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SUMO conjugation attenuates the activity of the *gypsy* chromatin insulator

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Chromatin insulators have been implicated in the establishment of independent gene expression domains and in the nuclear organization of chromatin. Post-translational modification of proteins by Small Ubiquitin-like Modifier (SUMO) has been reported to regulate their activity and subnuclear localization. We present evidence suggesting that two protein components of the *gypsy* chromatin insulator of *Dorsophila melanogaster*, Mod(mdg4)2.2 and CP190, are sumoylated, and that SUMO is associated with a subset of genomic insulator sites. Disruption of the SUMO conjugation pathway improves the enhancer-blocking function of a partially active insulator, indicating that SUMO modification acts to regulate negatively the activity of the *gypsy* insulator. Sumoylation does not affect the ability of CP190 and Mod(mdg4)2.2 to bind chromatin, but instead appears to regulate the nuclear organization of *gypsy* insulator complexes. The results suggest that long-range interactions of insulator proteins are inhibited by sumoylation and that the establishment of chromatin domains can be regulated by SUMO conjugation.

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Introduction

Eukaryotic genomes are thought to be organized into independent domains of gene expression. The maintenance of this autonomy is considered to be important for the proper execution of complex developmental programs and cellular responses to stimuli. Chromatin insulators are characterized by two key properties suggestive of an involvement in the organization of independent expression domains. First, insulators are able to block enhancer–promoter communication when positioned between these elements (Geyer and Corces, 1992; Kellum and Schedl, 1992). Second, insulators can protect transgenes from the effects of surrounding chromatin, allowing for their position-independent expression (Kellum and Schedl, 1991; Chung *et al*, 1993).

The *gypsy* insulator of *Dorsophila melanogaster* consists of a ~350 bp DNA sequence that binds a protein complex of at least three components, Su(Hw), Mod(mdg4)2.2 and CP190. Su(Hw) and CP190 can bind DNA directly via their zinc-finger domains (Spana *et al*, 1988; Pai *et al*, 2004), whereas Mod(mdg4)2.2 does not bind DNA directly but is recruited to the *gypsy* insulator sequence through physical interactions with Su(Hw) and CP190 (Gerasimova *et al*, 1995; Pai *et al*, 2004). The *gypsy* insulator was identified originally as the enhancer-blocking element within the *gypsy* retrotransposon (Geyer and Corces, 1992), but hundreds of endogenous binding sites for the *gypsy* protein complex exist throughout the *Drosophila* genome (Gerasimova and Corces, 1998). Analysis of highly replicated polytene chromosomes reveals that *gypsy* insulator proteins are found preferentially at the borders between condensed and decondensed chromatin, suggestive of their role in partitioning independent chromatin domains (Labrador and Corces, 2002; Pai *et al*, 2004). However, in diploid nuclei, *gypsy* insulator proteins coalesce into large complexes, termed insulator bodies (Gerasimova and Corces, 1998; Byrd and Corces, 2003). These bodies are thought to represent the meeting places of distant insulator complexes, which loop out the chromatin fiber and thus delineate chromatin domains. Both Mod(mdg4)2.2 and CP190 contain a conserved BTB/POZ domain capable of self-interactions (Dhordain *et al*, 1995; Ghosh *et al*, 2001), which has been proposed to mediate clustering of insulator complexes. The integrity of insulator bodies has been correlated functionally with *gypsy* insulator activity. For instance, mutations in insulator components that disrupt the enhancer-blocking activity of *gypsy* also interfere with insulator body formation (Gerasimova and Corces, 1998).

Chromatin insulators may thus play an important role in structurally demarcating domains of independently occurring transcriptional activity. Expectedly, such domains are often subject to developmental or environmental regulation, which implies that insulators may themselves be regulated to allow for a variety of gene expression programs of an organism. Regulatory mechanisms that can influence insulator activity have been described for the vertebrate insulator protein CTCF. The parent-specific enhancer-blocking activity of CTCF at the H19/Igf2 locus is controlled by differential methylation of its binding sites within this imprinted locus (Bell and Felsenfeld, 2000; Hark *et al*, 2000). Specifically, methylation of binding sites on the paternal chromosome only prevents the binding of CTCF to allow activation of Igf2 expression. In this case, the insulator activity of CTCF is regulated at the level of its DNA-binding ability. A second regulatory mechanism of CTCF activity employs post-translational modification by poly-ADP-ribose (PAR), which appears to be involved in positive regulation of insulator activity (Yu *et al*, 2004). Conjugation of PAR to CTCF does not alter its DNA-binding properties, but may be necessary for protein–protein interactions involved in setting up chromatin domains (Klenova and Ohlsson, 2005).

Conjugation to Small Ubiquitin-like Modifier (SUMO) serves as another post-translational modification that

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regulates the activity of multiple nuclear factors. Similar to ubiquitin, SUMO is covalently attached to target proteins by a cascade of enzymes, including the activating enzyme E1, which is a heterodimer of Aos1/SAE1 and Uba2/SAE2, the conjugating enzyme E2, also known as Ubc9, and a variety of the specificity-enhancing E3 ligases (Dohmen, 2004; Hay, 2005). SUMO conjugation has been detected most often within a sumoylation consensus motif ψKxE (where ψ is a large hydrophobic amino-acid residue and x is any residue). SUMO attachment has been linked to a variety of functional outputs, including regulation of transcriptional activity, subnuclear targeting and formation of nuclear compartments. Frequently, sumoylation of proteins has been shown to alter their ability to bind DNA or other protein factors.

