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The Ubiquitin Ligase dTopors Directs the Nuclear Organization of a Chromatin Insulator

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Summary

Chromatin insulators are gene regulatory elements implicated in the establishment of independent chromatin domains. The *gypsy* insulator of *D. melanogaster* confers its activity through a protein complex that consists of three known components, Su(Hw), Mod(mdg4)2.2, and CP190. We have identified a factor, *Drosophila* Topoisomerase I-interacting RS protein (dTopors) that interacts with the insulator protein complex and is required for *gypsy* insulator function. In the absence of Mod(mdg4)2.2, nuclear clustering of insulator complexes is disrupted and insulator activity is compromised. Overexpression of dTopors in the *mod(mdg4)2.2* null mutant rescues insulator activity and restores the formation of nuclear insulator bodies. dTopors associates with the nuclear lamina, and mutations in lamin disrupt dTopors localization as well as nuclear organization and activity of the *gypsy* insulator. Thus, dTopors appears to be involved in the establishment of chromatin organization through its ability to mediate the association of insulator complexes with a fixed nuclear substrate.

Introduction

A major challenge for the eukaryotic genome is to regulate the proper expression of its independent gene units. To accomplish this goal, genomes are proposed to be organized into structural chromatin domains that promote the autonomy of gene activity. One class of regulatory elements that has been implicated in establishing such domains is chromatin insulators or boundary elements. Chromatin insulators are operationally defined by two properties: they are able to interfere with enhancer-promoter communication (enhancer blocking activity) (Geyer and Corces, 1992; Kellum and Schedl, 1992) and they shield integrated transgenes from the effects of the surrounding chromatin (barrier activity) (Chung et al., 1993; Kellum and Schedl, 1991). Chromatin insulators have now been characterized in a variety of species, suggesting their importance in the global regulation of gene expression. Some of the best-characterized insulators include the chicken β-*globin* insulator, the *Fab-7* insulator element found in the *bithorax* complex of *Drosophila*, and the *scs* and *scs'* insulators that flank one of the fly *hsp70* loci (West et al., 2002).

Similar to other transcription regulatory elements, insulators consist of a specific DNA sequence and associated proteins. The ~350 bp DNA sequence of the *gypsy* insulator of *Drosophila* was originally identified in the 5′ UTR of the *gypsy* retrotransposon, based on the enhancer blocking activity found in that region (Geyer and Corces, 1992). This *gypsy* insulator sequence includes 12 tandemly repeated binding sites for the zinc finger protein Suppressor of Hairy-wing (Su[Hw]), which interacts with Modifier of mdg4 2.2 (Mod[mdg4]2.2), a second component of the *gypsy* insulator complex (Gause et al., 2001; Ghosh et al., 2001). Both proteins associate with an additional DNA binding factor, Centrosomal Protein 190 (CP190), which has been recently characterized as a third component of the *gypsy* insulator (Pai et al., 2004).

Although originally isolated from a retrotransposon, the *gypsy* insulator protein complex is present at hundreds of endogenous sites throughout the fly genome, as established by the analysis of the *Drosophila* polytene chromosomes (Gerasimova and Corces, 1998). Insulator proteins may be involved in similar processes of establishing chromatin boundaries at their endogenous loci. This idea is supported by the observation that the *gypsy* complex is preferentially localized to the borders between condensed and decondensed chromatin (Labrador and Corces, 2002; Pai et al., 2004). One such endogenous binding site has been recently identified as a 432 bp sequence in the 3′ region of the *yellow* gene (Golovnin et al., 2003; Parnell et al., 2003). This sequence possesses an enhancer blocking activity and may constitute a boundary between the regulatory regions of *yellow* and *achaete* genes.

In the nuclei of diploid cells, insulator proteins coalesce into large foci, termed insulator bodies (Gerasimova and Corces, 1998). These nuclear macro-complexes are hypothesized to bring distant insulator sites into physical proximity by looping out the intervening chromatin fiber to establish isolated chromatin domains (Gerasimova et al., 2000; Labrador and Corces, 2002). The self-interacting properties of the BTB/POZ domain, which is present in both Mod(mdg4)2.2 and CP190, are thought to mediate this clustering. This is supported by the observed dissolution of the insulator bodies in mutants that disrupt either the *mod(mdg4)* or *CP190* genes (Gerasimova and Corces, 1998; Pai et al., 2004). The ability of the *gypsy* insulator complexes to bring separate genomic loci together is further suggested by the observation that two *gypsy* insulators positioned in tandem lose their enhancer-blocking activity (Cai and Shen, 2001; Muravyova et al., 2001). This property has been proposed to reflect the ability of *gypsy* insulators to mediate long-distance chromatin interactions through physical clustering of its proteins. In addition to *gypsy*, evidence for physical association of insulators has been reported for other boundary elements, such as the contact observed between the *scs* and the *scs'* sequences (Blanton et al., 2003) and the recruitment of transgenic *Fab-7* elements by the endogenous *Fab-7* insulator of the Bithorax complex (Bantignies et al., 2003).

The establishment of higher-order chromatin domains by insulators may result from self-interactions or

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from association with a relatively fixed nuclear substrate (Capelson and Corces, 2004; West et al., 2002). The importance of utilizing a nuclear scaffold for chromatin attachment has been described for the chicken *β-globin* insulator (Yusufzai et al., 2004) and for the barrier elements of yeast (Ishii et al., 2002). Likewise, in addition to self-associations via Mod(mdg4)2.2 and CP190, *gypsy* insulator activity has been proposed to involve interactions with a nuclear scaffold (Byrd and Corces, 2003), though factors involved in mediating these interactions have not yet been identified. Here, we report the identification of a factor required for *gypsy* insulator activity, dTopors. dTopors, which has been previously reported to possess an E3 ubiquitin ligase activity (Secombe and Parkhurst, 2004), interacts with known *gypsy* insulator proteins and promotes the enhancer blocking function of *gypsy*. Furthermore, dTopors associates with the nuclear lamina and facilitates the coalescence of insulator bodies. We propose that the role of dTopors in *gypsy* insulator activity is to direct the establishment of chromatin domains by mediating the association between nuclear insulator complexes and the nuclear lamina.

Results

dTopors Interacts with Components of the *gypsy* Insulator Complex

To identify factors involved in insulator activity, we employed the yeast two-hybrid approach to screen for interactors with one of the *gypsy* insulator protein components, Mod(mdg4)2.2. Mod(mdg4)2.2, fused to the DNA binding protein LexA, was used as bait in the screen of a *Drosophila* embryonic cDNA library consisting of cDNA clones fused to the transcriptional activator VP16. We identified 256 positive clones that activated both the *HIS3* nutritional and *β-galactosidase* color indicator reporters in the initial screen of the cDNA library. To select further for reproducible interactions, the bait plasmid loss-reintroduction procedure was performed, resulting in six true positive clones, which were then sequenced for identification. Two of these clones were found to encode the cDNA of CG15104 or dTopors, the *Drosophila* homolog of the Topoisomerase I binding RS protein Topors. The mammalian Topors protein has been shown to interact with Topoisomerase I and p53 (Haluska et al., 1999; Zhou et al., 1999) and is a potential tumor suppressor protein downregulated in several cancer cell lines (Saleem et al., 2004). dTopors harbors an evolutionarily conserved RING domain, which is highly homologous to the RING domain of the viral E3 ubiquitin ligase ICP0, a factor involved in the transcriptional regulation of the herpes viral genome (Everett, 2000). Recently, dTopors was found to regulate the levels of Hairy, a *Drosophila* transcriptional repressor (Secombe and Parkhurst, 2004).

To test whether the newly isolated factor interacts with other components of the *gypsy* complex, we assayed the growth of yeast strains expressing the VP16-dTopors and each of the LexA-insulator protein constructs on plates with and without histidine. Full-length dTopors was found to interact with Su(Hw), in addition to Mod(mdg4)2.2 (Figure 1A). The interaction between VP16-dTopors and LexA-Su(Hw) was slightly less robust than that observed for the VP16-dTopors and LexA-Mod(mdg4)2.2 combination, as judged from the average growth rate and colony size for the two strains. Both strains grew distinctly above the levels of the negative controls LexA-Mod(mdg4)2.2 and LexA-Su(Hw) coexpressed with VP16 alone, as well as VP16-dTopors coexpressed with LexA-ENT1, a normally cytoplasmic protein (Figure 1A). We did not detect any significant interaction between dTopors and CP190 in this assay (data not shown).

