Effete, a *Drosophila* Chromatin-Associated Ubiquitin-Conjugating Enzyme That Affects Telomeric and Heterochromatic Position Effect Variegation

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ABSTRACT *Drosophila* telomeres are elongated by the transposition of telomere-specific retrotransposons rather than telomerase activity. Proximal to the terminal transposon array, *Drosophila* chromosomes contain several kilobases of a complex satellite DNA termed telomere-associated sequences (TASs). Reporter genes inserted into or next to the TAS are silenced through a mechanism called telomere position effect (TPE). TPE is reminiscent of the position effect variegation (PEV) induced by *Drosophila* constitutive heterochromatin. However, most genes that modulate PEV have no effect on TPE, and systematic searches for TPE modifiers have so far identified only a few dominant suppressors. Surprisingly, only a few of the genes required to prevent telomere fusion have been tested for their effect on TPE. Here, we show that with the exception of the *effete* (*eff*; also called *UbcD1*) mutant alleles, none of the tested mutations at the other telomere fusion genes affects TPE. We also found that mutations in *eff*, which encodes a class I ubiquitin-conjugating enzyme, act as suppressors of PEV. Thus, *eff* is one of the rare genes that can modulate both TPE and PEV. Immunolocalization experiments showed that Eff is a major constituent of polytene chromosomes. Eff is enriched at several euchromatic bands and interbands, the TAS regions, and the chromocenter. Our results suggest that Eff associates with different types of chromatin affecting their abilities to regulate gene expression.

TELOMERES are specialized nucleic acid–protein complexes that counteract incomplete terminal DNA replication and shield chromosome ends from inappropriate DNA repair, which might result in end-to-end fusion (Palm and De Lange 2008; Jain and Cooper 2010; Raffa *et al.* 2011). In most organisms, telomeres terminate with tandemly repeated G-rich sequences, which are added to chromosome ends by the telomerase holoenzyme (Blackburn *et al.* 2006). These telomere short repeats associate with sequence-specific binding factors, which in turn recruit additional telomeric proteins, forming multiprotein complexes that are crucial for

chromosome end homeostasis. A well-known example of such terminal complexes is shelterin, the six-protein assembly that coats human chromosome ends, allowing cells to distinguish between natural chromosome ends and DNA breaks (Palm and De Lange 2008).

Drosophila lacks telomerase and fly telomeres are elongated by transposition of three specialized retrotransposons—HeT-A, TART, and TAHRE—that form terminal arrays (HTT arrays) of complete and incomplete elements. Consistent with this situation, *Drosophila* telomeres are assembled independently of the sequence of terminal DNA (Mason *et al.* 2008; Pardue and Debaryshe 2011). Studies carried out in the past 15 years have detected many factors required to prevent telomere fusions (TFs) (for reviews see Cenci *et al.* 2005; Rong 2008; Raffa *et al.* 2011). The isolation and characterization of mutants displaying frequent TFs in larval brain cells has led to the identification of 10 genes required for telomere capping: *effete/UbcD1*, which encodes an E2 ubiquitin-conjugating

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enzyme (Cenci *et al.* 1997); the *Drosophila* homologs of the *ATM*, *RAD50*, *MRE11*, and *NBS1* DNA repair genes (Bi *et al.* 2004, 2005; Ciapponi *et al.* 2004, 2006; Oikemus *et al.* 2004, 2006; Silva *et al.* 2004; Song *et al.* 2004); *Su(var)205* and *caravaggio (cav)*, which encode heterochromatin protein 1 (HP1) and HP1/ORC-associated protein (HOAP), respectively (Fanti *et al.* 1998; Cenci *et al.* 2003); *without children (woc)*, which specifies a putative transcription factor (Raffa *et al.* 2005); *modigliani (moi)*, which encodes a non-conserved HOAP-binding protein (Raffa *et al.* 2009); and *verrocchio (ver)*, which specifies an OB-fold-containing protein structurally homologous to STN1 (Raffa *et al.* 2010). An additional telomere-capping protein called HP1-HOAP interacting protein (HipHop), was identified among the polypeptides that coprecipitate with HOAP and shown to prevent TFs in S2 cultured cells (Gao *et al.* 2010).

HOAP, Moi, Ver, and HipHop are nonconserved fast-evolving proteins that localize and function only at telomeres. These proteins form a capping complex we call terminin, which is functionally analogous to shelterin, although it binds chromosome ends independently of the DNA sequence (Raffa *et al.* 2011). The other proteins that protect *Drosophila* telomeres from fusion events (Eff, HP1, ATM, Rad50, Mre11, Nbs, and Woc) are evolutionarily conserved, do not localize only at telomeres, and do not play telomere-specific functions (Raffa *et al.* 2011). It has recently been proposed that concomitant with telomerase loss, *Drosophila* rapidly evolved terminin to bind chromosome ends in a sequence-independent fashion, and that the nonterminin telomere protection factors correspond to ancestral telomere-associated proteins that did not evolve as rapidly as terminin because of the functional constraints imposed by their involvement in diverse cellular processes (Raffa *et al.* 2009, 2010, 2011; Gao *et al.* 2010).

A particularly versatile nonterminin protein is the class I E2 ubiquitin-conjugating enzyme encoded by the *eff* gene. Eff is extraordinarily conserved. In 12 recently sequenced *Drosophila* species, the Eff proteins are 100% identical (Clark *et al.* 2007; Raffa *et al.* 2011). UBC4/UBCH5B and UBC5p, the human and yeast orthologs of Eff, are 89 and 82% identical to Eff, and Eff can functionally substitute for UBC4/UBCH5B (Treier *et al.* 1992). In addition, expression in flies of the human *UBC4/UBCH5B* gene rescues the telomere fusion phenotype elicited by *eff* mutations (our unpublished results). Eff has been implicated in ubiquitin-mediated degradation of several *Drosophila* proteins, including the *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1) (Ryoo *et al.* 2002) and CyclinA (Chen *et al.* 2009), and has been shown to interact with chromatin components such as the Ph component of the Polycomb complex (Fauvarque *et al.* 2001) and the nucleosome remodeling factor ISWI (Arancio *et al.* 2010). In addition, recent studies have also shown that Eff is a general component of *Drosophila* chromatin. A genome-wide analysis of the localization of 53 proteins has recently shown that the *Drosophila* genome is segmented into five major chromatin types (dubbed GREEN, BLUE, BLACK, RED, and YELLOW) defined by unique combinations of proteins (Filion *et al.* 2010). Eff is preferen-

tially enriched in the GREEN, BLUE, and BLACK chromatin, which are thought to have repressive properties (Filion *et al.* 2010).

