K12 and K16 histone H4 acetylations), which represent the main histone targets of Rpd3 (Kadosh and Struhl, 1998). Interestingly, loss of \textit{Rpd3} caused a substantial increase in the global levels of both acetylated H3 and H4 histones on polytene chromosomes (Fig. 3A–D), without affecting chromatin loading or expression of non-acetylated histones (supplementary material Fig. S5). Furthermore, western blot analysis on chromatin extracts derived from salivary gland cells, confirmed that chromosomes are loaded with higher levels of both acetylated histones H3 and H4 upon \textit{Rpd3} knockdown when compared with that on wild-type chromosomes (Fig. 3E,F).

Remarkably, upon \textit{Rpd3} RNAi high-magnification images of telomeres immunostained for AcH3 and AcH4, revealed that, whereas the levels of histone H3 acetylation staining slightly increased, the levels and extension of histone H4 acetylation were greatly reduced (Fig. 3B, D’. white arrowhead and double-arrowed bar). To confirm these findings we measured the levels of histone acetylation specifically associated with telomeric \textit{HeT-A}. To this aim, we optimized a chromatin immunoprecipitation (ChIP) technique on chromatin isolated from salivary glands using the telomere-associated protein HP2 (Fig. 4A). As expected, using antibodies directed against AcH3 and AcH4 we found a substantial increase in \textit{HeT-A}-associated histone H3 acetylation upon \textit{Rpd3} RNAi with respect to that in wild type (Fig. 3G,H, lanes 2 and 6). By contrast, the histone H4 acetylation associated with \textit{HeT-A} sequences was dramatically reduced (Fig. 3G,H, lanes 3 and 7) when compared with that in wild type. These ChIP data are consistent with our immunostaining results and collectively indicate that \textit{Rpd3} RNAi has different effects on histone acetylation at telomeres.

In male polytene chromosomes histone H4 lysine 16 acetylation (AcH4K16) specifically and exclusively localizes to the X chromosome (Gelbart et al., 2009) and is excluded from the male X telomere tip (Fig. 5A, white arrowhead). In \textit{Rpd3} knockdown polytene chromosomes AcH4K16 is still excluded from X chromosome ends but this loss of localization seems much wider than that for wild-type X chromosome tips (Fig. 5B,C, double-arrowed bar). Interestingly, ectopic AcH4K16 localization starts to appear on some autosomal chromatin loci (Fig. 5B,C, green arrowheads), probably as a consequence of loss of global \textit{Rpd3} histone deacetylase activity. ChIP analysis also confirmed a marked reduction of AcH4K16 on \textit{HeT-A} telomeric sequences upon \textit{Rpd3} RNAi (Fig. 5D,E). Taken together, our analyses on histone acetylation suggest that \textit{Rpd3} knockdown causes a significant increase of global H3 and H4 histone acetylation. However, although histone H3 acetylation levels are higher also within telomeric sequences upon \textit{Rpd3} RNAi, acetylation of histone H4 (including that at K16) appeared substantially and specifically decreased at the level of HTT sequences.
Ppd3 regulates heterochromatin marks on polytene telomeres

To investigate whether the marked decrease in histone H4 acetylation levels associated with telomeric HeT-A upon Ppd3 RNAi affected other telomere-associated epigenetic modifications, we analyzed the distribution of three common heterochromatin histone modifications: trimethylated histone H4K20 (trimeH4K20), trimethylated histone H3K9 (trimeH3K9) and trimethylated histone H3K27 (trimeH3K27). Typically, trimeH4K20 is found in the heterochromatic chromocenter and in numerous euchromatic bands (Fig. 5H) (Ebert et al., 2006), trimeH3K27 is associated with the chromocenter, telomeric TASs and a subset of about ~100 bands (Fig. 5J) (Andreyeva et al., 2005), whereas trimeH3K9 localizes at pericentric heterochromatin, at telomeres and at a number of sites along chromosome arms (Fig. 5F). We found that Ppd3 knockdown decreased the global localization of all three modified histones on polytene chromosome arms (Fig. 5G,I,K) when compared with wild type. However, although the levels of trimeH4K20 and trimeH3K27 were reduced also at telomeres upon Ppd3 RNAi (Fig. 5I,H11032, white arrowheads), the amount of H3K9 trimethylation increased in Ppd3-knockdown telomeres (Fig. 5G,H11032, white arrowhead). As it has been previously shown, trimeH3K9 localizes at the telomeric HTT domain (Andreyeva et al., 2005) and HTT telomeric sequences are not amplified in telomeres upon Ppd3 RNAi (Fig. 2), the observed trimeH3K9 accumulation combined with the reduced trimeH3K27 levels could reflect a change in heterochromatin structure in telomeres upon Ppd3 RNAi.