We next tested whether the interactions between dTopors and insulator proteins could be confirmed in vitro. Recombinant GST-dTopors, His_6-tagged Mod(mdg4)2.2 (His-Mod[mdg4]2.2), and His_6-tagged Su(Hw) (His-Su[Hw]) were purified from *E. coli* and tested for interaction. In contrast to GST alone, GST-dTopors bound to glutathione Sepharose was able to coprecipitate both His-Mod(mdg4)2.2 and His-Su(Hw) (Figure 1B). Therefore, the association between dTopors and the two insulator proteins is likely to be direct.

To determine the relevance of these interactions in vivo, a polyclonal antibody was raised against a recombinant purified dTopors and used to coimmunoprecipitate insulator proteins from extracts prepared from third instar larval tissues. Mod(mdg4)2.2 and, to a lesser extent, Su(Hw) and CP190 are immunoprecipitated by using α-dTopors antisera, but not with the preimmune serum (Figure 1C). Because no interaction was observed between CP190 and dTopors in the yeast two-hybrid, the association between CP190 and dTopors may be indirect. The reverse immunoprecipitation, using antibodies directed against Mod(mdg4)2.2, similarly resulted in retention of dTopors in the IP fraction. Together, these findings confirm the in vivo association of dTopors with all three known *gypsy* insulator protein components.

dTopors Facilitates *gypsy* Insulator Activity

To investigate the possible involvement of dTopors in insulator function, we analyzed the effects of varying dTopors levels on *gypsy* insulator enhancer blocking activity. To this end, alleles generated by the insertion of the *gypsy* retrotransposon, such as *yellow-2* (y^2) and *optomotor blind-P1-D11* ($omb^{P1\text{-}D11}$), were used as phenotypic markers for insulator activity. In the y^2 allele, the *gypsy* insulator disrupts the communication between the body enhancer and the promoter of the *yellow* gene, resulting in a drastic reduction of *yellow* gene expression and a concomitant decrease in the production of black pigment in the body cuticle (Geyer and Corces, 1992). The $omb^{P1\text{-}D11}$ mutation is caused by the insertion of *gypsy* between a silencer element present in the regulatory region of the *omb* gene and the promoter of the *white* gene, found in the P-element inserted in the region (omb^{P1}). This results in a characteristic distribution of patches of red pigment in the dorsal and ventral regions of the eye. The size of the red patches is dependent on the activity of the *gypsy* insulator, such that a less functional insulator produces smaller patches (Tsai et al., 1997).

Reduced levels of dTopors were found to disrupt *gypsy* insulator activity, as judged from changes in the

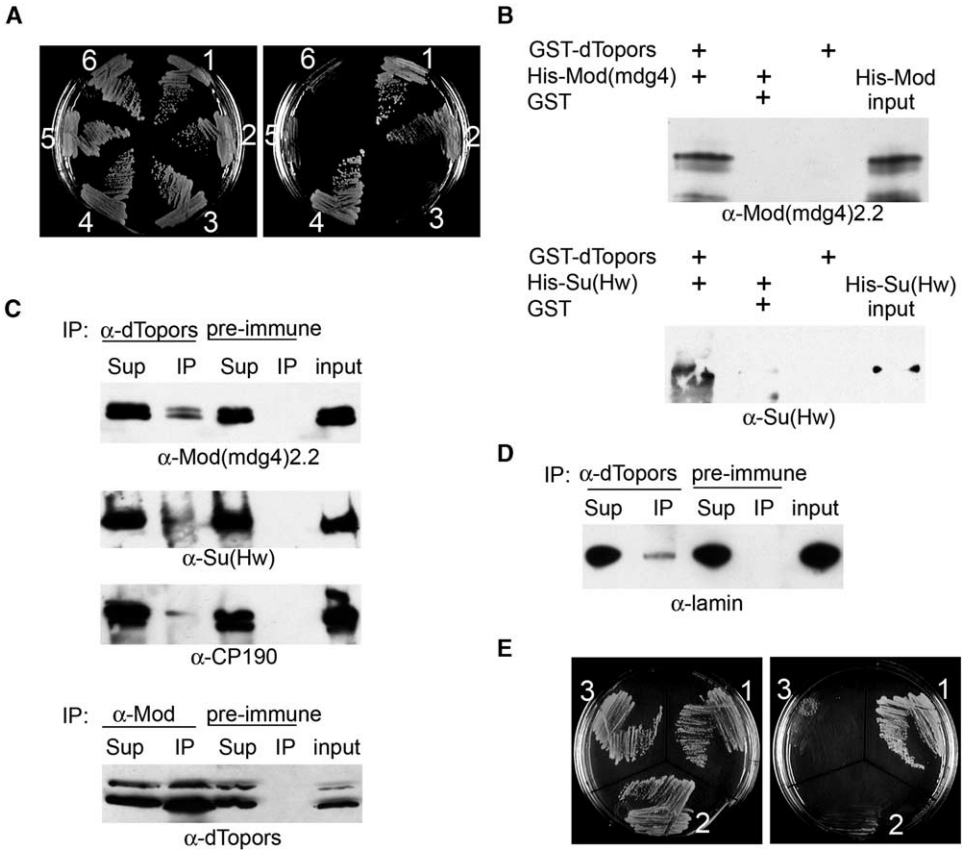

Figure 1. dTopors Interacts with Proteins of the *gypsy* Insulator Complex

(A) Growth of yeast strains expressing dTopors, insulator proteins, and controls in various combinations, on media nonselective (+Histidine) (left) or selective (–Histidine) (right) for the *HIS3* reporter gene. (1) Yeast expressing dTopors-VP16 and Mod(mdg4)2.2-LexA. (2) dTopors-VP16 and Su(Hw)-LexA. (3) dTopors-VP16 and ENT1-LexA. (4) Su(Hw)-VP16 and Mod(mdg4)2.2-LexA. (5) VP16 and Mod(mdg4)2.2-LexA. (6) VP16 and Su(Hw)-LexA.

(B) GST-dTopors or GST bound to glutathione beads was incubated with or without His-Mod(mdg4)2.2 (top) or His-Su(Hw) (bottom) and Western blotted with indicated antibodies.

(C and D) Larval protein extracts were incubated with rabbit α-dTopors, rabbit α-Mod(mdg4), or preimmune antisera. Lysates (input), precipitated fractions (IP), and unbound fractions (Sup) were Western blotted with rat antibodies against indicated proteins.

(E) Growth of yeast strains expressing lamin and dTopors, Su(Hw), or control on nonselective (+Histidine) (left) or selective (–Histidine) (right) media. Lamin-LexA and (1) dTopors-VP16 or (2) VP16 or (3) Su(Hw)-VP16.

phenotype of *gypsy*-induced mutations, suggesting that dTopors is required for proper function of the insulator. A genomic deletion, *Df(2R)P34*, which spans the genetic locus of dTopors in addition to other genes in the region, was found to disrupt the function of the insulator at both the y^2 and the $omb^{P1\text{-}D11}$ loci in a dominant fashion. This is manifested by increased production of black pigment in the abdomen of y^2; *Df(2R)P34*/+ flies compared to y^2 controls, indicating a reduced ability of the insulator to block the body enhancer from activating the *yellow* gene (Figure 2A). Similarly, reduced production of red pigment in the eyes of $omb^{P1\text{-}D11}$; *Df(2R)P34*/+ flies relative to the $omb^{P1\text{-}D11}$ controls signifies a decreased ability of the *gypsy* insulator to prevent the action of the nearby silencer on the promoter of the *white* gene. To confirm that the effects of *Df(2R)P34* originate from the disruption of dTopors, we generated an inducible RNAi construct in which a fragment of the dTopors cDNA was cloned in a tail-to-tail orientation under the control of a UAS element. The production of the disruptive hairpin dsRNA was induced by a GAL4 driver under the control of a constitutive *Actin* promoter, Actin-GAL4. When induced, the *UAS-dTopors-RNAi* construct similarly resulted in increased production of the *yellow* gene product or darker abdomen (Figure 2A).