Many studies have shown that telomeres modulate the expression of genes located in their proximity, a phenomenon known as telomere position effect (TPE). This form of transcriptional regulation is conserved from yeast to humans and has been implicated in numerous human pathologies (reviewed in Ottaviani *et al.* 2008). Studies in *Saccharomyces cerevisiae* have identified >50 telomere-associated proteins involved in TPE. Most of these proteins are bound to telomeres and their deletion reduces or abrogates telomere-induced gene silencing (Mondoux and Zakian 2007; Ottaviani *et al.* 2008). In *Drosophila*, TPE was first identified as a silencing effect on *white*⁺ transgenes inserted into the telomeric regions of the chromosomes (Gehring *et al.* 1984; Hazelrigg *et al.* 1984; Levis *et al.* 1985; Biessmann *et al.* 2005b). Subsequent studies showed that these variegating *white*⁺ transgenes were inserted within or next to a cluster of subtelomeric repeats, designed as telomere associated sequences (TASs) (Karpen and Spradling 1992; Levis *et al.* 1993; Cryderman *et al.* 1999; Mason *et al.* 2000; Golubovsky *et al.* 2001).

TASs can be subdivided into two classes: the 2L and 3L TASs, which contain 40–60 tandemly arranged copies of a 458-bp sequence; the XL, 2R, and 3R TASs, which contain a 400-bp repeat derived from the Invader 4 retrotransposon intercalated with a telomere-specific repeat that differs between the XL TAS and the TASs of 2R and 3R (Mason *et al.* 2008; Antao *et al.* 2012). Although the TASs are molecularly divergent, they appear to share some gene silencing properties, as deletions of the 2L TAS suppress TPE at nonhomologous telomeres (Golubovsky *et al.* 2001; Mason *et al.* 2004). In contrast to transgenes inserted into the TAS, reporter gene insertions into the HTT array of retrotransposons are not repressed (Biessmann *et al.* 2005a). However, it is currently unclear whether transgenes inserted in the most distal chromosome regions coated by terminin are negatively regulated.

Superficially, TPE resembles position effect variegation (PEV) of euchromatic genes placed next to heterochromatin by chromosome rearrangements (Weiler and Wakimoto 1995). However, TPE and PEV respond differently to genetic modifiers; PEV modifiers do not generally affect TPE of transgenes inserted into the second or third chromosome subtelomeric regions, but do affect TPE at the telomeres of the fourth chromosome, which shares properties with centric heterochromatin (e.g., it binds HP1) (Wallrath and Elgin 1995; Weiler and Wakimoto 1995). In addition, several studies have shown that dominant suppressors of TPE are relatively rare compared to the plethora of PEV dominant suppressors (Mason *et al.* 2004; Doheny *et al.* 2008; Antao *et al.* 2012). Unambiguous identification of TPE suppressors has been a difficult task due to the widespread presence of TAS deficiencies among *Drosophila* stocks and the allele-specific differences among the mutations that suppress TPE (Boivin *et al.* 2003; Mason *et al.* 2004; Doheny *et al.* 2008).

So far, only a few *bona fide* TPE suppressors have been identified. They include *grappa* (*grp*), which encodes histone H3 lysine 79 methyltransferase (Mason *et al.* 2004; Shanower *et al.* 2005; Doheny *et al.* 2008), *Su(var)3-9*, which encodes a histone H3 lysine 9-specific methyltransferase (Doheny *et al.* 2008), the *Polycomb* group genes *Polycomb* (*Pc*), *Posterior sex comb* (*Psc*), *Polycomblike* (*Pcl*), *polyhomeotic* (*ph*), and *Suppressor of zeste 2* [*Su(z)2*], which encode chromatin remodeling proteins (Wallrath and Elgin 1995; Cryderman *et al.* 1999; Boivin *et al.* 2003; Mason *et al.* 2004; Doheny *et al.* 2008), and *Su(var)3-26/Hdac1/RPD3*, which encodes a histone deacetylase that also affects telomere organization and behavior in polytene nuclei (Doheny *et al.* 2008; Burgio *et al.* 2011). Other genes that might be involved in TPE modulation are *Su(var)2-10*, which encodes a PIAS family protein that interacts with lamin and mediates proper telomere clustering (Hari *et al.* 2001; Doheny *et al.* 2008), *male sex lethal 3* (*msl3*), and *kismet* (*kis*), which specify chromodomain-containing proteins that are likely to interact with Hdac1/RPD3 (Doheny *et al.* 2008). Dominant suppressors of both TPE and PEV are even more rare; they include *Su(var)3-9* and possibly *Su(var)3-26/Hdac1* and *Su(var)2-10* (Doheny *et al.* 2008).

Mutations in genes required to prevent telomeric fusions have not systematically been assayed for their effect on TPE. Here we tested mutations in 10 of these genes and found that only those in *eff* suppress TPE. In addition, we found that mutations in *eff* suppress PEV. These results prompted us to ask to investigate whether Eff associates with specific genomic sites and whether these sites are related to PEV and TPE. Immunolocalization experiments on polytene chromosomes showed that Eff localizes to several discrete euchromatic sites, the TASs, and the chromocenter. These results are consistent with studies showing that Eff is a component of the GREEN, BLUE, and BLACK repressive chromatin types (Filion *et al.* 2010) and they suggest that loss of Eff disrupts proper organization of the GREEN and BLUE chromatin, leading to suppression of PEV and TPE, respectively.

Materials and Methods

Drosophila stocks

The *eff*^{Δ112} and *eff*^{Δ73} (formerly *UbcD1*^{Δ112} and *UbcD1*^{Δ73}) mutations (Cenci *et al.* 1997) and the other mutations causing telomere fusions (*cav*¹, *moi*¹, *moi*^{M12}, *ver*¹, *mre11*, *nbs*¹, *rad50*^{D5.1}, *Su(var)205*^{O5}, *Su(var)205*^{O4}, *tefu*^{atm3}, *tefu*^{atm6}, *woc*^{B111}, and *woc*⁹⁶⁴) were described previously. Most likely, *eff*^{Δ73}, *cav*¹, *moi*¹, *ver*¹, *mre11*, *rad50*^{D5.1}, *nbs*¹, *tefu*^{atm6}, *woc*^{B111}, and *woc*⁹⁶⁴ are amorph alleles, while *Su(var)205*^{O5}, *Su(var)205*^{O4}, *moi*^{M12}, *tefu*^{atm3}, and *eff*^{Δ112} are strong hypomorphs (Cenci *et al.* 2003; Bi *et al.* 2004; Ciapponi *et al.* 2004; Silva *et al.* 2004; Raffa *et al.* 2005, 2009, 2010). The Δ112 and Δ73 *eff* alleles have been generated by imprecise excision of a *P*-element inserted into the *eff* locus; Δ73 carries a deletion that removes almost the entire *eff* transcription unit (Cenci *et al.* 1997). Δ73 and Δ112 homozygotes

die during embryogenesis and late larval/pupal stages, respectively; Δ73/Δ112 heterozygotes die at late larval stages and exhibit frequent telomeric fusions (Cenci *et al.* 1997).