As we were unable to find ChIP primers specific for TAS sequences (data not shown), we conducted FISH analysis in order to obtain some low-resolution information about global chromatin organization of TAS in both wild-type and telomeres and upon Ppd3 RNAi. The FISH data indicate that, whereas in wild-type strains the TASs showed a wide punctuated localization at telomeres (Fig. 6A, double arrowed bars in 6A/H11032 and 6A/H11033), in Ppd3-knockdown TAS FISH signals on telomeres were slightly more compacted (Fig. 6B, double arrowed bar on the 3L–X chromosome fusion and white bar on the 2L unfused chromosome in B/H11032). This apparent slightly compacted TAS localization pattern in Ppd3-knockdown polytene chromosomes was not due to a reduction of TAS copies, as their number did not appear different from that observed in the w1118 control strain (data not shown). Thus, it is probably a...
consequence of a change in chromatin compaction that affects the organization of the sequences and/or probe accessibility to its specific complementary sequence.

**Loss of Rpd3 causes an accumulation of telomeric HP2**

It is widely accepted that trimethylation of H3K9 at chromosome ends is a key conserved mark for a proper localization of telomeric capping proteins (Schoeftner and Blasco, 2009). Thus, we wanted to verify whether the loss of Rpd3 RNAi influenced the localization of proteins known to have essential functions in *Drosophila* telomere maintenance. Immunofluorescence using antibodies directed against specific telomere capping proteins showed that Rpd3 knockdown did not affect the localization or abundance of HOAP (encoded by the *caravaggio* gene) (Fig. 7A–C), HP1 (Fig. 7D–F) (Fanti et al., 1998; Perrini et al., 2004), or Woc (Fig. 7G–I) (Raffa et al., 2005) in either fused or non-fused telomeres, indicating that capping proteins localize normally upon Rpd3 RNAi. The fact the HP1 localization and function is not affected as consequence of changes in telomeric levels of trimethylation of H3K9 strengthens previous observations that the interaction between the HP1 chromodomain and trimethylation of H3K9 is not required for the capping function of HP1 (Ebert et al., 2004; Perrini et al., 2004).

Because specific histone modifications are crucial for the localization of heterochromatin proteins, we sought to determine whether alterations in both acetylation and trimethylation of H3K9 caused by loss of Rpd3 RNAi influenced the distributions of heterochromatin proteins that, like HP1, associate with chromosome ends in *Drosophila*. We therefore analyzed the telomere localization pattern of HP2, an HP1-interacting factor with a role in the structural organization of chromosomes and in heterochromatin-induced gene silencing (Shaffer et al., 2006). In polytene chromosomes HP2 localizes at the chromocenter, at distinct chromosomal bands and at the cap of telomeres (Andreyeva et al., 2005; Shaffer et al., 2006). However, unlike HP1, HP2 is not a capping protein and is not required to prevent chromosome fusions (Shaffer et al., 2006). Surprisingly, immunofluorescence (Fig. 5L–M; Fig. 7J) and ChIP (Fig. 4B) analyses revealed a striking accumulation of HP2 at fused and non-fused telomeres upon Rpd3 RNAi, although the localization of HP2 at the chromocenter and other chromosomal regions remained indistinguishable from wild type (data not shown).

**Discussion**

We have identified the Rpd3–Sin3A histone deacetylase complex as a factor establishing proper chromatin structure of *Drosophila* telomeres. Strikingly, loss of Rpd3 dramatically affected the organization of polytene chromosome ends resulting in telomere prone to fusions. Interestingly, the observations that upon Rpd3 RNAi telomeric fusions are not seen in mitotic cells and that...
telomere capping proteins localize normally further suggest that the activity of Rpd3 is not required for chromosome end protection. Fused polytene telomeres have been always associated with an increase in HTT copies. Surprisingly, our FISH analysis, along with dot-blot results, clearly indicated that in Rpd3 knockdown telomeres the amount of the HeT-A, TART and TAHRE elements remained as high as in wild type. The apparent telomere elongation we observed upon Rpd3 RNAi probably reflects a change in telomere chromatin condensation in Rpd3 mutants, underlying changes in histone acetylation. As a result of this change in telomere chromatin condensation, Rpd3 mutants possess telomere tips that appear ‘longer’ than usual by DAPI staining. A combination of immunostaining and ChIP data indicated that loss of Rpd3 by RNAi leads to profound global, as well as telomeric, chromatin epigenetic changes. Upon specific Rpd3 RNAi telomere epigenetic changes included a decrease in H4 acetylation, including H4K16Ac, and an increase of trimethH3K9 at the level of telomere tips. Moreover, the HP2 protein, which normally binds to the telomere cap, was highly enriched upon Rpd3 RNAi. Because we were unable to perform ChIP on TAS sequences, we could not directly assess whether the epigenetic changes induced by Rpd3 RNAi also affected TAS sequences. Nevertheless, the apparent slight compaction of TAS organization detected by FISH in polytene chromosomes upon Rpd3 RNAi suggests that loss of Rpd3 affects the structural chromatin organization of this region.

