DNA Topoisomerase II Modulates Insulator Function in Drosophila

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Abstract

Insulators are DNA sequences thought to be important for the establishment and maintenance of cell-type specific nuclear architecture. In Drosophila there are several classes of insulators that appear to have unique roles in gene expression. The mechanisms involved in determining and regulating the specific roles of these insulator classes are not understood. Here we report that DNA Topoisomerase II modulates the activity of the Su(Hw) insulator. Downregulation of Topo II by RNAi or mutations in the Topo2 gene result in disruption of Su(Hw) insulator function. This effect is mediated by the Mod(mdg4)2.2 protein, which is a unique component of the Su(Hw) insulator complex. Co-immunoprecipitation and yeast two-hybrid experiments show that Topo II and Mod(mdg4)2.2 proteins directly interact. In addition, mutations in Topo2 cause a slight decrease of Mod(mdg4)2.2 transcript but have a dramatic effect on Mod(mdg4)2.2 protein levels. In the presence of proteasome inhibitors, normal levels of Mod(mdg4)2.2 protein and its binding to polytene chromosomes are restored. Thus, Topo II is required to prevent Mod(mdg4)2.2 degradation and, consequently, to stabilize Su(Hw) insulator-mediated chromatin organization.

Introduction

Eukaryotes use complex mechanisms to regulate spatial and temporal patterns of gene expression. At the chromatin level, much research has focused on the role of histone modifications and chromatin remodeling complexes in promoting or preventing transcription [1,2]. In addition, the highest order of chromatin structure, the chromosome, also participates in the establishment and maintenance of gene expression patterns [3,4,5]. Organization of chromatin at this level is complex, requiring an intricate balance between DNA compaction and accessibility to the transcription and replication machineries. A wealth of information accumulated during the last few years implicates chromatin insulators in the establishment of higher-order chromatin structure through the formation of chromatin loops and subsequent regulation of gene expression [6].

Enhancer-blocking insulators are DNA sequences defined by their ability to interfere with enhancer-promoter communication whereas barrier insulators have the ability to shield transgenes from position effects caused by surrounding chromatin [7,8]. The properties of enhancer blocking insulators can be explained by their role in mediating inter- and intra-chromosomal interactions that result in the establishment and/or maintenance of chromatin loops [9,10,11,12,13]. The formation of these loops can result in multiple insulators from distinct genomic loci coalescing via protein-protein interactions to form multi-complex entities termed insulator bodies. These insulator bodies may form functional chromatin domains isolating sequences within different loops and preventing interference from regulatory regions in one loop on genes located in other loops [14,15]. Recently, the nature of insulator bodies as entities formed by multiple insulator sites coalescing at a specific nuclear location has been brought into question based on the identification of a mutation in mod(mdg4) that affects insulator function without visibly disrupting the integrity of insulator bodies [16]. As an alternative, these authors suggest that insulator bodies may be protein aggregates. Nevertheless, the results can be also explained if the mod(mdg4) allele is a hypomorph that affects the insulator activity of the gypsy retrotransposon insulator, which has 12 copies of the Su(Hw) binding site, but not the function of endogenous insulators present in the Drosophila genome, which only contain 1–2 copies of this sequence.

Evidence from genome-wide association studies suggests that insulators may create a cell-type specific nuclear architecture that is important for the establishment and/or maintenance of lineage specific gene expression and genome organization [17,18,19,20,21]. Recent results suggest that three different Drosophila insulators utilize different DNA binding proteins to recognize different sites in the genome but share CP190, which is the main component responsible for inter-insulator interactions [17]. These studies indicate that these three insulator subclasses may serve unique functions in the cell. As a consequence, cells may have mechanisms to independently regulate the function of each insulator subclass. However, the mechanisms underlying the regulation of the activity of each of these insulators are not well understood.

The main Drosophila insulators characterized to date are defined by their DNA binding proteins, Su(Hw), dCTCF and BEAF [13]. The Su(Hw) insulator has two other core protein components,
Mod[mdg4]/2.2 and CP190 [15,22,23]; in addition, this insulator complex contains RNA as well as other proteins that may serve a regulatory function [24,25]. Here we present evidence suggesting that DNA Topoisomerase II (Topo II) is also required to modulate the activity of the Su(Hw) insulator. Topo II has been shown to be important for critical cellular functions such as transcription, replication, recombination and genome stability in addition to its function in the organization of chromatin architecture [26,27,28].