Because reduced levels of dTopors were found to disrupt insulator activity, we postulated that elevated levels of this protein may improve it. We thus tested overexpression of dTopors by generating transgenic flies that can express inducible UAS-driven dTopors fused to GFP (*UAS-dTopors*). In order to be able to assay for improved insulator activity, we overexpressed dTopors in flies with a null mutation in Mod(mdg4)2.2, $mod(mdg4)^{u1}$, which displays reduced insulator activity (Mongelard et al., 2002). When dTopors is overexpressed in the $mod(mdg4)^{u1}$ background using the Actin-GAL4 driver, we observe a dramatic rescue of the

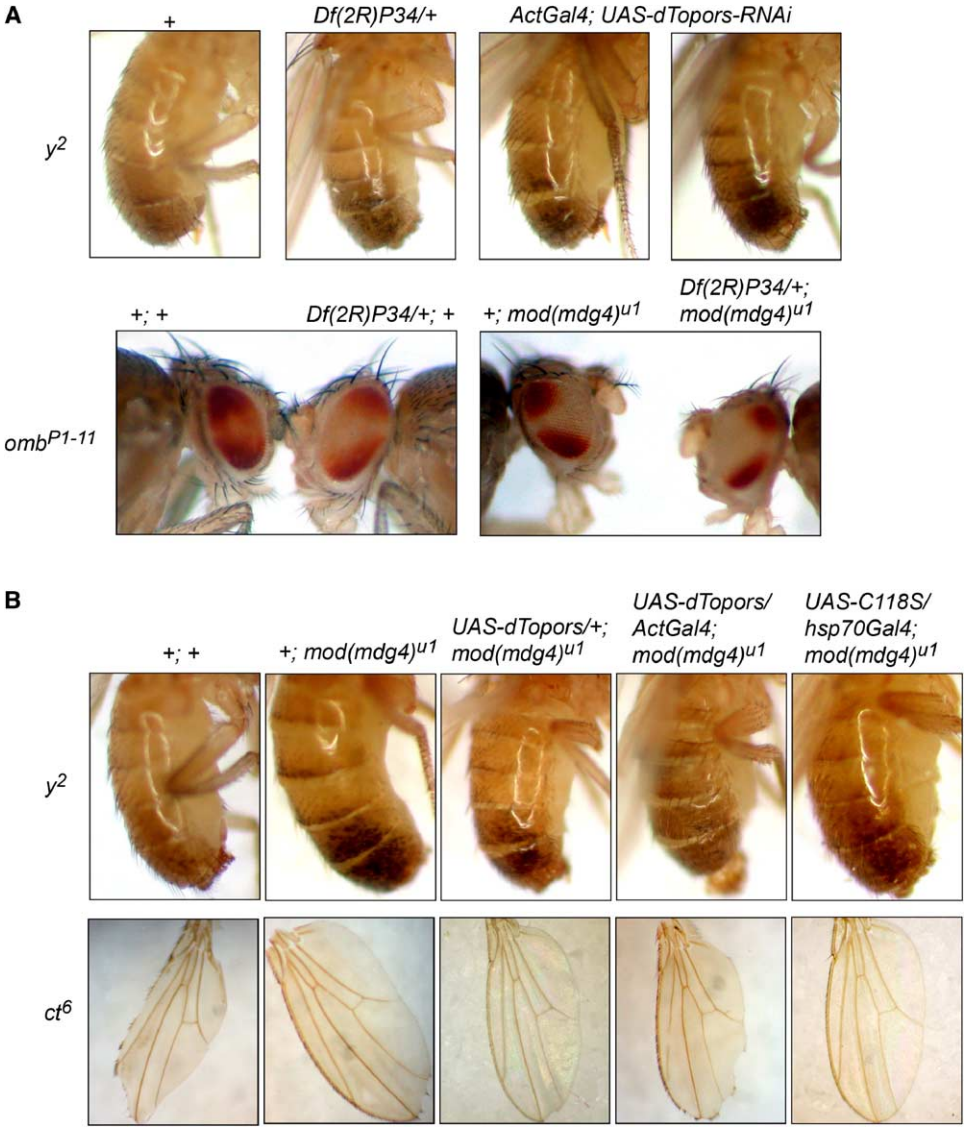

Figure 2. dTopors Promotes *gypsy* Insulator Activity

(A) Abdomens of male flies of the genotypes y^2; +, y^2; *Df(2R)P34/+* and y^2; *actinGAL4/+; UAS-dTopors-RNAi/+* (two independent lines for the RNAi construct) (top). Eyes of male flies of the genotypes $omb^{P1\text{-}D11}$;+; +, $omb^{P1\text{-}D11}$; *Df(2R)P34/+;* +, $omb^{P1\text{-}D11}$; +; *mod(mdg4)*^{u1} and $omb^{P1\text{-}D11}$; *Df(2R)P34/+; mod(mdg4)*^{u1} (bottom).

(B) Abdomens (top) and wings (bottom) of male flies of the genotypes y^2ct^6; +; +, y^2ct^6; +; *mod(mdg4)*^{u1}, y^2ct^6; *UAS-dTopors/+; mod(mdg4)*^{u1}, y^2ct^6; *UAS-dTopors/actinGAL4; mod(mdg4)*^{u1}, and y^2ct^6; *UAS-C118S/hsp70GAL4; mod(mdg4)*^{u1}.

insulator function at y^2, as judged by very low levels of pigment in the abdomen (Figure 2B). This effect is also observed with *cut-6* (ct^6), another phenotypic marker for insulator activity, caused by insertion of *gypsy* between the wing margin enhancer and the promoter of the *cut* gene that results in a jagged appearance of the wing edge (Gause et al., 2001). The disruption of insulator activity due to lack of the Mod(mdg4)2.2 protein allows for reactivation of *cut* expression and, consequently, results in a rounded wing edge (Figure 2B). Overexpression of dTopors in the *mod(mdg4)*^{u1} background produces a more notched edge of the wing compared to *mod(mdg4)*^{u1}, indicating an improvement in enhancer blocking by *gypsy* at ct^6 (Figure 2B). These effects are entirely dependent on induction by the Actin-GAL4 driver and are observed for three independent insertions of the *UAS-dTopors* transgene. Thus, overexpression of dTopors can augment insulator activity in the absence of the Mod(mdg4)2.2 protein, suggesting that although the two proteins exhibit a strong interaction, dTopors can affect the insulator through a Mod(mdg4)2.2-independent mechanism.

To test whether the evolutionarily conserved RING domain of dTopors is required for this effect, we generated a dTopors transgenic construct carrying a point mutation, which changes a highly conserved cysteine of the RING domain to a serine (*UAS-C118S*). This type of substitution has been shown to abolish the ubiquitin

ligase activity of a homologous RING domain, as it is thought to interfere with its folding (Honda and Yasuda, 2000). When expressed at similar levels as the wild-type dTopors, the *UAS-C118S* construct was not able to rescue the phenotype of the *mod(mdg4)^{u1}* mutant (Figure 2B). The ability of dTopors to promote insulator activity thus appears to be RING-domain dependent, suggesting that ubiquitin conjugation may be important for its effect on the function of *gypsy*.

dTopors Is Present at Chromosomal and Nuclear Insulator Sites

To further test the possibility that dTopors is a component of the *gypsy* insulator, we determined its subcellular localization relative to the *gypsy* insulator proteins. One well-characterized location of the *gypsy* insulator is the insertion of the *gypsy* retrotransposon at the *y^2* locus at the tip of the X chromosome (Gerasimova et al., 1995). Using α-dTopors antibodies, we found that dTopors is present at the *y^2* locus on polytene chromosomes, revealed by colocalization between dTopors and CP190 at that site (Figure 3A). Additionally, dTopors colocalizes with many of the endogenous genomic binding sites of Su(Hw) and CP190, the two DNA binding components of the *gypsy* insulator complex (Figures 3A and 3B). CP190 is found at the majority of the Su(Hw) binding sites on polytene chromosomes, yet additional binding sites of CP190 alone are present in the genome (Pai et al., 2004). We estimate that dTopors is present at approximately 30%–40% of the endogenous Su(Hw) binding sites and at approximately 70%–80% of the genomic CP190 binding sites.