The question of how the accumulation of HP2 on telomeric chromatin with reduced H4Ac and increased trimethH3K9 at the level of telomere tips, is linked to the fusion of telomeres upon Rpd3 RNAi still remains open. One intriguing possibility is that an overload of HP2 drives excessive heterochromatization at chromosome ends upon Rpd3 RNAi, inducing telomeric stickiness reminiscent of chromocenter heterochromatin coalescence. Alternatively, an excess of
heterochromatization might also impair telomere replication leading to stalled replication forks, which are known to cause fusigenic telomerases (Ferreira et al., 2004).

In conclusion, our work provides an important insight into the epigenetic determination of telomere chromatin organization in higher eukaryotes. As polytene chromosomes are known to be useful cytogenetic tools to address the question of structural and functional organization of telomeres in Drosofila, our results might represent a foothold for further studies to understand the interplay existing between histone acetylation and deacetylation events and telomere homeostasis.

Materials and Methods

Drosophila stocks and genetic crosses

Flies were raised on cornmeal-yeast-agar medium containing Tegosent. Drosophila RNAi strains and w1118;P[UAS-dicer, w+] were obtained from the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at/control/main).

To monitor the effects of Sin3A RNAi and Rpd3 RNAi on polytene chromosomes structure, larval salivary glands, were cultured by reaggregating yw, w, eyeGAL4 virgins (Hazelett et al., 1998) with homoygous UAS-Sin3A-RNAi (VDRC 10808) or heterozygous UAS-Rpd3-RNAi/T(2;3) CyO, Hu, Tb flies, recognizing the larvae of interest by the absence of the dominant marker Tb. The UAS-Rpd3-RNAi/T(2;3) CyO, Hu, Tb stock was obtained from Rpd3 RNAi strain VDRC 30599, and the UAS-Sin3A-RNAi/T(2;3) CyO, Hu, Tb stock was obtained from Rpd3 RNAi strain VDRC 30599, both with Tb as a marker. The eyeGAL4 driver, beside its specific expression in the brain (neuroblasts), allowing the analysis of Rpd3 RNAi on polytene chromosomes (Corona et al., 2007). For the analysis of mitotic chromosomes, heterozygous UAS-Rpd3-RNAi/T(2;3) CyO, Hu, Tb and homoygous UAS-Sin3A-RNAi males were crossed with eyeGAL4 virins (Corona et al., 2007). The eyeGAL4 driver is expressed ubiquitously in embryos and continues its expression in many other larval tissues, including the brain (neuroblasts), allowing the analysis of Rpd3 RNAi flies on mitotic chromosomes (Corona et al., 2007).

Analysis of polytene and mitotic chromosomes

diffyey, eyGAL4/UAS-Sin3a-RNAi and eyGAL4/UAS-Rpd3-RNAi mutant polytene chromosomes were prepared from third-instar larvae grown at 20°C. For single staining, polytene chromosome were processed as described previously (Burgio et al., 2008; Raffa et al., 2005). The primary antibodies used for immunostainings include rabbit antibodies against Sin3A (Pile and Wassarman, 2000), Rpd3 (Pile and Wassarman, 2000), acetylated histone H3 and H4 (Active Motif), trimethylated lysine 9 of H3 and trimethylated lysine 20 of H4 (UBI) (Schotta et al., 2004), a mouse antibody against trimethylated lysine 27 of H3 (Abcam) and antibodies against HOAP, HP1 and Woc (Raffa et al., 2005). For fluorescence in situ hybridization (FISH) we used the pSC-23Zn plasmid containing the 23ZnORF of HeT-A and the probes were obtained from PCR reactions on genomic DNA using T1 and H11032 primers (Perrini et al., 2004). Images were captured with a DC360 FX camera on a Leica DM 4000B Personal Molecular Imager (BioRad).