Mechanistically, Topo II functions to alter DNA topology by catalyzing the ATP-dependent passage of one DNA double helix through another by breaking and religating one of the DNA strands while transporting the second [26,29,30,31]. Empirical studies have demonstrated that Topo II may function to relieve supercoiling attributed to RNA polymerase-driven transcription [32,33]. Data presented here suggest that Topo II is also needed for proper Su(Hw) insulator function in Drosophila. Loss of Topo II leads to a reversion of gypsy-induced phenotypes along with the inability of Su(Hw)/Mod[mdg4]/2.2 to form insulator bodies. This effect is specific for the Su(Hw) insulator subclass and does not affect insulator bodies involving other insulator proteins. The effect of Topo II on Su(Hw) insulators appears to be mediated by the Mod[mdg4]/2.2 protein; decrease of Topo II function leads to the degradation of Mod[mdg4]/2.2 through a proteasome-dependent pathway. These results suggest a novel mechanism used to regulate a specific subset of insulator-mediated chromatin organization.

**Results**

**Reduction of Topo II via RNAi affects gypsy-induced phenotypes**

To test the role of Topo II in insulator function we used RNAi to decrease the amount of Topo II (Top2) gene expression in flies. This allowed us to examine the effect of downregulation of this protein on the phenotype of y^{wcd} flies carrying the gypsy retrotransposon inserted into the yellow (y) and cut (c) genes. The gypsy insertion affects the communication between upstream enhancers and downstream promoters, causing yellow body and cut wing phenotypes when insulator proteins bind to the gypsy insulator sequence [15] (Figure 1). In a y^{wcd} background, a transgenic fly expressing a UAS-Top2RNAi construct was used with various Gal4 drivers to reduce Topo II expression in different tissues. The UAS-Top2RNAi transgenic fly specifically targets the Top2 gene and has no off target sites [34]. First, to test effects on the y^{wcd} phenotype, the weak but ubiquitously expressed driver Arm-Gal4 [35] was used to knockdown Top2 expression. Quantitative real-time PCR (qRT-PCR) analysis shows that the transcript levels of Top2 are reduced in the Arm-Gal4;UAS-Top2RNAi flies (Figure S1A). As a positive control for the effects on the y^{wcd} phenotype, we used the Arm-Gal4 line in combination with a UAS-Su(Hw)RNAi line, which results in a decrease in gypsy insulator function manifested by a change in body color from yellow to black (Figure 1A). Loss of Su(Hw) provides an example of the phenotype expected by a reversion of the insulator caused by the gypsy insertion [36]. Similar results are seen when the Arm-Gal4 driver is crossed to the UAS-Top2RNAi line, with a reversion from a yellow body to a black body phenotype (Figure 1A). This suggests that loss of Topo II has an effect on the function of the gypsy insulator similar to that of Su(Hw). To confirm this finding, we further tested whether loss of Topo II also affects the cut wing phenotype in the ct^{f} allele by crossing the UAS-Top2RNAi and UAS-Su(Hw)RNAi lines to a C96-Gal4 driver. C96-Gal4 is expressed in the prospective wing margin region of the developing wing disc where the ct gene is expressed and, in combination with the UAS-Top2RNAi line, reduces the level of Topo II in the wing imaginal disc cells that give rise to the dorsal-ventral margin (Figure S1B). When the C96-Gal4 driver is crossed with the UAS-Su(Hw)RNAi control, the ct phenotype reverts to wild type (Figure 1B). A similar change in phenotype is observed when the C96-Gal4 driver is combined with the UAS-Top2RNAi line (Figure 1B), again suggesting that Topo II interacts with gypsy insulator components to regulate insulator function.

**Loss of Topo II in Drosophila diploid cells affects insulator body formation**

In Drosophila the insulator proteins Su(Hw), Mod[mdg4]/2.2, dCTCF, and CP190 co-localize to punctate foci within the nucleus termed insulator bodies. These insulator bodies are thought to result from several insulators from different genomic locations coming together and looping the intervening chromatin fiber [14,15,37,38,39]. Since insulator function correlates with the formation of insulator bodies, and downregulation of Topo II affects the activity of the gypsy insulator, we examined whether loss of Topo II has an effect on insulator body formation using RNAi to knockdown this protein in Drosophila cultured cells [40]. Using ampiclons targeting the second and fourth exons of Top2, double stranded RNAs (dsRNAs) were made and used for the knockdown assays. A schematic representation of the Top2 locus indicating the location of the ampiclons is shown in Figure S2A. Both the second and fourth exon ampiclons have no off target sites and only target transcripts made by the Top2 gene. As a control for these experiments, a lacZ ampiclon was used to make dsRNAs of lacZ to initiate the RNAi machinery and rule out any RNAi pathway specific effects. Similar to previous data by the Carmena group [41], we find that dsRNAs targeting Drosophila Top2 greatly reduce Topo II levels by 72 hours (Figure S2B) and cells can survive without this protein for longer than 6 days.