In diploid cell nuclei, *gypsy* insulator proteins coalesce into large nuclear complexes termed insulator bodies. dTopors colocalizes extensively with insulator bodies in the nuclei of diploid cells of imaginal discs and brains of third instar larvae (Figure 3C). A similar nuclear localization pattern was observed for transgenic *UAS-GFP-dTopors*, visualized by direct detection of GFP fluorescence or by using α-GFP antibodies (data not shown).

In addition to its presence at the nuclear locations of insulator bodies, dTopors is also distributed throughout the nuclear periphery, a pattern reminiscent of nuclear lamina proteins. Therefore, we compared the nuclear localization of dTopors and lamin and found that dTopors colocalizes with the nuclear lamina in diploid cell nuclei (Figure 3D), although the staining pattern of dTopors appears more punctate. The colocalization between dTopors and lamin can also be observed in a gentle preparation of partially intact polytenized nuclei (Figure 3E). The anti-lamin staining is observed in the nuclear envelope fraction and is entirely absent from chromatin, whereas dTopors appears to associate with both nuclear lamina and chromatin. This staining pattern appears to be specific to dTopors (Figure 3F, where Mod(mdg4)2.2 is found only on chromosomes, prepared with the same technique).

Due to their substantial colocalization, dTopors and lamin were also tested for direct interaction. Lamin was found to coimmunoprecipitate with dTopors from larval protein extracts, using α-dTopors antibodies (Figure 1D), confirming the association between dTopors and nuclear lamina in vivo. Additionally, their interaction was verified by a yeast two-hybrid assay, which showed a robust interaction between LexA-lamin and VP16-dTopors, but not between LexA-lamin and VP16-Su(Hw) or VP16 alone (Figure 1E).

dTopors Does Not Ubiquitinate Known Insulator Proteins

dTopors, its mammalian homolog Topors, and the viral homolog ICP0 possess a RING domain-dependent E3 ubiquitin ligase activity (Boutell et al., 2002; Rajendra et al., 2004; Secombe and Parkhurst, 2004). Thus, it is possible that the observed effect exerted by dTopors on the *gypsy* insulator is mediated through the dTopors-directed ubiquitin conjugation of insulator proteins. In general, RING E3 ligases are believed to act as adaptors, which bring together the catalytic E2 enzyme and the substrate protein to promote the specificity of ubiquitin transfer (Kim et al., 2002). In the absence of a substrate, the ligase activity of RING E3 enzymes can be monitored in vitro by their autoubiquitination, which is a result of their interaction with the E2 enzymes. In agreement with previously reported results, we observed a robust self-ubiquitination activity of recombinant purified dTopors in vitro in the presence of UbcH5a, the E2 enzyme identified for ICP0 (Boutell et al., 2002) (Figure 4A). However, none of the three known components of the insulator complex, Mod(mdg4)2.2, Su(Hw), or CP190, were found to be ubiquitinated in the absence or presence of dTopors in vitro (Figure 4B and data not shown). Addition of the other two components of the insulator complex to any one given insulator protein being assayed as a substrate similarly did not result in dTopors-promoted ubiquitin conjugation. For example, addition of either CP190 or Mod(mdg4)2.2 or both did not promote ubiquitination of Su(Hw) (Figure 4B and data not shown).

To further address the possibility of dTopors acting as an E3 ubiquitin ligase for insulator proteins, we assessed whether dTopors could affect the protein levels of Su(Hw), Mod(mdg4)2.2, or CP190 in vivo. Ubiquitination of proteins is often, though not always, associated with their proteasome-dependent degradation. As reported previously, dTopors targets Hairy for degradation by promoting its ubiquitination, such that higher levels of dTopors produce a decrease in the levels of Hairy protein (Secombe and Parkhurst, 2004). No significant reduction in the amounts of Su(Hw), Mod(mdg4)2.2, or CP190 was detected in protein extracts from third instar larvae overexpressing dTopors in either wild-type or *mod(mdg4)^{u1}* genetic backgrounds (Figure 4C). Additionally, insulator protein levels were not altered in S2 culture cells transfected with either a GFP-dTopors or a GFP-C118S construct and enriched for a GFP-positive population by flow-cytometry, as compared to control cells transfected with GFP alone (data not shown). Therefore, unlike its mechanism of action on Hairy, dTopors does not have any effect on the stability of insulator proteins. This result is not unexpected because dTopors exerts a positive effect on insulator activity, whereas dTopors-mediated targeting of insulator proteins for degradation would be expected to disrupt the activity of the *gypsy* insulator.

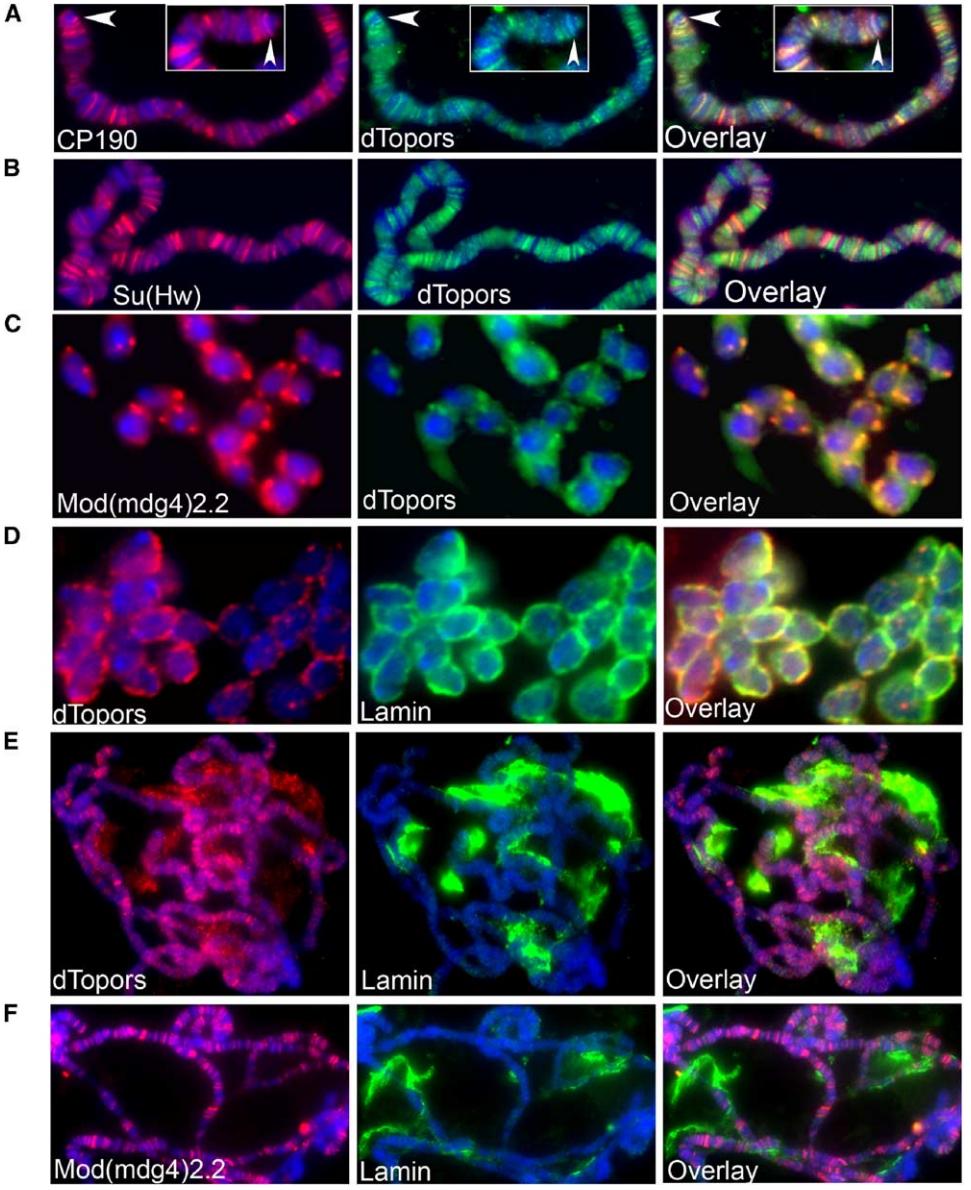

Figure 3. dTopors Is Present at Genomic Insulator Sites and in Nuclear Insulator Bodies