The insertion lines 39C-X (X euchromatin, region 2D), 39C-5 (2L TAS), 39-C58 (2R TAS), 39C-31 and 118E-26 (3R TAS), and 118-E15 (fourth chromosome telomeric region) were previously described (Wallrath and Elgin 1995; Cryderman *et al.* 1999) and kindly provided by L. Wallrath (University of Iowa, Iowa City, IA). All these lines carry a transgenic construct containing the *hsp26* gene fused to the sequence of the barley *SIP1* gene (designated as *hsp26-pt*), followed by the *hsp70* promoter fused to a mini-white⁺ (Figure 1A). In the 39C-5 line, the *hsp26-pt-hsp70-w* transgene is flanked on both sides by 4–5 kb of a 0.4-kb TAS satellite sequence. In the 39C-31 and 118E-26 lines the same transgene is flanked by a 1.0-kb TAS sequence (Cryderman *et al.* 1999). The transgene in 39C-58 is flanked by subtelomeric repetitive elements designated as 0.8-kb TAS and 1.0-kb TAS, respectively (Wallrath and Elgin 1995). The 118-E15 insert in the fourth chromosome is surrounded by 1.1 kb of unique DNA (Cryderman *et al.* 1999).

The *P[ro-eff⁺]* transgenic line 064 that carries a wild-type copy of the *eff* gene (Wu *et al.* 1999) was kindly provided by J. Fischer (University of Texas, Austin, TX). This transgene, located on chromosome 2, rescued the telomere fusion phenotype elicited by the *eff*^{Δ112} mutation, lowering the telomeric fusion frequency from ~40 to ~5% (130 cells analyzed; our unpublished results). The *mre11* null allele (Bi *et al.* 2004) was a gift from Y. Rong [National Cancer Institute, National Institutes of Health (NIH), Bethesda]. The variegating line *Tp(3;Y)BL2* (Lu *et al.* 1998) was provided by J. Eissenberg (Saint Louis University, St. Louis). *In(1)w^{m4}* and *bw^D* flies have been kept in our laboratory for many years and are described in detail in FlyBase (<http://flybase.bio.indiana.edu/>) together with all genetic markers and balancers used here. Stocks were maintained and crosses were made on standard *Drosophila* medium at 25°.

Eye pigment quantification

For quantification of eye drospterin by spectrophotometric analysis, 20 males were collected in a 15-ml centrifuge tube, frozen in liquid nitrogen, and rapidly vortexed to detach heads from thoraxes. Heads were incubated for 24 h at 25° in 1 ml of a 30% ethanol solution, pH 2. Tubes were then spun for 30 min at 3500 rpm to pellet the heads; the eye pigment-containing supernatants were then analyzed with a spectrophotometer with λ set at 480 nm. Five samples of 20 flies were analyzed for each genotype; the measures reported in Table 1 and Figure 3A are the mean values of these samples ± SEM.

Fluorescent *in situ* hybridization

Polytene chromosome preparation and fluorescent *in situ* hybridization (FISH) were carried out as described previously (Berghella and Dimitri 1996). A 6-kb fragment of the 2L TAS array (Kurenova *et al.* 1998), kindly provided by

Figure 1 *eff* mutations suppress variegation of subtelomeric *white*⁺ transgenes. (A) Schematic representation of the *hsp26-pt-hsp70-w*⁺ construct (top) and the endogenous *hsp26* gene (bottom). The triangles indicate the positions of the primers used for the sqRT-PCR. (B) The *eff*^{Δ73} and *eff*^{Δ112} mutant alleles behave as dominant suppressors of variegation of the mini-*white* gene carried by the *hsp26-pt-hsp70-w*⁺ construct inserted into the 2L (39C-5), 2R (39C-58), or 3R (39C-31 and 118E-26) TASs or near the fourth chromosome telomere (118E-15). (C) Mutations in *eff* lower the expression of the *hsp26-pt-hsp70-w*⁺ transgene. The columns represent the amounts of PCR products (mean ± SEM; *n* = 4) obtained using the P1 and P2 primers indicated in A, relative to the amount of the endogenous *hsp26* transcript obtained with primers P1 and P3 (see A). *P[eff⁺064]* is a transgene that carries a wild-type copy of *eff*. All values were normalized to the value of the 39C-X line (carrying a euchromatic insertion of *hsp26-pt-hsp70-w*⁺), which has been set at 100% expression. Asterisks indicate statistically significant differences (Student's *t*-test; **P* < 0.01; ***P* < 0.05).

J. Mason (National Institute of Environmental Health Sciences, NIH, Triangle Park, NC) was used as probe. The slides were mounted in Vectashield medium H-1200 with 4,6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to stain DNA.

Transcription analysis by semi-quantitative RT-PCR

Five samples of 20 larvae were analyzed for each genotype. Larvae were heat-shocked for 45 min at 37° to drive the expression of both the endogenous *hsp26* gene and the *hsp26-pt-hsp70-w*⁺ construct; total RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacture's instructions. A total of 100 ng of RNA per sample were converted into cDNA using the Access RT-PCR System kit (Promega). For cDNA amplification we used the same primers previously used by Cryderman *et al.* (1999): P1 5'-CCTTTGCTTACAAGTCAAACAAGTTC-3', which is common to the endogenous *hsp26* gene and the *hsp26-pt-hsp70-w*⁺ transgenes; P2 5'-CTCAAGATATGGAACATGAACAAGTGC-3', which corresponds to the barley sequence; P3 5'-CTGGTGT TTACGAATGGGTCTTACC-3, which is specific for the endogenous *hsp26* gene (Figure 1A). PCR amplification was performed with an Eppendorf MasterCycler (Eppendorf) using the reaction conditions previously described (Cryderman *et al.* 1999). However the P1 and P2 primers worked only for

the 39C-58 insertion line, but failed to amplify a *hsp26-pt* transcript in the 39C-5, 39C-31, and 118E-26 lines. We were not able to amplify this transcript even using the following primers (kindly suggested by D. E. Cryderman): 5'-ACAA CACCGACATGCTCTACAG-3' (forward) and 5'-CGAGGAAG AGCGTGTGTAGG-3' (reverse). PCR products obtained from the 39C-58 insertion line were run on a 0.7% agarose gel and transferred to a Hybond-N membrane (Amersham). Filters were probed with an *hsp26* PCR product that was ³²P dATP-labeled by random priming (using the DIG DNA labeling and detection kit; Roche), and then hybridized as described in Raffa *et al.* (2005). The blots were optically scanned and the density of signals was determined with the OpitQuant 3.10 software (Packard Instruments).