To examine the effect of Topo II knockdown on insulator body formation, Drosophila S2 cells were fixed 3–4 days after treatment with dsRNAs and then immunostained with a combination of antibodies against control and insulator proteins. Downregulation of Top2 disrupts the formation of Mod[mdg4]/2.2 and Su(Hw) insulator bodies whereas the distribution of these two proteins is not affected in the control lacZ knockdown experiment (Figure 2A). However, normal insulator bodies form in a few cells (yellow arrows) where presumably the knockdown of Top2 is incomplete. Interestingly, loss of Topo II seems to have no effect on the distribution of CP190 or dCTCF insulator bodies (Figure 2B). Thus, the loss of Topo II negatively affects the ability of only Su(Hw) and Mod[mdg4]/2.2 to form insulator bodies. Together these results support findings suggesting that Su(Hw) and dCTCF are part of distinct subfamilies of insulators that are independent of each other but can colocalize in the cell nucleus [13,37,42]. In addition, these results suggest that the effects of Topo II on insulator function extend beyond those observed for the gypsy retrotransposon and apply to the endogenous Su(Hw) sites that are likely present at insulator bodies.

**Characterization of Drosophila Top2 mutants**

To further elucidate the role of Topo II in insulator function we examined the effect of mutations in the Top2 gene. We obtained five transgenic lines with P-element insertions in the Top2 locus from the Bloomington Drosophila Stock Center at Indiana University, none of which had been characterized previously. These transgenic strains are Top2^d05388 (Top2^d), Top2^d05377 (Top2^d), Top2^d01247 (Top2^d), Top2^d04070 (Top2^d), Top2^d04073 (Top2^d), and Top2^dMB04073 (Top2^d). Each of these transgenic lines has a P-element inserted in the first intron of the Top2 gene (Figure S2A). To determine if any of these
P-element insertions causes a mutant phenotype, each of the Top2 alleles was crossed with a CyO, Act-GFP balancer line and homozygous Top2 progeny were identified by the lack of GFP expression beginning at embryogenesis. Using this approach we determined that lines Top2c and Top2f are homozygous lethal while the other Top2 strains are not. qRT-PCR was then used to characterize these Top2 alleles. Top2c and Top2f have no detectable Top2 transcript while the amount of the Top2 RNA in the other alleles varies from approximately 40% to 90% of wild type levels (Figure S3A). Western analysis indicates that Topo II levels are only slightly affected in homozygous Top2d, Top2LA and Top2MB individuals while no Topo II protein is detectable in Top2c and Top2f mutant alleles (Figure S3).

Top2c displays the strongest lethal effect when homozygous, with most flies dying as first instar larvae and very few (1-3%) escaping to second instar. The weaker Top2f allele, on the other hand, has its highest mortality rate during second instar with fewer than 5% individuals escaping into the third instar larval stage. To further confirm that the lethality seen with homozygous Top2c and Top2f is due to the loss of Topo II function, we placed Top2c and Top2f over the deficiency allele Df(2L)Exel9043 that has a complete deletion of the Top2 gene and displays lethality during the first instar larval stage. In this combination, both Top2c and Top2f die as first instar larvae, similar to what is seen with homozygous Top2c mutants and Actin5C-Gal4/UAS-Top2RNAi animals. Transheterozygous combinations of the Top2c and Top2f alleles also show lethality during first instar larvae. Homozygous Top2c and Top2f mutants progresses normally through embryogenesis, presumably due to maternal contribution of Topo II, but once hatched, Top2c and Top2f animals show a 2-3-day delay in development when
compared to wild type larvae reared under identical conditions. The Top2f third instar escapers are smaller than wild type larvae and have less developed salivary glands and central nervous system while having extremely reduced to completely absent imaginal tissue. A summary of this information is shown in Table 1. Based on the fact that Top2c and Top2f are homozygous lethal, do not complement the Top2 deficiency allele and have undetectable transcript and protein levels, we conclude that these mutations affect the Top2 gene and could thus be used to study the role of Topo II in insulator protein function.