ChIP

Chromatin was extracted from 40 pairs of diffyey and eyGAL4/UAS-Rpd3-RNAi salivary glands and ChIP was conducted with 2 μg of anti-AcH3 and anti-AcH4 antibodies (Upstate; specifically recognizing histone H3 acetylations on K9 and K14 and histone H4 acetylations on K5, K8, K12 and K16), anti-H4K16Ac (Active Motif), and 1 μg of anti HP2, as described previously (Laursch et al., 2007), adapting reaction volumes to the small chromatin sample. In all experiments presented, the 'Input' chromatin corresponds to 15% of chromatin used for the ChIP. Input and immunoprecipitated DNA fragments (PCR purification kit; Quiagen) were amplified with the primers used for RT-PCR described above (Perrini et al., 2004). PCR products were analyzed on agarose gels stained with ethidium bromide and images were acquired with the Personal Molecular Imager (BioRad). Quantification of bands was performed with Quantity One software (Bio-Rad).

We thank the Bloomington and VDRC Stock Center for the Drosophila strains used in this work. We are grateful to Lori Pile for providing the anti-Sin3A and anti-Rpd3 antibodies, Thomas Jenuwein for the anti-trimeH3K9, anti-trimeH3K27 and anti-trimeH4K20 antibodies, Jim Kadonaga for the anti-H1 antibody, Sarah Elgin for the A-box antibody, Laura Ciapponi for the anti-HOAP antibody, Grazia Cenci, G., Siriaco, G., Raffa, G. D., Kellum, R. and Gatti, M. (2005). Three distinct chromatin domains in telomere ends of polytene chromosomes in Drosophila melanogaster Tel mutants. J. Cell Sci. 118, 299-309.

References

Ahiringer, I. (2000). NuRD and SIN3 histone deacetylase complexes in development. Trends Genet. 16, 351-356.

Albacete, I., Olmo, R. T., Lacoste, N., Tan, S., Briggs, S. D. and Cote, J. (2007). Interplay of chromatin modifiers on a short basic patch of histone H4 tail defines the boundary of telomeric heterochromatin. Mol. Cell 28, 1002-1014.

Andreyeva, E. N., Belyaeva, E. S., Semeshin, V. F., Pokholkova, G. V. and Zhimulev, I. F. (2005). Three distinct chromatin domains in telomere ends of polytene chromosomes in Drosophila melanogaster Tel mutants. J. Cell Sci. 118, 5465-5477.

Blasco, M. A. (2007). The epigenetic regulation of mammalian telomeres. Nat. Rev. Genet. 8, 299-308.

Bougeret, G., La Rocca, G., Sala, A., Arancio, W., Di Gesu, D., Collesano, M., Sperling, A. S., Armstrong, J. A., van Heeringen, S. J., Logie, C. et al. (2008). Genetic identification of a network of factors that functionally interact with the nucleosome remodeling ATPase BSIW1. PLoS Genet. 4, e1000089.

Cenci, G., Siriaco, G., Raffa, G. D., Kellum, R. and Gatti, M. (2003). The Drosophila HOAP protein is required for telomere capping. Nat. Cell Biol. 5, 82-84.

Cenci, G., Ciapponi, L. and Gatti, M. (2005). The mechanism of telomere protection: a comparison between Drosophila and humans. Chromosoma 114, 135-145.
Ciapponi, L. and Cenci, G. (2008). Telomere capping and cellular checkpoints: clues from fruit flies. Cytogenet. Genome Res. 122, 365-373.

Corona, D. F., Siriaco, G., Armstrong, J. A., Snarskaya, N., Mc Clemont, S. A., Scott, M. P. and Tamkun, J. W. (2007). ISWI regulates higher-order chromatin structure and histone H1 assembly in vivo. PLoS Biol. 5, e232.

De Lange, T. (2005). Telomere-related genome instability in cancer. Cold Spring Harb. Symp. Quant. Biol. 70, 197-204.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K. and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature 384, 589-591.

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448, 151-156.

Ebert, A., Schotta, G., Lein, S., Kubicek, S., Krauss, V., Jenuwein, T. and Reuter, G. (2004). Su(var) genes regulate the balance between euchromatin and heterochromatin in Drosophila. Genes Dev. 18, 2973-2983.

Ebert, A., Lein, S., Schotta, G. and Reuter, G. (2006). Histone modification and the control of heterochromatin gene silencing in Drosophila. Chromosome Res. 14, 377-392.

Fanti, L., Giovinazzo, G., Berloco, M. and Pimpinelli, S. (1998). The heterochromatin protein 1 prevents telomere fusions in Drosophila. Mol. Cell 2, 527-538.

Ferreira, M. G., Miller, K. M. and Cooper, J. P. (2004). Indecent exposure: when genes marked by H3K36 trimethylation using a sequence-independent mechanism. Mol. Cell. Biol. 24, 5121-5127.