Multiple SUMO E3 ligases, which do not seem to be required for but enhance sumoylation of specific substrates both *in vivo* and *in vitro*, have been characterized. Members of the Siz/PIAS family of SUMO E3 ligases contain a domain homologous to the RING domain of ubiquitin E3 ligases (Johnson and Gupta, 2001; Schmidt and Muller, 2002). Recently, dTopors, which harbors a RING domain, has been characterized as a E3 ubiquitin ligase involved in *gypsy* insulator activity (Capelson and Corces, 2005), but several reports also implicate homologs of dTopors, human Topors and viral ICP0, in the SUMO pathway (Muller and Dejean, 1999; Weger *et al*, 2003, 2005; Lee *et al*, 2004). We thus investigated the possibility that *gypsy* insulator proteins are regulated by SUMO modification as well as the potential involvement of dTopors in this process, perhaps as an E3 SUMO ligase. Here, we present evidence suggesting that two components of the *gypsy* insulator complex, Mod(mdg4)2.2 and CP190, are sumoylated *in vivo* and *in vitro*, and that sumoylation negatively attenuates *gypsy* insulator activity. Specifically, SUMO conjugation interferes with nuclear coalescence of insulator bodies, suggesting that establishment of higher-order chromatin domains can be regulated by post-translational modification of insulator proteins.

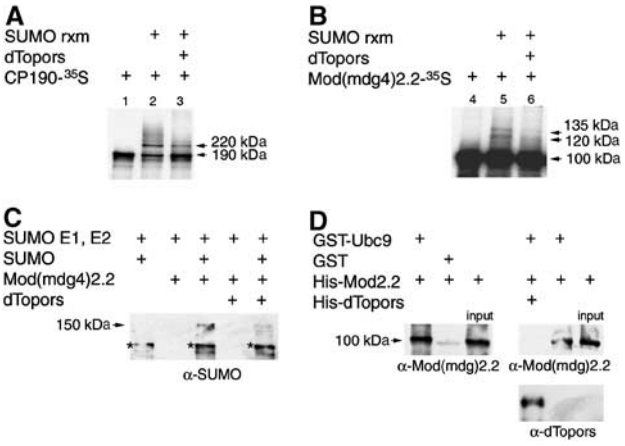

Figure 1 Mod(mdg4)2.2 and CP190 are modified by SUMO *in vitro*. The size of various protein bands is indicated in kDa. The unmodified form of Mod(mdg4)2.2 runs at 100 kDa, and the unmodified form of CP190 is 190 kDa. The sumoylated form of CP190 is 220 kDa and Mod(mdg4)2.2 shows two different sumoylated bands of 120 and 135 kDa. (**A, B**) *In vitro* sumoylation reactions with ^{35}S-labeled CP190 (A) or Mod(mdg4)2.2 (B) used as substrate, in the presence or absence of SUMO reaction components (SUMO rxm), including E1, E2 enzymes, SUMO and ATP, or of dTopors. Lane 1, CP190 alone; lane 2, CP190 with SUMO rxm; lane 3, CP190 with SUMO rxm and *in vitro*-generated dTopors; lane 4, Mod(mdg4)2.2 alone; lane 5, Mod(mdg4)2.2 with SUMO rxm; lane 6, Mod(mdg4)2.2 with SUMO rxm and *in vitro*-generated dTopors. Arrows point to sumoylated forms of CP190 and Mod(mdg4)2.2. (**C**) *In vitro* sumoylation reactions in the presence or absence of SUMO E1 and E2, SUMO, Mod(mdg4)2.2 or dTopors monitored with α-SUMO antibodies. The arrow points to the Mod(mdg4)2.2-specific SUMO-GST conjugate. The lower molecular weight band marked with an asterisk corresponds to Ubc9-SUMO-GST. (**D**) GST-Ubc9 or GST, bound to glutathione beads, were mixed with His_6-Mod(mdg4)2.2 in the presence or absence of His_6-dTopors. The precipitated fractions and input proteins were resolved by SDS–PAGE and Western blotted with α-Mod(mdg4)2.2 or α-dTopors antibodies.

Results

Insulator proteins are sumoylated in vitro

Topors, the mammalian homolog of dTopors, has been recently shown to interact with the SUMO E2-conjugating enzyme Ubc9 (Weger *et al*, 2003). This association appears to be evolutionarily conserved, as we have also detected an interaction between *Drosophila* Ubc9 and dTopors in the yeast two-hybrid assay (data not shown). To determine whether dTopors functions as an E3 SUMO ligase for insulator proteins, Su(Hw), Mod(mdg4)2.2 and CP190 were tested as substrates in an *in vitro* sumoylation reaction, in the presence or absence of dTopors. All three proteins contain lysines found within a SUMO modification consensus motif ψKxE, and can thus be potentially modified by SUMO. For each sumoylation reaction, *in vitro*-transcribed and -translated ^{35}S-labeled substrate protein was incubated with the E1, E2 enzymes, SUMO and *in vitro*-generated or -purified recombinant dTopors. Both CP190 and Mod(mdg4)2.2 can be modified by SUMO, as higher molecular weight bands appear when the SUMO conjugation machinery is added (Figures 1A and B). The addition of dTopors not only does not promote, but appears to disrupt the sumoylation of CP190 and Mod(mdg4)2.2, as assessed by decreased levels of the higher migrating modified forms of either protein in the presence of dTopors (Figures 1A and B). No disruption of sumoylation is observed upon introduction of equivalent amounts of another substrate protein in the reaction, such as the addition of Mod(mdg4)2.2 to a reaction with CP190 as a substrate (data not shown). The same pattern can be observed when detection of SUMO conjugation is carried out by using anti-SUMO antisera (Figure 1C). Again, the appearance of Mod(mdg4)2.2-specific SUMO conjugates is counteracted by the addition of recombinant purified dTopors protein.