Generation of an anti-Eff antibody and Western blotting

The *eff* reading frame, omitting the start codon, was amplified by PCR using the 5' primer GCATGGATCCGCGTTAAA AAGAATC and the 3' primer GCATGAATTCTCACATAGCATAC. The amplified fragment was cloned into pGEX-2T and expressed in BL21 (pLysS) cells after induction with 2 mM IPTG. The glutathione-Eff fusion protein was extracted as described previously (Smith and Johnson 1988). The purified protein was injected into rabbits to produce the antibody,

Table 1 Effects on TPE of mutations in genes required for telomere capping

Genotype	OD values ^a				
	Insertions on Ch 2		Insertions on Ch 3		Insertions on Ch 4
	<i>yw 39C-5</i>	<i>yw 39C-58</i>	<i>yw 39C-31</i>	<i>yw 118E-26</i>	<i>yw 118E-15</i>
+ ^b	0.052 (±0.002)	0.086 (±0.003)	0.094 (±0.003)	0.085 (±0.006)	0.096 (±0.003)
<i>TM6C</i> ^b	0.058 (±0.002)	0.080 (±0.003)	0.101 (±0.003)	0.080 (±0.003)	0.102 (±0.004)
<i>eff</i> ^{Δ73}	0.198 (±0.003) ^c	0.288 (±0.008) ^c	0.224 (±0.010) ^c	0.264 (±0.005) ^c	0.186 (±0.002) ^c
<i>eff</i> ^{Δ112}	0.103 (±0.002) ^c	0.120 (±0.010) ^c	0.248 (±0.001) ^c	0.270 (±0.006) ^c	0.178 (±0.005) ^c
<i>cav</i> ¹	0.042 (±0.001)	0.100 (±0.001)	0.104 (±0.005)		
<i>tefu</i> ^{atm3}	0.058 (±0.003)	0.062 (±0.009)	0.102 (±0.003)		
<i>tefu</i> ^{atm6}	0.064 (±0.002)	0.086 (±0.002)	0.122 (±0.003)		
<i>moi</i> ¹	0.045 (±0.003)	0.102 (±0.003)	0.082 (±0.003)		
<i>moi</i> ^{M12}	0.048 (±0.002)	0.058 (±0.002)			
<i>ver</i> ¹	0.060 (±0.002)	0.094 (±0.004)			
<i>woc</i> ^{B111}	0.042 (±0.004)	0.080 (±0.006)			
<i>woc</i> ⁹⁶⁴	0.035 (±0.002)	0.095 (±0.005)			
<i>nbs</i> ¹	0.045 (±0.003)	0.082 (±0.005)			
<i>rad50</i> ^{D5.1}	0.061 (±0.001)	0.082 (±0.001)			
<i>mre11</i>	0.054 (±0.006)	0.088 (±0.008)			
<i>Su(var)205</i> ⁰⁵	0.055 (±0.004)	0.056 (±0.001)			
<i>Su(var)205</i> ⁰⁴	0.050 (±0.003)	0.081 (±0.002)			

^a All values refer to the average optical density ± SEM of five samples (containing 20 heads each) from flies heterozygous for a TF mutation-bearing chromosome (or a control chromosome) and carrying a single copy of either 39C-5, 39C-58, 39C-31, 118E-26, or 118E-15.

^b Wild-type background; eyes from F1 males obtained by crossing Oregon R females to males from the insertion lines.

^c Values significantly different from those found in +/+ and *TM6C*/+ control flies (Student's t-test; P < 0.01).

which was purified by standard methods. Western blotting was performed as previously described (Somma *et al.* 2002), using protein extracts from larval brains. The anti-Eff antibody was diluted 1:1000; the anti-Giotto antibody (Giansanti *et al.* 2006), used as a loading control, was diluted 1:4000.

Immunostaining of polytene chromosomes

To obtain polytene chromosomes for immunostaining with anti-Eff, anti-HOAP, and anti-Pc, salivary glands from third instar larvae were dissected in 0.7% NaCl, fixed for 5 min with 2% formaldehyde in 45% acetic acid, and squashed in the same fixative. Slides were frozen in liquid nitrogen and, after flipping off the coverslip, immediately immersed in cold TBS for 5 min. Slides were then washed in TBS-T (TBS containing 0.1% Tween 20) and incubated overnight with rabbit anti-Eff (diluted 1:50), goat anti-Pc (1:50; Santa Cruz Biotechnology), and mouse anti-HOAP (a gift from L. Ciapponi; diluted 1:50). For Eff and HP1 immunostaining, polytene chromosomes were fixed as previously described (Raffa *et al.*, 2005). Slides were incubated overnight with rabbit anti-Eff (1:50) and mouse anti-HP1 (1:20). Secondary antibody incubation was carried out at room temperature for 2 hr, using FITC-conjugated donkey anti-goat or FITC-conjugated goat anti-mouse (Jackson Laboratories), and AlexaFluor 555-conjugated donkey anti-rabbit (Invitrogen). Slides were then mounted in Vectashield medium H-1200 with DAPI to stain DNA.

Microscopy

FISH and indirect immunofluorescence on polytene chromosome preparations were detected using a Zeiss Axioplan epifluorescence microscope equipped with a cooled Charge-

Coupled Device (CCD) camera (CoolSnap HQ, Photometrics). Pictures of variegating eyes and lac-Z stained tissues were taken using a Leica MZ 12 dissecting microscope (Leica) equipped with a Nikon Coolpix 990 (Nikon).

Results

Mutations in *eff* suppress TPE

To assay whether mutations that cause telomere fusion affect TPE, we used the *y w*; *p[w+]39C-5*, *y w*; *p[w+] 39C-58*, *y w*; *p[w+]39C-31*, and *y w*; *p[w+]118E-26* reporter strains described previously (Wallrath and Elgin 1995; Cryderman *et al.* 1999). These strains bear a construct containing a mini-*white*⁺ reporter gene, driven by an *hsp70* promoter, embedded in the TAS satellite sequences of 2L (39C-5), 2R (39C-58), and 3R (39C-31 and 118E-26). In addition, this construct contains a molecular tag, the barley *SIP1* gene, that allows measuring TPE by analysis the *hsp26-SIP1* (designated as *hsp26-pt*) transcript levels (Figure 1A). In a *white* background, the *p[w+] 39C-5*, *p[w+] 39C-58*, *p[w+] 39C-31*, and *p[w+] 118E-26* transgenes, when heterozygous with chromosomes carrying normal telomeres, result in a pale yellow or orange eye color with occasional red facets (Figure 1B).