Galbert, M. E., Larschan, E., Peng, S., Park, P. J. and Kuroda, M. I. (2009). Drosophila MSL complex globally acetylates H4K16 on the male X chromosome for dosage compensation. Nat. Struct. Mol. Biol. 16, 825-832.

Hazelett, D. J., Bourouis, M., Walldorf, U. and Treisman, J. E. (1998). decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. Development 125, 3741-3751.

Johansen, K. M., Johansen, J., Jin, Y., Walker, D. L., Wang, D. and Wang, Y. (1999). Chromatin structure and nuclear remodeling. Crit. Rev. Eukaryot. Gene Expr. 9, 267-277.

Kadosh, D. and Struhl, K. (1998). Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol. Cell. Biol. 18, 5121-5127.

Larschan, E., Aleksyenko, A. A., Gorchakov, A. A., Peng, S., Li, B., Yang, P., Workman, J. L., Park, P. J. and Kuroda, M. I. (2007). MSL complex is attracted to genes marked by H3K36 trimethylation using a sequence-independent mechanism. Mol. Cell 28, 121-133.

Mason, J. M., Frydrychova, R. C. and Biessmann, H. (2008). Drosophila telomeres: an exception providing new insights. BioEssays 30, 25-37.

Nugent, C. I. and Lundblad, V. (1998). The telomerase reverse transcriptase: components and regulation. Genes Dev. 12, 1073-1085.

Palm, W. and de Lange, T. (2008). How shelterin protects mammalian telomerases. Annu. Rev. Genet. 42, 301-334.

Perrini, B., Piacentini, L., Fanti, L., Altieri, F., Chiarchielli, S., Berloco, M., Turano, C., Ferraro, A. and Pimpinelli, S. (2004). HP1 controls telomere capping, telomere elongation, and telomere silencing by two different mechanisms in Drosophila. Mol. Cell 15, 467-476.

Pile, L. A. and Wassarman, D. A. (2000). Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. EMBO J. 19, 6131-6140.

Raffa, G. D., Cenci, G., Siriaco, G., Goldberg, M. L. and Gatti, M. (2005). The putative Drosophila transcription factor woc is required to prevent telomeric fusions. Mol. Cell 20, 821-831.

Raskova, S., Karam, S. E., Kellum, R. and Pardue, M. L. (2002). Gag proteins of the two Drosophila telomeric retrotransposons are targeted to chromosome ends. J. Cell Biol. 159, 397-402.

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M. and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA 93, 14503-14508.

Schoeftner, S. and Blasco, M. A. (2009). A ‘higher order’ of telomere regulation: telomere heterochromatin and telomeric RNAs. EMBO J. 28, 2323-2336.

Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D. and Jenuwein, T. (2004). A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. 18, 1251-1262.

Shaffer, C. D., Cenci, G., Thompson, B., Stephens, G. E., Slawson, E. E., Adu-Wusu, K., Gatti, M. and Elgin, S. C. (2006). The large isoform of Drosophila melanogaster heterochromatin protein 2 plays a critical role in gene silencing and chromosome structure. Genetics 174, 1189-1204.

Shareef, M. M., King, C., Damaj, M., Badagu, R., Huang, D. W. and Kellum, R. (2001). Drosophila heterochromatin protein 1 (HIP1) origin recognition complex (ORC) protein is associated with HP1 and ORC and functions in heterochromatin-induced silencing. Mol. Biol. Cell 12, 1671-1685.

Shpiz, S., Kwon, D., Uneva, A., Kim, M., Klenov, M., Rozovskv, Y., Georgiev, P., Savitsky, M. and Kalmykova, A. (2007). Characterization of Drosophila telomeric retroelement TAHRIE: transcription, transpositions, and RNAi-based regulation of expression. Mol. Biol. Evol. 24, 2533-2545.

Siriaco, G. M., Cenci, G., Haoudi, A., Champion, L. E., Zhou, C., Gatti, M. and Mason, J. M. (2002). Telomere elongation (Tel), a new mutation in Drosophila melanogaster that produces long telomeres. Genetics 160, 235-245.

Steitz, M. C., Wickenheisser, J. K. and Siegfried, E. (1998). Overexpression of zeste white 3 blocks wingless signaling in the Drosophila embryonic midgut. Dev. Biol. 197, 218-233.

Stephens, G. E., Craig, C. A., Li, Y., Wallrath, L. L. and Elgin, S. C. (2004). Immunofluorescent staining of polytene chromosomes: exploiting genetic tools. Methods Enzymol. 376, 372-393.