Like many identified substrates for SUMO conjugation, CP190 and Mod(mdg4)2.2 do not seem to require the presence of an E3 ligase *in vitro* in order to be sumoylated, suggesting that they are able to bind Ubc9 directly. We confirmed this association for Mod(mdg4)2.2 and Ubc9 using a GST pull-down assay (Figure 1D, left panel). SUMO E3 ligases are thought to function as adaptor surfaces, binding the catalytic E2 and the target protein simultaneously to promote conjugation. If dTopors acts as a SUMO E3 ligase, it should be able to form a complex with both Ubc9 and Mod(mdg4)2.2 and facilitate the interaction between them. However, in agreement with the results of *in vitro* sumoylation assays, addition of dTopors to a mixture of purified GST-Ubc9 and Mod(mdg4)2.2 interferes with their interaction and

results in decreased levels of Mod(mdg4)2.2 recovered in the pulled down fraction (Figure 1D, right panel). These results suggest that dTopors may antagonize sumoylation by disrupting the association between Ubc9 and Mod(mdg4)2.2 or CP190. The ability of dTopors to reduce sumoylation of CP190 and Mod(mdg4)2.2 *in vitro* is reminiscent of the *in vivo* properties of its viral homolog ICP0, which inhibits the modification of PML and Sp100 proteins by SUMO (Muller and Dejean, 1999). Together, these findings suggest that CP190 and Mod(mdg4)2.2 are sumoylated and argue against dTopors functioning as an E3 SUMO ligase for insulator proteins. Instead, dTopors may regulate the modification of these proteins by inhibiting their sumoylation.

CP190 and Mod(mdg4)2.2 are sumoylated in vivo

To determine whether CP190 and Mod(mdg4)2.2 are sumoylated *in vivo*, we analyzed endogenous proteins from larval protein extracts prepared with and without two inhibitors of SUMO isopeptidases, *N*-ethylmalemide (NEM) and iodoacetamide (IAA) (Eloranta and Hurst, 2002). Lysis of cells in the presence of NEM and IAA results in higher levels of recovered SUMO conjugates compared to extracts prepared without the inhibitors, as demonstrated by Western analysis with SUMO antisera (Figure 2A, left panel). Similarly, this treatment results in the appearance of higher molecular weight conjugates of CP190 and Mod(mdg4)2.2 (Figure 2A, right panels). We normally detect one or two additional bands of Mod(mdg4)2.2 in protein extracts or in *in vitro* assays, which is consistent with the presence of two strong consensus sumoylation sites in the amino-acid sequence of Mod(mdg4)2.2. CP190 appears to have one predominant sumoylated form *in vivo* or *in vitro*, although its sequence contains five predicted consensus sites. No higher molecular weight forms of Su(Hw) were observed to correlate with the addition of the inhibitors, regardless of the length of the Western blot exposure.

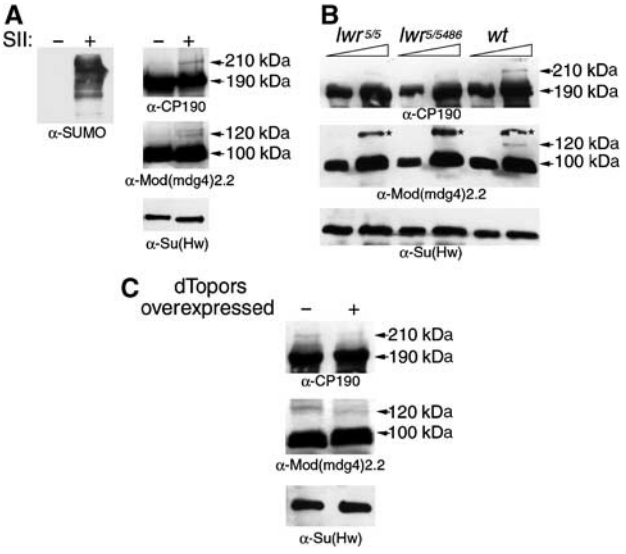

Figure 2 Mod(mdg4)2.2 and CP190 are sumoylated *in vivo*. The size of various protein bands is indicated in kDa. The unmodified form of Mod(mdg4)2.2 runs at 100 kDa, and the unmodified form of CP190 is 190 kDa. The sumoylated form of CP190 is 220 kDa and Mod(mdg4)2.2 shows two different sumoylated bands of 120 and 135 kDa. (**A**) Protein extracts from *Drosophila* larvae were prepared in the presence or absence of SUMO Isopeptidase Inhibitors (SII) NEM and IAA, resolved by SDS–PAGE and Western blotted with antibodies to SUMO and indicated *gypsy* insulator proteins. (**B**) Larval protein extracts from wild-type organisms or indicated *lwr* mutants were prepared in the presence of SII, resolved by SDS–PAGE and Western blotted with antibodies to indicated proteins. Triangles indicate increasing amounts of extracts loaded on the gels. Asterisk indicates a non-Mod(mdg4)2.2 band recognized by the antibody (Mongelard *et al*, 2002). (**C**) Protein extracts from wild-type (−) or *UAS-dTopors/ActGAL4* (+) larvae were prepared in the presence of SII, resolved by SDS–PAGE and Western blotted with antibodies to indicated proteins.

The identity of these slower migrating forms of CP190 and Mod(mdg4)2.2 was verified further by examining mutants for the SUMO conjugating E2 enzyme Ubc9, which in *Drosophila* is encoded by the gene *lesswright* (*lwr*). Protein extracts from larvae of two hypomorphic allelic combinations of *lwr*, the homozygous lwr^{5}/lwr^{5} and the transheterozyous lwr^{5}/lwr^{5486} (Apionishev *et al*, 2001), display a significant reduction in the higher molecular weight forms of CP190 and Mod(mdg4)2.2 as compared to wild type (Figure 2B). As expected, no variation in Su(Hw) was detected in these mutants.

Since dTopors was found to interfere with sumoylation *in vitro*, its ability to affect the sumoylated forms of CP190 and Mod(mdg4)2.2 was also examined *in vivo*. To this end, overexpression of dTopors was induced in larvae carrying a UAS-dTopors transgenic construct by an actin-GAL4 (*ActGAL4*) driver. Elevated levels of dTopors result in a reduction of the sumoylated forms of CP190 and Mod(mdg4)2.2, as judged by Western analysis of protein extracts obtained from larvae with induced versus uninduced dTopors (Figure 2C). Together, the observed decrease of these slower migrating protein forms in larvae mutant for the SUMO E2 ligase or larvae overexpressing dTopors reinforces the idea that they represent sumoylated forms of CP190 and Mod(mdg4)2.2.