Immunolocalization of: (A) dTopors (green) and CP190 (red) on polytene chromosomes. DNA is stained with DAPI (blue) in all immunofluorescence images. Arrows point to the y^2 locus. (B) dTopors (green) and Su(Hw) (red) on polytene chromosomes. (C) dTopors (green) and Mod(mdg4)2.2 (red) in diploid cell nuclei. (D) dTopors (red) and lamin (green) in diploid cell nuclei. (E) dTopors (red) and lamin (green) on polytene chromosomes from partially intact polytenized nuclei. (F) Mod(mdg4)2.2 (red) and lamin (green) on polytene chromosomes, prepared as in (E).

Monoubiquitination of proteins has been described as a regulatory modification that does not serve as a signal for degradation. To explore potential involvement of dTopors in ubiquitination of insulator proteins for nonproteolytic regulation, we tested whether insulator proteins are modified by ubiquitin in vivo. No forms of CP190, Su(Hw), or Mod(mdg4)2.2 were recognized by anti-ubiquitin antibodies in immunoprecipitates of insulator proteins, suggesting that they are not mono-ubiquitinated in vivo (data not shown). Together, these results imply that either dTopors affects insulator function by a mechanism that requires an intact RING domain, but not its associated ubiquitin ligase activity, or that it does so by ubiquitinating an uncharacterized component of the insulator complex or of the nuclear lamina.

dTopors Promotes the Association of Su(Hw) with Chromatin

As shown above, overexpression of dTopors is able to restore the enhancer blocking function of the *gypsy* insulator in the *mod(mdg4)*^{u1} mutant. To exercise this effect, dTopors would be expected not to require Mod(mdg4)2.2 for its recruitment to insulator sites. Thus, we initially tested whether Mod(mdg4)2.2 is nec-

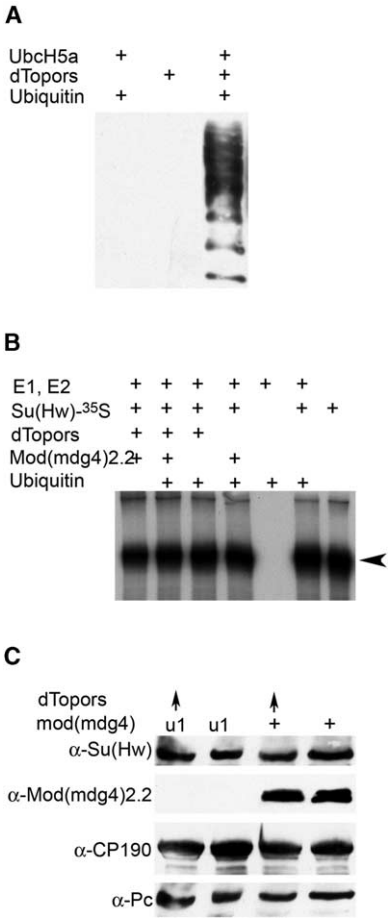

Figure 4. The E3 Ubiquitin Ligase Activity of dTopors Does Not Act Directly on Known Insulator Proteins and Does Not Promote Their Degradation

(A) Indicated combinations of dTopors, the E2 (UbcH5a) enzyme, and Ubiquitin were tested in an in vitro ubiquitination assay, and formation of polyubiquitin chains was detected by Western blotting analysis with α-Ubiquitin antiserum.

(B) Su(Hw), labeled with ^{35}S, was used as substrate in an in vitro ubiquitination assay containing indicated combinations of dTopors, Mod(mdg4)2.2, E1, UbcH5a, and Ubiquitin.

(C) Protein extracts from larvae of genotypes *UAS-GFP-dTopors/actinGAL4; mod(mdg4)^{u1}*, *+; mod(mdg4)^{u1}*, *+;UAS-GFP-dTopors/actinGAL4*, and *+;+* were Western blotted as indicated.

essary for the binding of dTopors to chromatin by immunostaining polytene chromosomes of *mod(mdg4)^{u1}* mutants for dTopors. dTopors is still observed on polytene chromosomes, including at the *y^2* locus, in the absence of Mod(mdg4)2.2 (Figure 5A).

The binding pattern of Su(Hw) on polytene chromosomes from wild-type larvae consists of approximately 500 distinct binding sites, located often at the borders between chromosomal bands and interbands that represent areas of more condensed and less condensed chromatin, respectively. In the *mod(mdg4)^{u1}* mutant, the binding pattern of Su(Hw) on polytene chromosomes is disrupted, with the majority of Su(Hw) binding sites becoming less localized and more diffuse (Figure 5B). Close inspection of polytene chromosomes revealed that the original binding sites of Su(Hw) lose their localized binding to the border regions and instead often spread into the interbands in chromosomes of *mod(mdg4)^{u1}* larvae. Interestingly, 10–15 Su(Hw) binding sites in the fly genome are not affected by lack of Mod(mdg4)2.2 and remain sharply localized as in wild-type chromosomes. Among them are the binding sites in the *gypsy* retrotransposon, such as those found at *y^2* or *omb$^{P1\text{-}D11}$*.

Overexpression of dTopors rescues the mislocalization of Su(Hw) in the *mod(mdg4)^{u1}* mutant. Instead of the few visibly brighter loci in the background of general diffuse binding in chromosomes of *mod(mdg4)^{u1}* larvae, all Su(Hw) binding sites are restored to a near wild-type binding pattern in mutant larvae also expressing the dTopors construct (Figure 5B). The diffuse mislocalization of Su(Hw) to interbands reverts to sharply localized binding sites, found predominantly at the borders between bands and interbands. The observed changes in Su(Hw) binding are not explained by variations in its protein levels, as the relative amount of Su(Hw) is not affected in these genotypes as compared to wild-type (Figure 4D). This rescue of Su(Hw) binding is also dependent on the integrity of the RING domain of dTopors, as overexpression of the C118S mutant did not result in improved localization of Su(Hw) (Figure 5B). Thus, elevated levels of dTopors restore the wild-type binding pattern of Su(Hw) in the absence of Mod(mdg4)2.2, suggesting that dTopors may be involved in stabilizing Su(Hw) insulator complexes on chromatin.

dTopors Facilitates the Nuclear Organization of Insulator Bodies

The ability of Mod(mdg4)2.2 to participate in homeotypic interactions is believed to mediate the clustering of distant insulator complexes. This model is supported by the fact that in the *mod(mdg4)^{u1}* mutant, the remaining insulator proteins fail to coalesce into large nuclear foci. In the absence of Mod(mdg4)2.2, the nuclear staining of CP190 that is used to mark insulator bodies is diffuse throughout the nucleus as compared to the wild-type punctate pattern (Figure 6A). This diffuse localization is replaced by a near wild-type pattern of distinct insulator foci when dTopors is overexpressed. dTopors is thus able to compensate for the loss of coalescence of insulator complexes and to promote reformation of nuclear insulator bodies.