To determine the effects of mutations in genes required to prevent telomere fusion (henceforth designated as TF genes) we crossed *y w*; *p[w+]39C-5*, *y w*; *p[w+] 39C-58*, *y w*; *p[w+] 39C-31*, or *y w*; *p[w+]118E-26* homozygous females to males bearing mutations in TF genes balanced over *TM6C* (*eff*^{Δ112}, *eff*^{Δ73}, *cav*¹, *moi*¹, *moi*^{M12}, *ver*¹, *nbs*¹, *tefu*^{atm3}, *tefu*^{atm6}, *woc*^{B111}, and *woc*⁹⁶⁴) or *CyO* (*mre11*, *rad50*^{D5.1}, *Su(var)205*⁰⁴, and *Su(var)205*⁰⁵). *y w* F₁ males heterozygous for

either 39C-5, 39C-58, 39C-31, or 118E-26, and each of the TF mutations were examined for eye color both by visual inspection and by measuring the amount of pigment with a spectrophotometer. *y w* males bearing the *TM6C* balancer and either the 39C-5, 39C-58, 39C-31, or 118E-26 insertion were used as control. Observation of the eyes (Figure 1B) and measurements of the eye pigment (Table 1) revealed that the *eff* mutant alleles ($\Delta 112$ and $\Delta 73$) suppress TPE associated with 39C-5, 39C-58, 39C-31, and 118E-26, whereas none of the mutations in the other TF genes has a detectable effect on TPE.

We also tested whether mutations in *eff* suppress the variegated eye phenotype associated with transgenes inserted near the fourth chromosome telomere. We crossed *y w* females homozygous for the 118E-15 fourth chromosome insertion (Cryderman *et al.* 1999) to *eff* $^{\Delta 112}$ /*TM6C* or *eff* $^{\Delta 73}$ /*TM6C* males. *y w* *F*₁ males heterozygous for both the 118E-15 insertion and each of the *eff* mutant alleles displayed darker eyes and higher levels of drosoterin compared to brothers bearing 118E-15 and the *TM6C* balancer (Figure 1B and Table 1). Thus, we conclude that mutations in *eff* dominantly relieve silencing of transgenes inserted near the telomeres of chromosome 4.

It has been reported that deficiencies of the 2L TAS dominantly suppress TPE (Mason *et al.* 2004). To ascertain whether the *eff*-mediated TPE suppression was not due to a deficiency of 2L TAS, we performed FISH on polytene chromosomes using a 2L TAS probe (Kurenova *et al.* 1998). Examination of 20 polytene nuclei from wild-type and *eff* $^{\Delta 112}$ /*eff* $^{\Delta 73}$ larvae revealed no detectable differences in FISH signal at the 2L tips (Figure 2). Mason *et al.* (2004) also noticed that most of the 2L TAS deficiencies that suppressed TPE also remove the terminal *l(2)gl* gene. The *eff* $^{\Delta 112}$ and *eff* $^{\Delta 73}$ mutations complemented mutations in *l(2)gl* (data not shown), confirming that the *eff* $^{\Delta 112}$ and *eff* $^{\Delta 73}$ retain intact 2L terminal regions. Thus, both FISH and complementation analysis indicate TPE suppression is a consequence of lesions in the *eff* locus and not a deficiency of the 2L TAS.

Mutations in *eff* enhance transcription of transgenes inserted in the TAS

To substantiate the finding that mutations in *eff* dominantly enhance the expression of genes embedded into the TAS, we used semi-quantitative RT-PCR to determine the transcription levels of the *hsp26-pt* transgene. We could only examine transcription of the 39C-58 and the 39C-X transgenes because we were not able to amplify the *hsp26-pt* transcript from the 39C-5, 39C-31, and 118E-26 lines. Amplification failed even using primers different from those originally designed by Cryderman *et al.* (1999) and used here to amplify the *hsp26-pt* transcript of the 39C-58 line. We generated larvae heterozygous for the 39C-58 insertion and either homozygous or heterozygous for each of the two *eff* mutant alleles. The 39C-58/+; *eff* $^{\Delta 112}$ /+ and 39C-58/+; *eff* $^{\Delta 73}$ /+ larvae were generated as described above; 39C-58/+; *eff* $^{\Delta 112}$ /*eff* $^{\Delta 112}$ and 39C-58/+; *eff* $^{\Delta 112}$ /*eff* $^{\Delta 73}$ larvae were gen-

Figure 2 The subtelomeric regions of the 2L chromosome arms of *eff* mutants retain the TAS. Fluorescent *in situ* hybridization with a 2L TAS-specific probe (red) shows that wild-type (A) and *eff* $^{\Delta 73}$ /*eff* $^{\Delta 112}$ (B) 2L polytene arms exhibit similar signals.

erated by crossing 39C-58/39C-58; *eff* $^{\Delta 112}$ /*TM6C* females to either *eff* $^{\Delta 112}$ /*TM6C* or *eff* $^{\Delta 73}$ /*TM6C*. The *eff* $^{\Delta 112}$ /+, *eff* $^{\Delta 73}$ /+, *eff* $^{\Delta 112}$ /*eff* $^{\Delta 112}$, and *eff* $^{\Delta 112}$ /*eff* $^{\Delta 73}$ larvae were distinguished from their *TM6C*-bearing siblings because of their non-*Tubby* phenotype.

All larvae were heat shocked for 45 min. We also heat shocked larvae heterozygous for the euchromatic insertion 39C-X, which served as control. Forty-five minutes after the heat shock, we extracted total RNA from these larvae and performed RT-PCR using primers that allow amplification of both the endogenous *hsp26* gene and the *hsp26-pt* transgenes (Figure 1A). The resulting RT-PCR products were then quantified after radioactive hybridization, and the amounts of *hsp26-pt* and *hsp26* transcripts were normalized to that observed for the 39C-X stock in which the level of the *hsp26-pt* transcript was 2.18-fold (± 0.44) higher than that of the endogenous *hsp26* transcript. If the expression level of *hsp26-pt* in the 39C-X/+ control line is set to 100%, in larvae heterozygous for the 39C-58 insertion, the *hsp26-pt* expression is reduced to 28% of control (Figure 1C). However, in 39C-58/+; *eff* $^{\Delta 112}$ /+ and 39C-58/+; *eff* $^{\Delta 73}$ /+ larvae, the *hsp26-pt* expression level was partially restored to 56 and 58% of the control level, respectively. Importantly, the presence of a wild-type *eff* transgene (indicated as 064) (Wu *et al.* 1999) in larvae heterozygous for both the 39C-58 insertion and *eff* $^{\Delta 112}$ (39C-58/+; 064/*eff* $^{\Delta 112}$) brought back the expression of *hsp26-pt* to approximately the same level of 39C-58/+ flies (36 vs. 28%, Figure 1C). Thus, our results collectively indicate that mutations in *eff* act as dominant suppressors of TPE.