SUMO is associated with chromatin binding sites of insulator proteins

To examine the functional relevance of the SUMO modification of insulator proteins, we analyzed whether SUMO is present at chromatin binding sites of the *gypsy* insulator complex. For this purpose, the distribution of SUMO on polytene chromosomes of *Drosophila* third instar larvae was compared to that of Mod(mdg4)2.2 and CP190. Consistent with its involvement in multiple nuclear processes and as described previously (Lehembre *et al*, 2000), SUMO localizes to hundreds of discrete binding sites throughout the genome. Furthermore, it appears to associate with different chromatin states of polytene chromosomes, including the heterochromatic chromocenter, the euchromatic decondensed interbands and the border regions between interbands and the condensed bands. A fraction of Mod(mdg4)2.2 sites, which are found exclusively at the border regions, colocalize with SUMO, as revealed by costaining of polytene chromosomes with anti-SUMO and anti-Mod(mdg4)2.2 antisera (Figure 3A). Similarly, SUMO is detected at a subset of CP190 chromatin binding sites (Figure 3B). As described previously, all binding sites of Mod(mdg4)2.2 overlap with CP190, but CP190 binds to additional sites that do not overlap with Mod(mdg4)2.2 and Su(Hw) (Pai *et al*, 2004). Although the total number of bands that overlap with SUMO is higher for CP190 than for Mod(mdg4)2.2, we estimate the percentage of sites that colocalize with SUMO is approximately

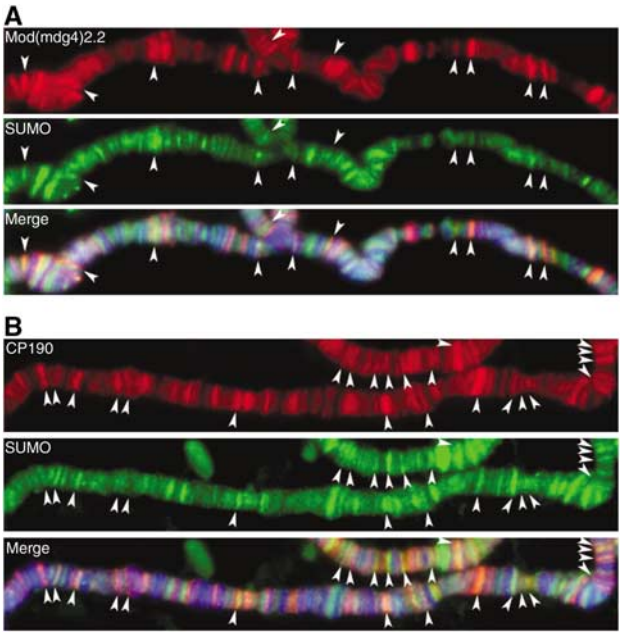

Figure 3 SUMO is associated with a fraction of *gypsy* protein complexes on chromatin. White arrows point to places of colocalization between SUMO and insulator proteins. DNA is stained with DAPI (blue). (**A**) Immunostaining of polytene chromosomes of third instar larvae with antibodies to Mod(mdg4)2.2 (red) and SUMO (green). (**B**) Immunostaining of polytene chromosomes with antibodies to CP190 (red) and SUMO (green).

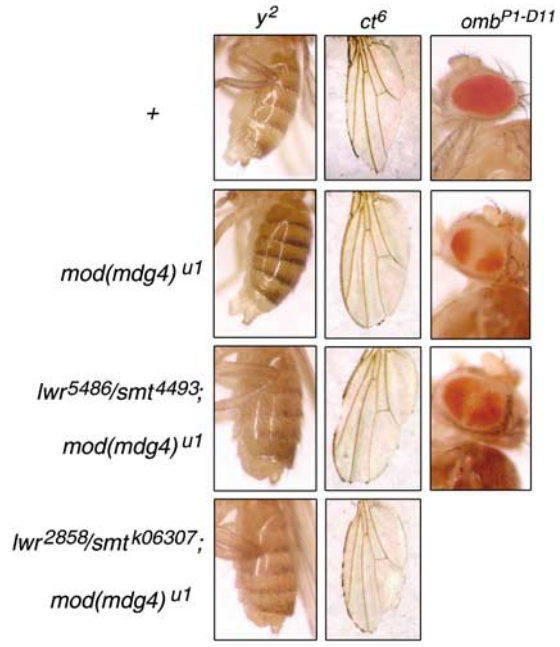

Figure 4 Mutations in components of the SUMO conjugation pathway promote activity of the *gypsy* insulator. Shown are abdomens, wings and eyes of $y^2omb^{P1\text{-}D11}ct^6$; +; +, $y^2omb^{P1\text{-}D11}ct^6$; +; $mod(mdg4)^{u1}$, $y^2omb^{P1\text{-}D11}ct^6$; $lwr^{5486}/smt3^{4493}$; $mod(mdg4)^{u1}$ and $y^2omb^{P1\text{-}D11}ct^6$; $lwr^{2858}/smt3^{k06307}$; $mod(mdg4)^{u1}$ female flies.

10–15% for both of the analyzed insulator proteins. These sites presumably correspond to regions where bound CP190 or Mod(mdg4)2.2 may be modified by SUMO. The presence of SUMO modification at a fraction of insulator sites suggests that sumoylation may be associated with a specialized insulator state.