One possible explanation for the rescue of the *mod(mdg4)^{u1}* phenotype by overexpression of dTopors is that this protein mediates the attachment of insulator complexes at a nuclear substrate (Figure 7). In the absence of Mod(mdg4)2.2, dTopors may provide additional sites of attachment for the remaining insulator components, which in turn results in the recovery of insulator bodies. If this is correct, increasing potential interaction sites between Su(Hw) and dTopors by raising the levels of Su(Hw) may similarly result in the rescue of insulator activity. To test this hypothesis, we introduced a transgene carrying the coding region of Su(Hw) under the control of the endogenous Su(Hw) promoter (Gerasimova et al., 2000) in the *mod(mdg4)^{u1}* background. The homozygous Su(Hw) transgene, which effectively increases Su(Hw) expression 2-fold, results

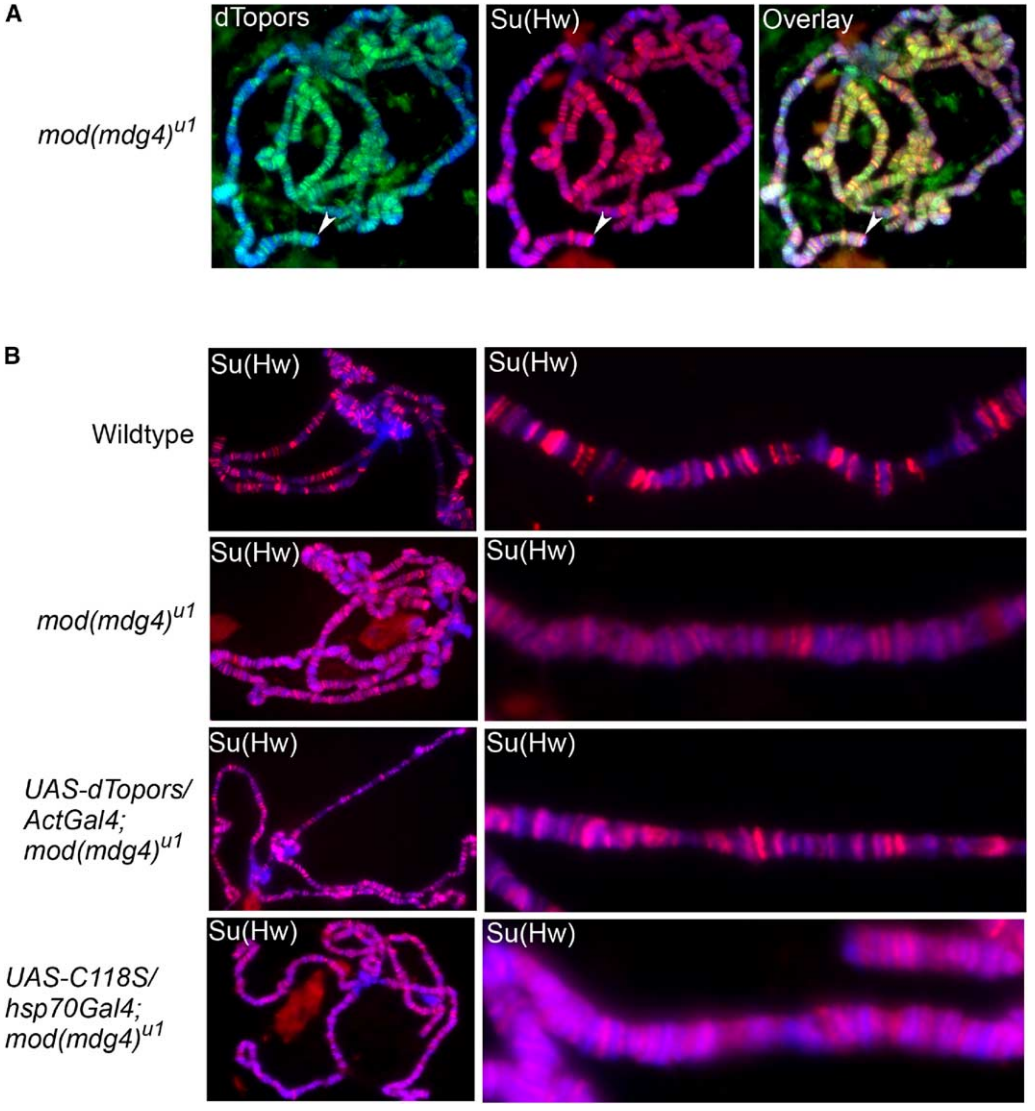

Figure 5. The Association of Su(Hw) with Chromatin Is Stabilized by dTopors

(A) Immunolocalization of dTopors (green) and Su(Hw) (red) on polytene chromosomes from y^2; +; *mod(mdg4)^{u1}* larvae.

(B) Polytene chromosomes from larvae of indicated genotypes stained with α-Su(Hw) antiserum (red). Images of entire chromosome spreads (left) or more detailed views of a region of chromosome arm 3R (right) are shown.

in a partial rescue of *gypsy* insulator activity that is comparable to that seen for the overexpression of dTopors, as judged from its effect on the y^2 allele (Figure 6B).

Disruption of the Nuclear Lamina Affects dTopors Localization and Insulator Function

Because dTopors was shown to associate with lamin, the nuclear lamina may serve as a substrate for dTopors-mediated organization of insulator bodies. To address this possibility, we analyzed a hypomorphic allele of the *Drosophila lamin* gene, *lamin^{4643}* (Guillemin et al., 2001), for effects on nuclear organization and activity of the insulator. Reduced levels of lamin resulted in a disruption of the nuclear organization of insulator bodies (Figure 6C), suggesting that lamin is necessary for the proper nuclear organization of the *gypsy* insulator. The nuclear localization of dTopors is also altered by the *lamin^{4643}* mutation, such that the wild-type peripheral staining of dTopors becomes diffuse throughout the nucleus in the *lamin^{4643}* mutant (Figure 6C).

Furthermore, the *lamin* mutation was found to exert a dominant disruptive effect on the phenotype of *gypsy*-induced mutations. The expression of the *yellow* gene from the y^2 allele is elevated in the abdomens of *lamin^{4643}/+; mod(mdg4)^{u1}* as compared to *mod(mdg4)^{u1}* flies (Figure 6D). A similar reduction of insulator activity in the heterozygous *lamin^{4643}* mutants was also observed for the ct^6 locus (data not shown). Additionally, although flies homozygous for the *lamin^{4643}* allele die in late third instar larvae stage and *mod(mdg4)^{u1}* mu-