The *eff* $^{\Delta 73}$ mutant allele carries a large deletion of the *eff* locus, which leads to embryonic lethality when homozygous. However, both *eff* $^{\Delta 112}$ homozygotes and *eff* $^{\Delta 112}$ /*eff* $^{\Delta 73}$ heterozygotes survive till the larval-pupal transition (Cenci *et al.* 1997). This enabled us to measure *hsp26-pt* transcription in 39C-58/+; *eff* $^{\Delta 112}$ /*eff* $^{\Delta 112}$ and 39C-58/+; *eff* $^{\Delta 112}$ /*eff* $^{\Delta 73}$ mutant larvae, which showed 74 and 103% of *hsp26-pt* transcription relative to the control level, respectively (Figure 1C). These results indicate the degree of TAS-induced gene silencing

is inversely related to the amount of Eff in the chromatin (see below).

Mutations in *eff* suppress PEV

The finding that *eff* mutations suppress variegation of transgenes inserted near the fourth chromosome telomere prompted us to ask whether these mutations also suppress gene silencing associated with pericentric heterochromatin (PEV). To assay whether *eff* mutations modify PEV, we used the *In (1)white^{m4}* (*w^{m4}*), *brown^D* (*bw^D*), and *Tp(3;Y)BL2* variegating strains. In *w^{m4}*, the *w⁺* gene, juxtaposed to the X heterochromatin by an inversion, is clonally inactivated, leading to a variegated pattern of eye pigmentation in hemizygous males and homozygous females (Weiler and Wakimoto 1995). The *bw^D* chromosome carries a transposition that places a megabase of 2L heterochromatin next to the *brown* gene in region 59E, giving rise to dominant silencing of *bw* in *bw^D/bw⁺* heterozygotes, which exhibit variegated eyes with pigmented ommatidia scattered in a very pale eye (Henikoff *et al.* 1995). *Tp(3;Y)BL2* carries an inducible *Hs-lacZ* transgene that can be used to detect PEV modifications in larval tissues upon staining for β -galactosidase activity (Lu *et al.* 1998).

To analyze PEV in the eye, we crossed *w^{m4}/w^{m4}* and *bw^D/bw^D* females to *eff^{Δ112}/TM3* or *eff^{Δ73}/TM3* males. We used the *TM3* to balance the *eff* mutations because previous studies showed that this balancer does not affect PEV (Weiler 2007; Zhu *et al.* 2008). We then compared *w^{m4}/Y; eff/+* and *bw^D/+; eff/+* F₁ males with their *w^{m4}/Y; TM3/+* and *bw^D/+; TM3/+* siblings for the eye pigment. This analysis revealed that males heterozygous for *eff* mutations and either hemizygous for *w^{m4}* or heterozygous *bw^D* exhibit a significant increase in eye pigment with respect to their brothers bearing the *TM3* balancer instead of the *eff* mutation (Figure 3A). We also compared *Tp(3;Y)BL2; +/+* and *Tp(3;Y)BL2; eff^{Δ112}/eff^{Δ73}* males for Lac-Z expression in salivary glands and larval brains (Figure 3). β -Gal staining revealed that homozygosity for *eff* mutations (*eff^{Δ112}/eff^{Δ73}*) strongly enhances Lac-Z expression. We note that the observed effects of *eff* mutations on *w^{m4}*, *bw^D*, or *Tp(3;Y)BL2* variegation cannot be the consequence of Y chromosome hyperploidy, as both during this study and our past studies (Cenci *et al.* 1997) we never observed *eff* mutant brains with two Y chromosomes. We thus conclude that *eff* represses the expression of both genes relocated next to heterochromatin and transgenes embedded into subtelomeric TASs. In addition, in both cases the degree of repression appears to be directly related to the amount of Eff associated with the chromatin.

Localization of Eff on polytene chromosomes

To localize the Eff protein along the polytene chromosomes, we generated a polyclonal antibody against the entire Eff polypeptide; this antibody recognized a band of the expected molecular weight (~19 kDa) in Western blots from larval brain extracts (Figure 4A). This band was strongly reduced in larval brain extracts from both *eff^{Δ112}/eff^{Δ112}* and *eff^{Δ112}/*

Figure 3 Mutations in *eff* suppress PEV. (A) Quantification of the eye pigment levels [mean optical density (OD) $\pm$ SEM; *n* = 5] for *w^{m4}; +/+*, *w^{m4}; +TM3*, *w^{m4}; eff^{Δ112}/+*, and *w^{m4}; eff^{Δ73}/+* males (red columns) and *bw^D; +/+*, *bw^D; +TM3*, *bw^D; eff^{Δ112}/+*, and *bw^D; eff^{Δ73}/+* males (green columns). Asterisks (*) indicate statistically significant differences (Student's *t*-test; *P* < 0.01). (B and C) Expression of the *Hs-lacZ* transgene of *Tp(3;Y)BL2* in salivary glands and brains (insets) from larvae with a wild-type genetic background (B) or carrying mutations in *eff* (*eff^{Δ73}/eff^{Δ112}*) (C).

eff^{Δ73} mutants, demonstrating the specificity of the antibody and confirming that *eff^{Δ112}* is a hypomorphic mutant allele (Cenci *et al.* 1997) (Figure 4A). Immunostaining of polytene chromosomes revealed that Eff localizes to many euchromatic regions along all chromosome arms (Figure 4B); polytene chromosomes from *eff^{Δ112}/eff^{Δ73}* mutants were not stained, consistent with Western blotting results (data not shown). Most Eff signals on polytene chromosomes did not coincide with brightly fluorescent DAPI bands. About 60% of the Eff signals corresponded to interbands that are not stained by DAPI, while the remaining 40% appeared to coincide with thin bands that were weakly stained by DAPI (Figure 4, B'–D). In addition, we observed a diffuse Eff staining of the chromocenter (Figure 4, B and B').