The SUMO modification pathway antagonizes gypsy insulator activity

In order to understand the functional consequences of sumoylation of *gypsy* insulator proteins, we analyzed mutations in components of the SUMO conjugation pathway for effects on the enhancer-blocking activity of *gypsy*. To this end, three *gypsy* retrotransposon-induced mutations were used as phenotypic indicators of *gypsy* insulator function. The y^2 allele contains an insertion of *gypsy* between the body enhancer and the promoter of the *yellow* (*y*) gene, which results in low expression of the *yellow* gene product in the cuticle of adult flies (Figure 4, top panel). Similarly, the cut^6 (ct^6) mutant allele is caused by the insertion of *gypsy* between the promoter and the wing margin enhancer of the *cut* gene. Phenotypically, this is manifested by a jagged or cut appearance of the fly wing edge (Figure 4, top panel). Finally, the $omb^{P1\text{-}D11}$ mutation results from the insertion of *gypsy* between the regulatory region of the *omb* gene and the promoter of a *white* transgene inserted at this locus (Tsai *et al*, 1997). Control of *white* gene expression by the *omb* regulatory region produces a characteristic expression of the red pigment in dorsal and ventral patches of the fly eye. Insertion of the *gypsy* insulator prevents a silencing element of the *omb* regulatory region from acting on the *white* gene promoter, resulting in a broader distribution of the red pigment throughout the eye (Figure 4, top panel).

In order to test for positive or negative effects of sumoylation on insulator activity, mutations in SUMO pathway components were examined in the sensitized background of a compromised insulator created by a null mutation in Mod(mdg4)2.2, $mod(mdg4)^{u1}$ (Mongelard *et al*, 2002). Lack of Mod(mdg4)2.2 partially disrupts insulator activity, thus increasing enhancer–promoter communication. At y^2 and ct^6, this is manifested by an increase in gene product expression, which results in a more pigmented abdomen or a smoother wing edge, respectively (Figure 4, second from top panel). At the $omb^{P1\text{-}D11}$ locus, the $mod(mdg4)^{u1}$ mutation yields an opposite effect on transcription, since in this case, the insulator interferes with a silencer–promoter communication. Thus, the result is less *white* gene product or smaller areas of red pigmentation in the eye (Figure 4, second from top panel).

Combined mutations of the SUMO E2-conjugating enzyme *lwr* and of the SUMO gene *smt3* suppress the effect of $mog(mdg4)^{u1}$ on *gypsy*-induced phenotypes, suggesting that SUMO conjugation reduces *gypsy* insulator activity. Two different heterozygous combinations of *lwr* and *smt3*, $lwr^{5486}/smt3^{4493}$ and $lwr^{2858}/smt3^{k06307}$, produced similar effects on y^2, ct^6 and $omb^{P1\text{-}D11}$. The phenotype of homozygotes of these alleles could not be assessed as none of them survive to adulthood. A decreased expression of *yellow* or a lighter abdomen in y^2; $lwr5486/smt3^{4493}$; $mod(mdg4)^{u1}$ or y^2; $lwr^{2858}/smt3^{k06307}$; $mod(mdg4)^{u1}$ flies was observed when compared to y^2; $mod(mdg4)^{u1}$ controls (Figure 4, bottom two panels), indicative of improved enhancer-blocking activity of the *gypsy* insulator. Similarly, increased insulator activity is detected for ct^6, judging from the more jagged

and discontinuous wing edge in flies that bear SUMO pathway mutations in the *mod(mdg4)^{u1}* background relative to *mod(mdg4)^{u1}* flies (Figure 4, bottom two panels). The eyes of *omb$^{P1\text{-}D11}$; lwr^{5486}/smt3^{4493}; mod(mdg4)^{u1}* mutants display broader distribution of red pigment than those of the *omb$^{P1\text{-}D11}$; +; mod(mdg4)^{u1}* controls (Figure 4, second from bottom panel), indicative of increased insulator activity, implying that the SUMO conjugation pathway is antagonistic to normal *gypsy* insulator function. The observed phenotypic changes are subtle due to the fact that only heterozygous combinations of these alleles could be tested. The effects observed in *ct^{6}* and *omb$^{P1\text{-}D11}$* are consistent within the population of mutant flies and the photographs shown in Figure 4 are a good representation of the observed effects. Phenotypic changes at *y^{2}* in the *lwr/smt3* mutants occur in approximately 30% of flies of either genotype (the remaining 70% display a continuum of less pronounced effects). Interestingly, all of these genetic interactions are particularly pronounced in females, although they are detectable in both sexes. The effects of the *smt3^{k06307}* mutation on *omb$^{P1\text{-}D11}$* could not be assessed because this mutation is caused by insertion of a P-element carrying the *white* gene. These results suggest that *lwr* and the SUMO conjugation pathway are involved in negative regulation of *gypsy* insulator function.

Sumoylation of insulator proteins does not regulate their binding to DNA

It has been reported that sumoylation can interfere with the DNA-binding ability of some transcription factors or with their recruitment to certain chromatin locations (Goodson *et al*, 2001; Chalkiadaki and Talianidis, 2005). Similarly, sumoylation of CP190 or Mod(mdg4)2.2 may disrupt their binding to chromatin. To investigate this possibility, we analyzed the effects of varying levels of Ubc9 on the binding of insulator proteins to polytene chromosomes. The localization of Mod(mdg4)2.2 and CP190 appears unchanged in polytene chromosomes of *lwr* mutant larvae or larvae overexpressing *UAS-lwr* induced by the *ActGAL4* driver, as compared to wild type (Figure 5A). No significant changes in the levels of chromatin-associated insulator proteins or in their global binding patterns were observed.

Since phenotypic consequences of mutations in the SUMO pathway were analyzed in the *mod(mdg4)^{u1}* background, we also examined the binding of CP190 to chromosomes in *lwr^{5}/lwr^{5486}; mod(mdg4)^{u1}* and *mod(mdg4)^{u1}* mutants. No variation in the overall binding of CP190 to polytene chromosomes was detected in larvae mutant for *lwr* and *mod(mdg4)* as compared to larvae mutant for *mod(mdg4)* alone (Figure 5B). Since we had not observed sumoylation of Su(Hw), its binding was not expected to be altered in the absence of the SUMO E2 ligase. Consequently, similar levels of Su(Hw) are found at the *y^{2}* locus in the *lwr^{5}/lwr^{5486}; mod(mdg4)^{u1}* genetic background relative to *mod(mdg4)^{u1}* (Figure 5B, inset). Likewise, CP190 is detected in comparable amounts at the *y^{2}* locus in these mutants, suggesting that sumoylation does not affect the ability of insulator proteins to associate with chromatin.