Mod(mdg4)2.2 is not present at insulator sites in Top2 mutants

To address how loss of Topo II may affect insulator function, polytene chromosomes of mutant Top2f larvae were used to examine the presence of insulator proteins on polytene chromosomes using immunofluorescence microscopy. Since loss of Top2 via RNAi in S2 cells affects the formation of Su(Hw) and Mod(mdg4)2.2 insulator bodies we wanted to determine whether loss of Topo II in Top2 mutants also has an effect on the binding of insulator proteins to chromosomes. Polytene chromosomes from Top2f third instar larval escapers were first immunostained with antibodies to Topo II; this protein is not present at detectable levels on the polytene chromosomes (Figure S4A). In addition, similar to the results seen with the Top2 dsRNA knockdown in S2 cells, localization of dCTCF (red) or CP190 (green) insulator bodies is unaffected by the loss of Top2 alleles (Figure 2A). dsRNA knockdown of Top2 using either amplicon targeting exon 2 or exon 4 causes a loss of Mod(mdg4)2.2 (red) and Su(Hw) (green) insulator bodies. (B) dsRNA knockdown of Top2 has no effect on dCTCF (red) or CP190 (green) insulator bodies as indicated by the formation of these structures in the LacZ control and Top2 knockdown.

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Table 1. Genetic analysis of P-element crosses.

<table>
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<tr>
<th>Alleles</th>
<th>Top2</th>
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<th>Top2d</th>
<th>Top2LA</th>
<th>Top2MB</th>
<th>Df(2L)</th>
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<td>2nd instar</td>
<td>Viable</td>
<td>Viable</td>
<td>2nd instar*</td>
<td></td>
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<tr>
<td>Top2f</td>
<td>3rd instar*</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td></td>
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</tr>
<tr>
<td>Top2f</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
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<tr>
<td>Top2f</td>
<td>Viable</td>
<td>Viable</td>
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<td></td>
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<tr>
<td>Df(2L)</td>
<td>2nd instar*</td>
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*Homozygous or trans-heterozygous lethal with some 2nd or 3rd instar escapers. Mutant alleles were crossed in all combinations to determine complementation. The term “viable” indicates that flies have no visible phenotypes, survive to adults and are fertile.

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Topo II (Figure 3A). However, unlike the S2 cell knockdown, results indicating that loss of Topo II inhibits the formation of Su(Hw) insulator bodies, Su(Hw) binding is not affected on polytene chromosomes from Topo II mutant larvae (Figure 3B–C). This suggests that the inability of Su(Hw) to form insulator bodies is not due to the failure of Su(Hw) to directly bind DNA but may be an effect on the integrity of the overall Su(Hw) complex. To test this possibility, we examined the distribution of Mod(mdg4)2.2 on polytene chromosomes of Topo II mutant larvae and found that loss of Topo II drastically reduced the levels of Mod(mdg4)2.2 present in polytene chromosomes (Figure 3C and Figure S4B). In addition, Mod(mdg4)2.2 is also absent in diploid cells of the Topo II mutant, whereas CP190 is still detectable in punctate bodies within the nucleus of Topo II mutant cells (Figure S4C). It is known that in flies carrying the null mod(mdg4)2.22T16, or the hypomorphic mod(mdg4)2.2 2F1 and mod(mdg4)2.2 27F alleles, the arrangement of Su(Hw) insulator bodies is greatly disrupted and the protein is randomly distributed throughout the nucleus [14,38]. Thus, it is possible that the lack of Su(Hw) insulator bodies in Topo II mutants is due to the inability of Mod(mdg4)2.2 to stably bind to Su(Hw) and bring together distant Su(Hw) insulator sites.

Topo II does not co-localize with insulator proteins

Since Topo II has been found to bind to the gypsy retrotransposon insulator in vitro [43], it is possible that this protein stabilizes the interaction of Mod(mdg4)2.2 with the insulator complex and that, in its absence, Mod(mdg4)2.2 is unable to bind to other components of the Su(Hw) insulator. To test this possibility, we examined the distribution of Topo II on polytene chromosomes of wild type larvae to determine if this protein could also be found in vivo at Su(Hw) insulator sites. To this end we used Topo II antibodies to conduct immunostaining on polytene chromosomes of y2 flies, which have a gypsy insertion in the yellow locus. In these flies, Topo II is found to localize to y2 as indicated by the co-staining with Mod(mdg4)2.2 (Figure 4A). However, the amount of Topo II at the y2 locus is low compared to other sites in the polytene chromosomes. In addition, Topo II seems to localize primarily at DAPI-stained bands rather than with Mod(mdg4)2.2 or Su(Hw) (Figure 4B and C), suggesting that, although Topo II may be able to bind to gypsy retrotransposon sequences, it is not present extensively at other Su(Hw) and Mod(mdg4)2.2 insulator sites in the genome (Figure S5A–B). Topo II also does not co-localize appreciably with dCTCF and CP190 (Figure 4D–E and Figure S5C–D). However, we do see some occurrence of Topo II co-localizing or juxtaposed to insulator bodies in diploid cells (Figure S4C), suggesting a possible transient interaction. Thus, these results suggest that Topo II is not a stable component of the Su(Hw) insulator but may function to modulate insulator activity possibly through a transient interaction.
Loss of Topo II affects Mod(mdg4)2.2 protein levels