To obtain additional insight into the Eff localization pattern we co-immunostained polytene chromosomes for Eff and either HP1 or Polycomb (Pc), a component of the repressive PRC1 complex (Saurin *et al.* 2001). Examination of 10 Eff/HP1- and 10 Eff/Pc-stained polytene nuclei revealed that Eff is enriched in ~110 clear-cut polytene bands. A total

Figure 4 Eff localizes in many bands and interbands of wild-type polytene chromosomes. (A) Western blots of protein extracts from wild-type and *eff* mutant larvae demonstrate the specificity of the antibody. Anti-Giotto (Giansanti *et al.* 2006) was used as loading control. (B and B') Localization of Eff in polytene chromosomes. (C and D) Close-ups of the insets indicated in B'. Note that Eff localizes mainly to interbands and weakly stained DAPI bands.

of 15% of these bands colocalized with Pc signals and 28% with HP1 signals (Figure 5). These findings indicate that Eff is highly enriched in both HP1- and Pc-containing chromatin domains and demonstrate that Eff localization is not restricted to a specific chromatin type.

Double immunostaining for Eff and HOAP revealed that Eff exhibits telomeric signals that partially overlap HOAP signals (Cenci *et al.* 2003) (supporting information, Figure S1). The apparent colocalization of Eff and HOAP at the telomere regions prompted us to ask whether Eff binds the very end of the chromosomes like the telomere-capping HOAP protein. To address this question, we immunostained for Eff the polytene chromosomes from flies bearing the *Telomere elongation* (*Tel*) dominant mutation. These flies exhibit a strong increase in the copy number of the HTT elements, which form homogeneously stained regions at the end of the chromosomes (Siriaco *et al.* 2002). Telomere-capping proteins such as HP1 and HOAP associate with the distal ends of these regions (Andreyeva *et al.* 2005) (Figure 6). Thus, in the polytene chromosomes of *Tel* mutant, the TAS are separated from the telomere by a long HTT array, allowing the TAS and the telomere cap to be spatially resolved (Andreyeva *et al.* 2005) (see also Figure 6). We found that the HOAP signals at the end of all chromosomes are distinct from the most distal Eff bands. Thus, Eff is not detectably enriched at the telomere but accumulates in subtelomeric bands that, based on the DAPI staining pattern, are likely to correspond to the TAS repeats (Figure 6).

To determine whether the subtelomeric Eff bands correspond to the TASs, we immunostained for Eff and Pc the polytene chromosomes of larvae from the *Tel*-bearing *Gaiano*

3 strain (Figure 7). Previous studies have shown that the TASs are enriched in Pc (Boivin *et al.* 2003; Andreyeva *et al.* 2005). Specifically, it has been shown that in polytene chromosomes from *Tel* mutants the most distal Pc bands correspond to the XL, 2L, 3L, and 3R TASs; in 2R, the most distal Pc band is distinct from and proximal to the TAS (Andreyeva *et al.* 2005). Our analyses of Eff and Pc doubly stained chromosomes revealed that in all chromosome arms but 2R, the most distal Pc band corresponds to an Eff band (Figure 7). These results indicate that the XL, 2L, 3L, and 3R TAS regions are enriched in Eff. The most distal Eff band in 2R is not enriched in Pc. However, based on previous Pc and TAS mapping studies (Andreyeva *et al.* 2005) it is likely to correspond to the TAS.

Discussion

We have tested mutations in 10 genes required to prevent telomere fusion for dominant effect on TPE. We found that mutations in *eff* suppress TPE. In contrast, mutations in *cav*, *moi*, *ver*, *woc*, *Su(var)205*, *mre11*, *rad50*, *nbs*, and *tefu/atm* did not display dominant effects on TPE. These results agree with most but not all previous studies on *Drosophila* TPE. Several studies have shown that mutations in the HP1-encoding *Su(var)205* gene do not affect TPE (Cryderman *et al.* 1999; Doheny *et al.* 2008). A deficiency screen for dominant suppressor of TPE revealed that deficiencies that uncover *cav*, *moi*, *ver*, *woc*, *Su(var)205*, or *mre11* do not relieve silencing of telomeric transgenes, while deficiencies that remove *rad50* or *nbs* behave as strong and weak TPE suppressors,

Figure 5 Eff colocalizes with both Pc- and HP1-enriched bands. (A and B) Immunostaining of wild-type polytene chromosomes for Pc (red) and Eff (green) (A) or for HP1 (red) and Eff (green) (B). Eff precisely colocalizes with 15% of the Pc and 28% of the HP1 bands; some of the Eff/Pc and Eff/HP1 bands are indicated by arrows.

respectively (Mason *et al.* 2004). This deficiency screen did not provide information on *eff* and *tefu/atm*, as deficiencies uncovering these genes were not tested (Mason *et al.* 2004). However, in contrast to our results, previous studies showed the *tefu*¹ mutant allele acts as dominant suppressors of TPE (Oikemus *et al.* 2004) and that the *eff*^{mer4} allele does not affect TPE (Doheny *et al.* 2008). Given that in our analyses we used different *tefu* and *eff* mutant alleles (*eff*^{Δ112}, *eff*^{Δ73}, *tefu*^{atm6}, and *tefu*^{atm3}), these discrepancies are not surprising as previous studies documented many allele-specific differences among the mutations that suppress TPE. For example, studies on *Su(var)3-9*, *Psc1*, and *Su(z)2* revealed that not all mutant alleles at each locus suppress TPE (Boivin *et al.* 2003; Doheny *et al.* 2008).

We believe that our results unambiguously demonstrate that mutations in *eff* relieve silencing of transgenes inserted into the TASs. We have shown that the strong *eff*^{Δ112} and *eff*^{Δ73} mutant alleles suppress the eye variegated phenotype of the 39C-5 and the 39C-58 insertion lines that contain mini-*white*⁺ transgenes embedded into the 2L and 2R TASs, respectively. The same *eff* mutations also suppress TPE associated with the 39C-31 and 118E-26 lines that carry mini-*white*⁺ transgenes inserted into the 3R TAS. We have also shown that *eff*^{Δ112} and *eff*^{Δ73} enhance transcription of the barley tag of the 39C-58 transgene. Importantly, this enhancement of

Figure 6 Eff does not colocalize with the telomeric HOAP signal on XL. XL polytene from wild type (wt, left) and *Tel* mutants (right) were stained for DNA (with DAPI), HOAP, and Eff. The top panels show XL chromosome tips stained for DAPI and HOAP (red); the bottom panels show Eff staining in black and white of the same chromosome tips. Note that in the wild-type X chromosome, the most distal Eff signal is apparently coincident with the HOAP signal. However, in the X chromosome from the *Tel*-bearing *Gaiano* strain, the Eff and HOAP signals are separated by the long HTT array (see schemes in the middle panels) that characterizes the *Gaiano* chromosomes.

transcription was suppressed by a wild-type *eff* transgene. It is thus clear that *eff* has the ability to modulate the expression of genes inserted into the TAS. The finding that none of the other TF genes tested here (*cav*, *moi*, *ver*, *woc*, *Su(var)205*, *mre11*, *rad50*, *nbs*, and *tefu/atm*) affects TPE is intriguing but not surprising. The simplest interpretation of this finding is that TPE is induced by the TAS sequences, which are physically separated from the telomere-capping complex. We believe that Eff suppresses TPE because in addition to protecting telomere from fusion events it is also enriched at the TASs.