Sumoylation disrupts nuclear clustering of insulator proteins

SUMO conjugation has also been described to regulate protein–protein interactions, where sumoylation of proteins causes them to lose certain associations or to gain new ones

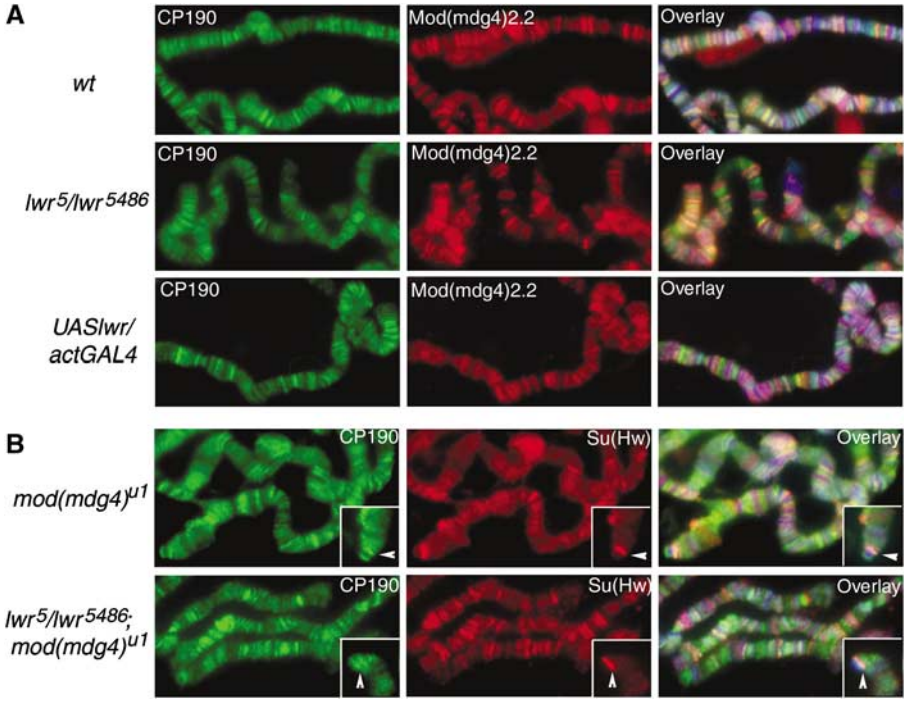

Figure 5 Sumoylation does not affect the binding of CP190 and Mod(mdg4)2.2 to chromatin. DNA is stained with DAPI (blue). (**A**) Immunostaining of polytene chromosomes from wild-type, *UASlwr/ActGAL4* and *lwr$^{5/5486}$* larvae with antibodies to Mod(mdg4)2.2 (red) and CP190 (green). (**B**) Immunostaining of polytene chromosomes from *y^{2}; +; mod(mdg4)^{u1}* and *y^{2}; lwr$^{5/5486}$; mod(mdg)^{u1}* larvae with antibodies to Mod(mdg4)2.2 (red) and CP190 (green). Arrows point to the *y^{2}* locus (insets).

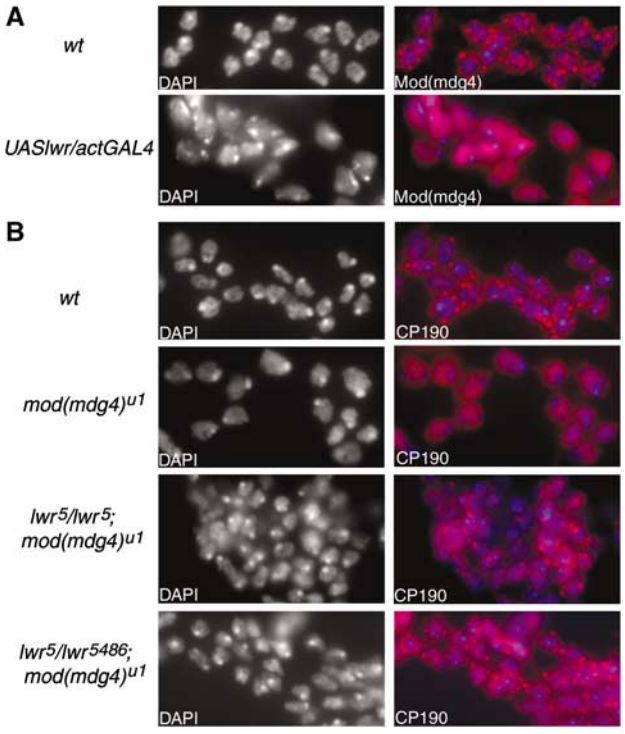

Figure 6 SUMO conjugation antagonizes nuclear coalescence of insulator proteins. (**A**) Immunostaining of diploid cells from brains and imaginal discs of wild-type and *UASlwr/ActGAL4* larvae with antibodies to Mod(mdg4) (red). (**B**) Immunostaining of diploid cells of wild-type, *mod(mdg4)^{u1}*, *lwr$^{5/5}$*; *mod(mdg)^{u1}* and *lwr$^{5/5486}$*; *mod(mdg)^{u1}* larvae with antibodies to CP190 (red). DAPI alone is shown at left and in blue in overlay.

(Seeler *et al*, 2001; Girdwood *et al*, 2003). As a consequence of this, sumoylation has been shown to induce subnuclear reorganization, such that sumoylated proteins are associated with nuclear compartments different from those of their unmodified counterparts (Kim *et al*, 1999; Ross *et al*, 2002). Since nuclear organization of *gypsy* insulator proteins is functionally linked to insulator activity (Gerasimova and Corces, 1998; Pai *et al*, 2004; Capelson and Corces, 2005), we wanted to investigate whether SUMO modification of Mod(mdg4)2.2 and CP190 can affect their ability to form insulator bodies.