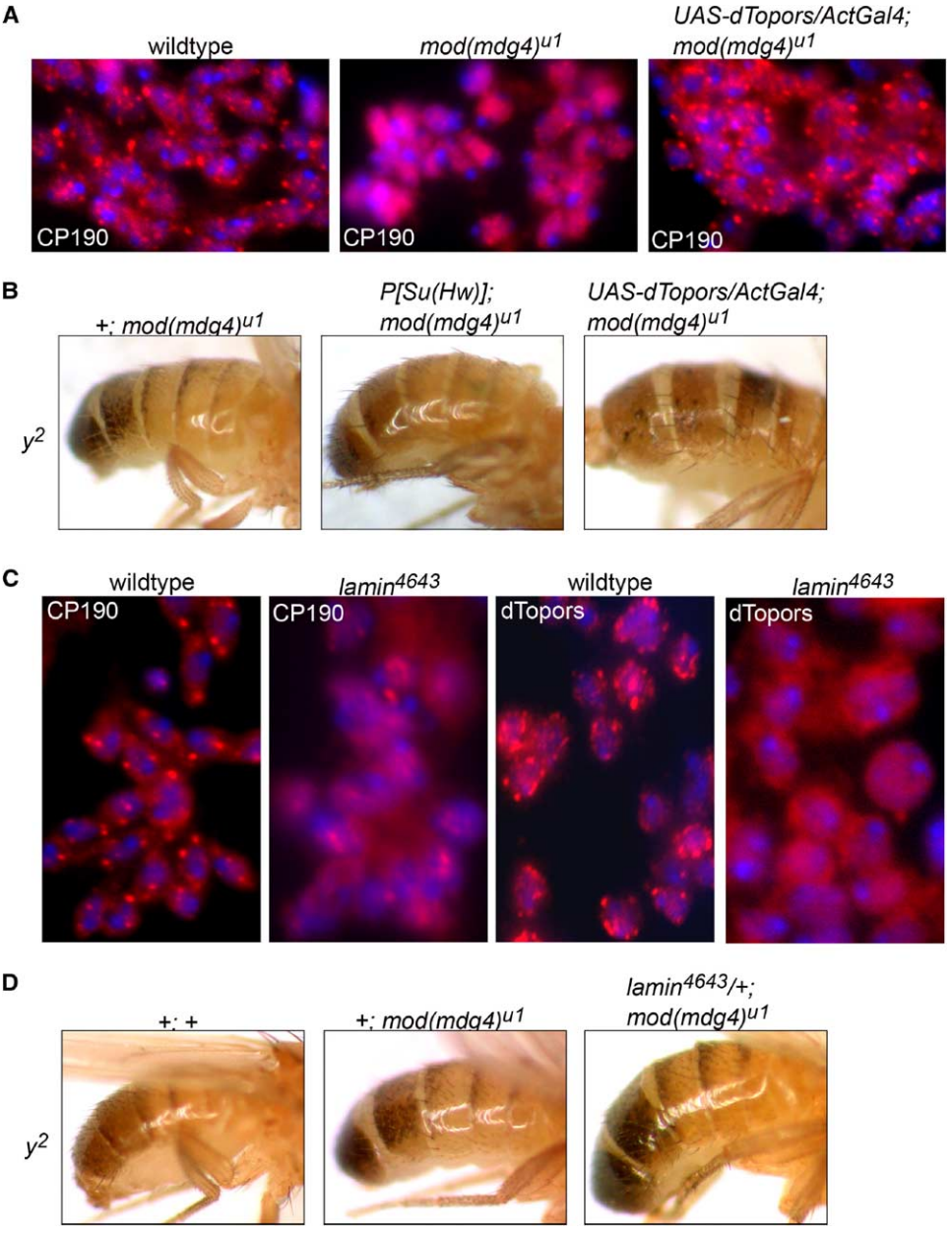

Figure 6. dTopors and Lamin Facilitate Formation of Nuclear Insulator Bodies

(A) Immunolocalization of CP190 (red), used to mark insulator bodies, in diploid cells from larvae of indicated genotypes.

(B) Abdomens of male flies of the genotypes y^2; +; *mod(mdg4)*^{u1}, y^2; *pSuHw-GFP; mod(mdg4)*^{u1}, and y^2; *UAS-GFP-dTopors/actinGAL4; mod(mdg4)*^{u1}.

(C) Immunolocalization of CP190 (red) (left two panels) and of dTopors (red) (right two panels) in diploid cells from wild-type versus *lamin*^{4643} mutant larvae.

(D) Abdomens of male flies of genotypes y^2; +; +, y^2; +; *mod(mdg4)*^{u1}, and y^2; *lamin*^{4643}/+; *mod(mdg4)*^{u1}.

tants are viable to adulthood, combining the two mutations results in embryonic lethality, such that *lamin*^{4643}; *mod(mdg4)*^{u1} animals die before entering larval stages of development. This genetic interaction between *lamin* and *mod(mdg4)* mutations supports the notion that the two proteins may function in the same process and partially compensate for each other.

Discussion

A yeast two-hybrid screen for proteins that interact with Mod(mdg4)2.2 resulted in identification of dTopors as a factor involved in the activity of the *gypsy* insulator. dTopors was found to interact with the three known insulator components, Su(Hw), Mod(mdg4)2.2, and CP190,

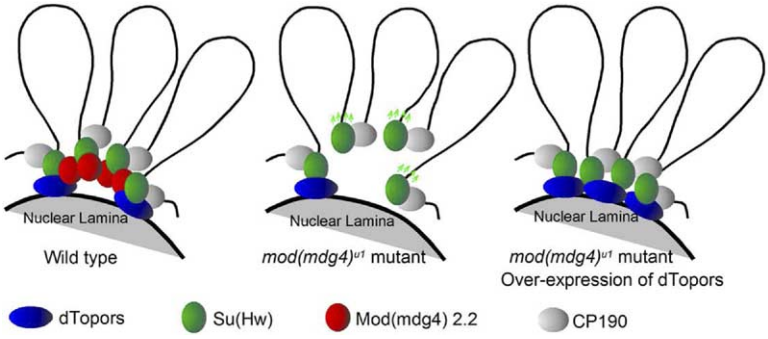

Figure 7. dTopors Regulates Nuclear Organization of the *gypsy* Insulator

Nuclear organization of insulator bodies and associated chromatin may involve both the coalescence of distant complexes through self-interactions of Mod(mdg4)2.2 (red) and the tethering of these complexes to the nuclear lamina via dTopors (blue). In a *mod(mdg4)* mutant, the nuclear organization is disrupted due to loss of clustering, and Su(Hw) binding to chromatin is destabilized (indicated by red arrows). Proper binding of Su(Hw) can be recovered by hyperactivating the second mechanism, mediated by dTopors and lamin, resulting in rescue of nuclear complex formation.

and to associate with the *gypsy* insulator complex on chromosomes and in diploid nuclei. Additionally, dTopors appears to physically associate with the nuclear lamina. Genetically, dTopors was shown to behave as a positive factor involved in *gypsy* insulator activity. Consistently, reduction in levels of dTopors, observed in the background of a dTopors-spanning deletion or of an inducible dTopors RNAi construct, results in the disruption of insulator activity. The effects of elevated levels of dTopors are particularly dramatic as they restore the activity of a compromised *gypsy* insulator on multiple levels. The enhancer blocking function of the insulator, the binding of Su(Hw) to chromatin, and the formation of insulator bodies in cell nuclei—all compromised in *mod(mdg4)^{u1}* mutants—are rescued by overexpression of dTopors.

These effects can be explained by a model in which dTopors acts as a nuclear lamina-associated factor that serves to tether the *gypsy* insulator complexes to a fixed substrate (Figure 7). In the wild-type situation, Mod(mdg4)2.2 mediates the coalescence of distant insulator sites and the subsequent establishment of chromatin compartments, whereas dTopors may be involved in further organization of insulator bodies at specific nuclear attachment points through its direct interaction with both Mod(mdg4)2.2 and Su(Hw). The absence of Mod(mdg4)2.2 leads to the breakdown of nuclear organization and the destabilization of Su(Hw)-chromatin association. Through tethering distant insulator sites to a nuclear substrate, dTopors, when present at elevated levels, may be able to compensate for the loss of a component such as Mod(mdg4)2.2. By stabilizing the nuclear organization of insulator complexes, dTopors may also promote the binding of Su(Hw) to chromatin. This explanation is further reinforced by the observed disruptive effects of a *lamin* mutation on the nuclear organization and the enhancer blocking activity of the *gypsy* insulator.

The connection between *gypsy* insulator activity and nuclear insulator bodies has relied predominantly on the effects of the mutations in Mod(mdg4)2.2 and CP190 on both enhancer blocking function and insulator body integrity (Gerasimova and Corces, 1998; Pai et al., 2004). The activity of dTopors provides further evidence for a functional relationship between insulators and their nuclear localization, as rescue of insulator phenotypes by dTopors is accompanied by the recovery of insulator bodies. Establishment of independent chromatin domains, which has been proposed as the main function of insulators, is thought to rely on structural partitioning of chromatin through physical interactions between distant loci or through interactions with a fixed nuclear substrate. It has been previously intimated that *gypsy* insulators may employ both types of structural organization to ensure the establishment of domain autonomy. Our work suggests that the *gypsy* insulator may undergo physical clustering through the BTB domains of Mod(mdg4)2.2 and of CP190 and may utilize the attachment to the nuclear lamina via dTopors. The interaction of the insulator with a nuclear substrate is further supported by a recent report that *gypsy* insulator proteins associate with the nuclear matrix, of which lamin is a principal component (Byrd and Corces, 2003). Tethering to a subnuclear surface has also been implicated in the activity of the chicken *β-globin* insulator, where *β-globin* insulator loci were observed to interact with the nucleolar surface, perhaps via a direct association between the insulator protein CTCF and the nucleolar component nucleophosmin (Yusufzai et al., 2004).