We have also shown that mutations in *eff* dominantly suppress the eye variegated phenotype of mini-*white*⁺ transgenes inserted near the fourth chromosome telomeres, and *white*⁺ genes placed next to the X chromosome heterochromatin. In addition, *eff*^{Δ112} and *eff*^{Δ73} suppress the *brown* dominant variegated phenotype and relieve silencing of the *Hs-lacZ* transgene in larval tissues. We thus conclude that *eff* is one of the rare genes that can modulate both TPE and PEV.

It has recently been shown that the *Drosophila* genome is segmented into five major chromatin types (GREEN, BLUE, BLACK, RED, and YELLOW) defined by unique combinations of proteins (Filion *et al.* 2010). These chromatin types are organized in large domains that extend over genomic regions >100 kb. Eff is present in all chromatin types but

Figure 7 The TAS regions are enriched in Eff. Immunostaining of *Gaiano* polytene chromosomes for both Eff (green) and Pc (red) shows that the most distal Pc bands of XL and 2L (that correspond to the TAS; see text) coincide with Eff bands. At the ends of the 2R arms, there is an Eff band just distal to the terminal Pc band. Previous studies have shown that the 2R TASs are also localized just distally to the terminal Pc band (see text). Thus, the 2R TAS regions are likely to be enriched in Eff.

preferentially binds the GREEN, BLUE, and BLACK chromatin. The GREEN chromatin, which is enriched in HP1 and the Su(var)3-9 histone methyltransferase but not in PcG proteins, is thought to correspond to classic heterochromatin. The BLUE chromatin does not contain HP1 or Su(var)3-9 but binds PcG proteins such as Pc, E(z), Pcl, and Sce. The BLACK chromatin covers 48% of the genome, is relatively gene poor, and is marked by histone H1, the D1 protein, the IAL kinase, SUUR, and lamin (Lam). The YELLOW and RED chromatin bind similar proteins, most of which are absent or underrepresented in the GREEN, BLUE, or BLACK chromatin types. BLUE and BLACK chromatin are transcriptionally inactive compared to the other chromatin types, and silencing of mini-*white*⁺ transgenes inserted in BLACK chromatin is more pronounced than BLUE or GREEN chromatin (Filion *et al.* 2010).

Our finding on the Eff distribution along polytene chromosomes are consistent with the hypothesis that chromatin is segmented into discrete domains that contain unique combinations of proteins (Filion *et al.* 2010). However, inferences on the types of chromatin contained in the Eff stained bands and interbands should be made with great caution. Subdivision of chromatin in multicolor domains was achieved by analyzing Kc167 tissue culture cells and does not apply to all tissues of developing flies. For example a survey of gene expression profiling indicated that some of the genes in BLACK chromatin of Kc167 cells can become active during *Drosophila* development (Chintapalli *et al.* 2007; Filion *et al.* 2010). With this in mind, we would like to suggest that the chromatin associated with the TAS is BLUE chromatin, as it contains PcG proteins and Eff, but it is not enriched in HP1 and SUUR (Boivin *et al.* 2003; Andreyeva *et al.* 2005; this report). We do not know the “color” of the chromatin associated with the Eff bands that do not contain Pc proteins. Most of these bands should contain BLACK chromatin but some might contain GREEN or even YELLOW or RED chromatin. Definition of the nature of these bands will require detailed analyses by double immunostaining for Eff and specific markers of the different chromatin color types.

Our finding that mutations in *eff* are dominant suppressors of both TPE and PEV are consistent with both molecular and cytological data on Eff distribution in chromatin domains. Eff is enriched in the chromocenter of the polytene chromosome that includes the fourth chromosome chromatin and pericentric heterochromatin, which are thought to correspond to GREEN chromatin (Filion *et al.* 2010). In addition, Eff is enriched in the TAS chromatin and in other Pc-containing bands; the TAS and these bands are likely to correspond to BLUE chromatin. Thus, one can easily envisage that loss or reduction of Eff would result in alterations of both heterochromatin/GREEN chromatin and TAS-associated BLUE chromatin, impairing the repressive properties of both chromatin types. However, the nature of the alterations caused by Eff depletion is currently unknown. Given that Eff is an E2 ubiquitin-conjugating enzyme (see Introduction), some of the Eff targets might not be properly mono- or polyubiquitinated, leading to defects in chromatin structure. Alternatively, Eff may be a structural component of the chromatin and serve an ubiquitination-independent function. It is even possible that Eff plays different roles in different chromatin types. Thus, an understanding of the molecular mechanisms through which Eff mediates TPE and PEV will require extensive studies aimed at defining the Eff interacting proteins and their possible post-translational modifications.

Even if Eff is required to prevent telomere fusion, surprisingly we did not observe any Eff accumulation at the telomere cap. This finding is subject to two alternative explanations. The most likely one is that Eff is present at the telomere caps in amounts that are not detected by ordinary immunofluorescence. Alternatively, the telomeric Eff target may be ubiquitinated in the cytoplasm or in the nucleus and then recruited to the telomeres. We have recently found that Eff physically interacts with HOAP (our unpublished results). However, this result does not discriminate between the above alternatives, as the biochemical Eff-HOAP interaction may occur either at the telomeres or in the cytoplasm. Whatever the alternative, the Eff targets at the telomere cap might

be different from those contained in the TAS-associated chromatin.

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Effete, a *Drosophila* Chromatin-Associated Ubiquitin-Conjugating Enzyme That Affects Telomeric and Heterochromatic Position Effect Variegation

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Figure S1 Eff partially co-localizes with HOAP at polytene chromosome ends. (A-C) Wild-type polytene chromosomes stained for DAPI, Eff and HOAP; (A) DAPI (white)/HOAP (red) merge; (B) Eff staining; (C) DAPI (white)/Eff (green)/HOAP (red) merge. (D-F) Close-ups of the 2R telomere (inset in C) showing that the Eff and HOAP signals partially co-localize.