Overexpression of Ubc9 by driving the expression of *UAS-lwr* with *ActGAL4* causes a dramatic dispersal of insulator bodies, as assessed by immunostaining of diploid cells of third instar larvae with anti-Mod(mdg4) antiserum (Figure 6A). Staining of DNA with DAPI is shown as a control for nuclear integrity. These findings are consistent with the observed genetic interference of sumoylation with the enhancer-blocking function of *gypsy*. Furthermore, they suggest that SUMO conjugation negatively regulates insulator activity by interfering with nuclear coalescence of insulator proteins.

This conclusion is further substantiated by the effect of lower levels of Ubc9 on the distribution of *gypsy* insulator bodies. In the absence of Mod(mdg4)2.2, the formation of insulator bodies is disrupted due to the loss of one of the bridging components of the insulator complex (Gerasimova and Corces, 1998). For both of the analyzed combinations of mutant alleles of *lwr* in the *mod(mdg4)^{u1}* background,

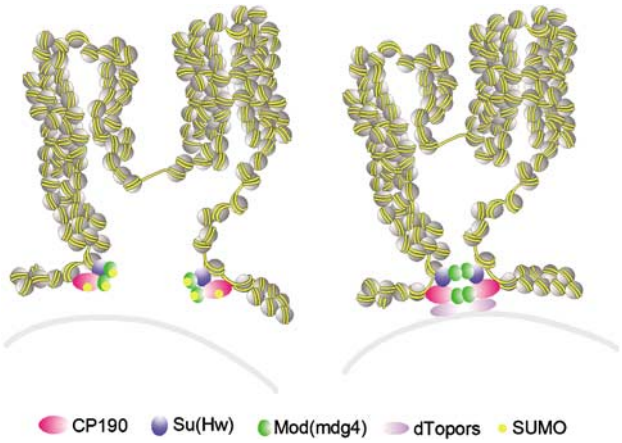

Figure 7 Model for the effect of SUMO modification on chromatin domain formation. Chromatin loop domains may be established through interactions via distant *gypsy* insulator complexes and further stabilized through the tethering function of dTopors (right). Sumoylation of CP190 and Mod(mdg4)2.2 interferes with their self-interactions, resulting in a breakdown of chromatin domains (left). Gray line represents the nuclear lamina, and yellow spheres represent nucleosomes.

lwr$^{5/}$lwr^{5}; mod(mdg4)^{u1} and *lwr$^{5/}$lwr^{5486}; mod(mdg4)^{u1}*, insulator bodies, marked with anti-CP190 antibodies, are seen to reform in a pattern similar to that observed in wild-type cells (Figure 6B). The correlation of improved nuclear clustering of insulator proteins with lower levels of their modification by SUMO suggests that sumoylation may interfere with self-interactions of distant insulator complexes (Figure 7). This is consistent with the presence of consensus sumoylation sites within the BTB domain of Mod(mdg4)2.2 and immediately preceding the BTB domain of CP190, since these domains are involved in mediating interactions between these two proteins (Pai *et al*, 2004).

Discussion

Two protein components of the *gypsy* chromatin insulator, Mod(mdg4)2.2 and CP190, were found to be modified by SUMO *in vitro* and *in vivo*. dTopors was observed to interfere with their sumoylation by possibly disrupting the contacts between the SUMO E2 enzyme Ubc9 and substrate insulator proteins. The inhibitory effect of dTopors, although relatively subtle, is consistent across the various assays utilized such that any time dTopors was introduced at higher levels, either by direct addition *in vitro* or by increasing expression *in vivo*, it was found to result in reduced sumoylation of Mod(mdg4)2.2 and CP190. Disruption of SUMO conjugation by mutations in genes coding for Ubc9 and SUMO exerts a positive effect on *gypsy* insulator activity, suggesting that the normal role of SUMO modification is to antagonize insulator function. A fraction of chromatin-bound insulator proteins appears to be associated with SUMO, yet mutations in the SUMO pathway are not seen to affect the chromatin-binding properties of CP190 or Mod(mdg4)2.2. Instead, sumoylation interferes with the formation of nuclear insulator bodies, such that overexpression of Ubc9 leads to breakdown of nuclear insulator structures, whereas lower levels of Ubc9 and sumoylation result in a partial recovery of coalescence lost in the absence of Mod(mdg4)2.2.

These findings suggest that modification of CP190 and Mod(mdg4)2.2 by SUMO may prevent self-association and thus interfere with long-range interactions between distant insulator complexes required to form insulator bodies (Figure 7). Thereby, sumoylation may preclude formation of closed chromatin loops and the consequent establishment of autonomous gene expression domains.

Multiple lines of evidence point to a role for SUMO modification in transcriptional repression. Sumoylation of histones has been characterized as a mark of repressed chromatin (Shiio and Eisenman, 2003), whereas SUMO conjugation to certain transcriptional regulators leads to their association with histone deacetylases, which remove the active acetylation marks from histones (Girdwood *et al*, 2003; Yang and Sharrocks, 2004). SUMO modification of the Polycomb group (PcG) protein SOP-2 is required for its function in stable repression of *Hox* genes (Zhang *et al*, 2004), and another PcG repressor, Pc2, acts as a SUMO E3 ligase (Kagey *et al*, 2003). Modification of *gypsy* insulator proteins by SUMO does not seem to associate them exclusively with transcriptional repression, as reduction of sumoylation in *lwr/smt3* mutants results in the upregulation of expression from the $omb^{P1\text{-}D1}$ locus, but in the downregulation of transcription at y^2 and ct^6. In these cases, transcriptional output appears to correlate only with the enhancer-blocking activity of the insulator. Nevertheless, it is possible that one of the roles of sumoylation involves association of selected insulator sites in the genome with transcriptional repression. Sumoylated insulator complexes may not participate in the formation of expression domains, but instead, could target silencing factors to the surrounding chromatin.