The E3 ubiquitin ligase activity of dTopors was not found to act directly on the known insulator proteins, yet the RING domain of dTopors appears to be essential for its positive effect on the *gypsy* insulator. It thus remains possible that an unknown factor involved in insulator activity may be a substrate for dTopors-mediated ubiquitination. A connection between the *gypsy* insulator complex and the ubiquitin conjugation pathway is also suggested by the presence of BTB domains in Mod(mdg4)2.2 and CP190, as BTB domain proteins have been proposed to act as substrate adaptors for the ubiquitin RING E3 ligases (Geyer et al., 2003; Pintard et al., 2003). It is feasible that BTB-containing insulator proteins and RING-containing dTopors are involved in ubiquitin conjugation with functional consequences for the insulator.

The association of dTopors with a subset of insulator binding sites on polytene chromosomes implies that its presence is not required by all insulator complexes. This may be a consequence of the proposed function of dTopors as a tethering factor, such that the interaction between distant insulator loci may alleviate the need for dTopors at every binding site of the insulator complex. Alternatively, it may suggest that endogenous insulator complexes are not all functionally equivalent, and that

the enzymatic properties of dTopors may be important for specific insulator complexes. The ubiquitin ligase activity of dTopors may be involved in regulation of insulator complexes, such that modification of a yet uncharacterized component by ubiquitin can lead to variation in function of endogenous insulators.

Experimental Procedures

Yeast Two-Hybrid

To generate the bait construct, Mod(mdg4)2.2 cDNA (accession number U30905) was cloned into the EcoRI site of the 2 μ pBTM116 vector, carrying the *TRP1* gene for selection. The *Drosophila* embryonic cDNA-VP16 fusion library (Poortinga et al., 1998), carrying the *LEU2* gene for selection, was transformed into LD40 yeast that contained the bait. Positive clones were selected on plates lacking tryptophan, leucine, and histidine and supplemented with 5 mM 3-AT and were assayed for β-galactosidase activity. Clones that displayed the highest β-galactosidase activity were subjected to the bait-loss reintroduction assay. Selected colonies with no bait were mated to the AMR70 yeast strain that carried either bait or ENT1-LexA vector. Diploids were assayed for activity of both reporter genes. The prey DNA from the resulting six positive clones was amplified by polymerase chain reaction (PCR) and sequenced. dTopors cDNA (EST clone LD43109) and Lamin cDNA (EST clone 38055) were obtained from Research Genetics and were cloned into the BglII-NotI sites of pVP16 and into the EcoRI site of pBTM116, respectively. The ENT1 cDNA for the ENT1-LexA construct was a gift from Dr. B. Wendland.

Fly Strains and Construction of Transgenic Flies

Fly stocks were maintained in standard medium at 25°C. The genomic deletion *Df(2R)P34* was obtained from the Bloomington Stock Center. The UAS-dTopors-RNAi construct was generated by cloning a fragment of dTopors cDNA in two opposing orientations into the pUAST vector that contained a *ftz* gene intron between the two fragments, using the NotI-EcoRI fragment for the 5′-3′ orientation and primers 5′-CATCTGGTACCCGCAAATGCAGAGGTGGC-3′ and 5′-CCACGTCTAGAATGCTCGTTGAAGAGTCCG-3′ for the 3′-5′ orientation. Two out of five generated *UAS-dTopors-RNAi* lines displayed the reported effect, when induced, and the reduction of dTopors protein levels was verified by Western blotting analysis. The UAS-GFP-dTopors construct was generated by cloning the EGFP cDNA into the EcoRI-BglII sites and the dTopors cDNA into the BglII-KpnI sites of pUAST. The UAS-GFP-C118S construct was cloned by PCR mutagenesis, using primers 5′-GTGCTTCACGG ACTCGTCCATGCACCAGTTC-3′ and 5′-GAACTGGTGCATGGACG AGTCCGTGAAGCAC-3′. The six obtained transgenic lines were lethal when driven by ActinGAL4, thus we used hsp70GAL4 to induce overexpression of C118S. The levels of mutant dTopors in *UAS-C118S/hsp70GAL4* were equivalent or higher than those of wild-type dTopors in *UAS-dTopors/ActinGAL4*, as assessed by α-GFP Western blotting analysis. We postulate that this mutation results in elevated levels of dTopors due to improved stability or lack of downregulation.

Protein Purification and Antibody Production

The dTopors coding cDNA was amplified by PCR with primers 5′-GACTAGATCTCGCCAGAATGGCGGAG-3′ and 5′-GAGCGGATA TCTTAATACGGCAGTAG-3′ and was cloned into the BglII-EcoRV sites of the PET30a vector in-frame to the N-terminal His_6 Tag. Cultures of His-dTopors in the Rosetta bacterial strain (Novagen) were induced by 1 mM IPTG, grown for 3 hr, lysed either under denaturing conditions for antibody production or under native conditions for in vitro assays, and purified by Ni chromatography. Purified His-dTopors was used to immunize rats and rabbits by standard procedures. The specificity of the antisera versus that of the preimmune was verified by Western blotting analysis of fly, yeast, and bacterial protein extracts. The GST-dTopors protein fusion was produced by cloning the dTopors coding sequence into the BglII-SmaI sites of the pGEX-2TK vector, induced as described above and purified by glutathione chromatography. His-Su(Hw) and His-Mod(mdg4)2.2 recombinant proteins were generated by cloning the coding sequences into the NotI and EcoRI sites of the PET30a vector, respectively, and purified under native conditions.

Immunohistochemistry

Immunostaining of polytene chromosomes and larval diploid cells was carried out as described previously (Gerasimova et al., 2000). Rabbit and rat α-dTopors antisera, described above, were used at a 1:20 dilution and a 1:10 dilution, respectively. Rabbit α-lamin antibody was a gift from Dr. P.A. Fisher and was used at a 1:100 dilution.

Protein Extracts, Immunoprecipitation, and Western Blotting Analysis

Protein extracts from third instar larvae were prepared by lysing the anterior tissues, containing salivary glands, brains, and imaginal discs, in RIPA buffer (1% NP40, 0.1% SDS, 150 mM NaCl, and 10 mM Tris [pH 7.4]) for 20 min followed by brief centrifugation to remove debris. For immunoprecipitation, 30 μl of protein A Sepharose beads, 0.2 mg of protein extract, 30 μl of rabbit α-dTopors, α-Mod(mdg4), or preimmune sera and interaction buffer (50 mM Tris [pH 8.0], 120 mM KCl, 0.1% NP40, 0.1 mM EDTA, and 3 mM $MgCl_2$) were added to 200 μl total volume and incubated overnight at 4°C. Western blots were probed with rat α-Mod(mdg4)2.2 at 1:3500 dilution, rat α-Su(Hw) at 1:5000 dilution, rat α-CP190 at 1:5000 dilution, and rat α-dTopors at 1:1000 dilution. The α-Pc antiserum (1:3000) was described previously (Gerasimova and Corces, 1998).

In Vitro Ubiquitination Assays

Ubiquitination assays were carried out in a buffer containing 50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mM DTT, 5 mM ATP, 1 mM creatine phosphate, and 15 units of creatine phosphokinase (Calbiochem) in the presence of 200 ng rabbit E1 enzyme (Calbiochem), 500 ng human E2 enzyme GST-UbcH5a, 10 μg ubiquitin, 100 ng ubiquitin aldehyde (all from AFFINITI Research Products Limited) and 500–1000 ng recombinant purified His-dTopors. For reactions containing known insulator proteins, 2–5 μl of in vitro transcribed and translated Mod(mdg4)2.2 and/or Su(Hw) and/or CP190 were used. Substrate proteins were labeled with ^{35}S-methionine. Reactions were incubated at 30°C for 90 min. Ubiquitin transfer was detected by either Western blotting analysis with α-Ubiquitin antiserum (Stressgen Biotechnologies Corporation, SPA-203; 1:1000) or by autoradiography of ^{35}S-labeled protein.

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