In mammalian nuclei, the homolog of dTopors localizes to PML bodies, which are enriched in the SUMO conjugation machinery (Rasheed *et al*, 2002). If inhibition of sumoylation is also a property of mammalian Topors, it may play a role in preventing further sumoylation of factors that are targeted to these nuclear compartments. In this manner, ICP0 also localizes to the PML bodies, where it causes desumoylation of two primary components, PML and SP100 (Muller and Dejean, 1999). It was recently reported that Topors may function as a SUMO E3 ligase for the tumor suppressor p53 protein (Weger *et al*, 2005). This apparent contradiction with our results may be due to several reasons. Topors and dTopors may have diverged their functions regarding the SUMO pathway, such that Topors functions as a SUMO E3 while dTopors interferes with SUMO addition due to its conserved interaction with Ubc9. Alternatively, the involvement of dTopors in the SUMO pathway may be substrate-specific, since it may bind to Ubc9 in ways that allow for interaction with a given target protein or prevent it. In the context of the *gypsy* insulator, the interference of dTopors with sumoylation is consistent with previous observations that dTopors promotes insulator activity (Capelson and Corces, 2005), whereas sumoylation appears to disrupt it.

It has been suggested that SUMO conjugation may affect the function of the modified protein even after the SUMO tag itself has been removed, creating a cellular memory for protein regulation (Hay, 2005). This idea has arisen partly to explain the commonly observed contradiction between the small percentage of a given protein that is modified by SUMO and the dramatic consequences of the modification on the protein's cellular function. Sumoylation may be needed for proteins to enter stable complexes or functional states, but the persistence of the SUMO modification may not be required after the initial establishment. Thus, the actual effect of sumoylation may far exceed that of the detectable sumoylated population since the function of a much larger proportion of molecules has been altered by SUMO conjugation and subsequent deconjugation. Similarly to other reported cases, the sumoylated forms of Mod(mdg4)2.2 and of CP190 represent a small fraction of the total pool of the insulator proteins, yet the phenotypic effects of the loss of these forms are quite striking. It is possible that SUMO attachment regulates the initial organization of chromatin domains, perhaps in earlier development or following mitosis, yet once established, the domains may be stably maintained without SUMO. Additionally, the rapid conjugation and deconjugation cycle of the SUMO tag implies that sumoylation may be used by processes that require reassembly upon signal. In that sense, SUMO modification seems particularly suitable for the regulation of gene expression domains as it can result in 'remembered' yet flexible states.

Materials and methods

In vitro sumoylation

For reactions with radioactively labeled substrates, CP190, Mod(mdg4)2.2 or Su(Hw) were *in vitro*-transcribed and -translated with ^{35}S-methionine (using TNT Coupled Rabbit Reticulocyte Lysate System; Promega). A measure of 3 μl of each TNT reaction were mixed with 150 ng of SAE1/SAE2, 1000 ng of Ubc9, 1000 ng of SUMO (all components of the sumoylation kit from LAE Biotech # K007), with or without 1000 ng of recombinant purified His_6-dTopors or 3 μl of *in vitro*-transcribed/translated dTopors. Reactions were carried out in a buffer containing 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP for 60 min at 37°C, resolved on 7.5% SDS–PAGE gels and visualized by autoradiography. To study the effect of dTopors on sumoylation of Mod(mdg4)2.2 and Cp190 *in vitro*, we used increasing concentrations of *in vitro*-transcribed/translated protein ranging from 1 to 5 μl. No effect was observed below 3 μl, and this was the concentration used in the experiments shown in Figure 1. For reactions to be detected by Western blotting, 400 ng of recombinant purified *Drosophila* Ubc9-GST (the pGEX-Ubc9 construct was a gift from Dr L Griffith) and 150 ng of SAE1/SAE2 (LAE # P006) were combined in a buffer containing 50 mM Tris, pH 7.5, 2.5 mM $MgCl_2$, 0.1 mM DTT, 5 mM ATP, with or without 3 μg of recombinant purified *Drosophila* SUMO-GST (the pGEX-SMT3gg construct was a gift from Dr A Courey), 400 ng of recombinant purified His_6-Mod(mdg4)2.2 and 1000 ng of recombinant purified His_6-dTopors, and the reactions were carried out for 120 min at 30°C. Western blotting was carried out with α-SUMO antibodies (a gift from Dr A Dejean) at 1/10 000 dilution.

Recombinant protein purification and GST pull-down assays

Cultures of GST-Ubc9, GST-SUMO, GST, His_6-Mod(mdg4)2.2 and His_6-dTopors, transformed into the Rosetta bacterial strain (Novagen), were induced with 0.1 mM IPTG, grown for 3 h, lysed by sonication in PBS with 1% Triton X and purified by either glutathione or Ni chromatography. For GST pull-down assays, 0.5 ml of lysate of cultures expressing GST-Ubc9 or GST were incubated with glutathione-conjugated beads for 3 h at 4°C, washed once with PBS and combined with purified His_6-Mod(mdg4)2.2, with or without equimolar amounts of His_6-dTopors. After overnight incubation, bound proteins were washed with PBS, eluted by boiling and analyzed by Western blotting.

Preparation of protein extracts and Western blot analysis

Protein extracts from third instar larvae were prepared as described (Capelson and Corces, 2005), with or without SUMO isopeptidase inhibitors NEM at 80 mM and IAA at 0.2 mM. Proteins were resolved on 7.5% SDS–PAGE and transferred to PVDF membranes

in glycine buffer with 7% methanol. Blots were probed as described (Capelson and Corces, 2005).

Fly strains and crosses

Fly stocks were maintained in standard medium at 25°C. Stocks of *smt3*^{4493} and *smt3*^{k06307} mutations were obtained from the Bloomington Stock Center. The *lwr*^{5486}, *lwr*^{5}, *lwr*^{2858} mutant strains and the *UAS-lwr* transgenic strain were a gift from Dr S Tanda.

Immunohistochemistry

Immunostaining of polytene chromosomes and diploid cells of larval imaginal discs and brains was carried out as described previously (Gerasimova and Corces, 1998; Gerasimova *et al*, 2000). Rabbit and rat α-CP190 antibodies were used at 1:400 and at 1:100 dilutions, respectively, and rabbit α-SUMO at 1:50 dilution.

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