Since loss of Topo II affects the presence of Mod(mdg4)2.2 at insulator sites and these two proteins do not extensively co-localize on polytene chromosomes, we examined whether the amount of Mod(mdg4)2.2 protein is affected by loss of Topo II. Western blot analysis of Top2c, Top2f mutants and Top2 knockdown in S2 cells were conducted to address this question. Lack of Topo II in Top2c and Top2f mutants results in a complete loss of Mod(mdg4)2.2 when compared to a wild type sample (Figure 5A), explaining the absence of Mod(mdg4)2.2 on polytene chromosomes of Top2f mutant larvae. The other Top2 alleles show little Mod(mdg4)2.2 protein reduction (Figure S6A). In addition, the protein levels of Su(Hw), CP190, and dCTCF are unaffected in Top2c and Top2f alleles (Figure 5A). However, prior to the lethal stage of Top2c and Top2f, Mod(mdg4)2.2 and TopoII levels can be detected (Figure S6B). Levels of Mod(mdg4)2.2 are also decreased in Drosophila cultured cells in which Topo II expression was downregulated using dsRNAs and in Arm-Gal4;UAS-Top2RNAi strains (Figure 5B and Figure S6C). The efficiency of Topo II knockdown in these assays ranges between 75%–95% for cultured cells and approximately 85% in the Arm-Gal4;UAS-Top2RNAi lines. Consequently, the amount of Mod(mdg4)2.2 is greatly reduced but not completely eliminated in these experiments (Figure 5B and Figure S6C). However, the levels of Su(Hw), CP190, and dCTCF

Figure 4. Topo II and insulator proteins do not co-localize on polytene chromosomes. (A) Immunofluorescence staining using α-Topo II and α-Mod(mdg4)2.2 antibodies; the red arrows point to the yellow locus indicating that Topo II is present at this location in the y2 allele (red arrow) but Topo II staining is more intense elsewhere in the chromosome. (B) Localization of Topo II and Su(Hw) on polytene chromosomes from wild type larvae. (C) Localization of Topo II and Mod(mdg4)2.2. (D) Localization of Topo II and dCTCF. (E) Localization of Topo II and CP190. The merged images are shown at the right and DAPI is in blue in all panels.

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are unchanged in the Top2 knockdowns. Therefore, the inability of S2 cells lacking Topo II to form Su(Hw) insulator bodies may be due to the loss of Mod(mdg4)2.2 protein. It is possible that in the absence of Topo II there is a decrease in the transcription of the mod(mdg4)2.2 gene or that the Mod(mdg4)2.2 protein is unable to properly interact with other insulator components and may be directed for degradation.

Targeted degradation of Mod(mdg4)2.2 in Top2 mutants

To distinguish between a possible role for Topo II in the transcription of the mod(mdg4)2.2 gene, translation of the mRNA, or the stability of the encoded protein, we first analyzed the effects of loss of Topo II on the expression of the mod(mdg4)2.2 gene. qRT-PCR was used to determine transcript levels of the Top2 and mod(mdg4)2.2 genes in wild type and mutant Top2\textsuperscript{f} larvae. As expected, we see a loss of Top2 in the Top2\textsuperscript{f} mutants while mod(mdg4)2.2 RNA levels are only reduced to an average of 60% of those present in wild type (Figure 6A). This reduction in mod(mdg4)2.2 transcription only accounts for a fraction of the decrease in Mod(mdg4)2.2 protein observed by western blot analysis and cannot explain the total absence of Mod(mdg4)2.2 protein in Top2\textsuperscript{f} larvae. Still, it is possible that Topo II affects Mod(mdg4)2.2 protein levels through inhibition of the translation machinery. However, this is less likely due to the fact that a general inhibition of the translation machinery would cause levels of all proteins to diminish, but western analysis only shows a reduction of Mod(mdg4)2.2 while levels of other insulator proteins are unaffected (Figure 5). Furthermore, qRT-PCR analysis using primers to the BTB domain shared by all mod(mdg4) isoforms shows a reduction of all mod(mdg4) RNAs and not just mod(mdg4)2.2 (Figure S6D). However, only the Mod(mdg4)2.2 isoform is currently known to interact with the Su(Hw) insulator complex.

To investigate the possible role of Topo II in the degradation of Mod(mdg4)2.2, we inhibited proteasome-dependent degradation using the proteasome inhibitor MG-132, which reduces the degradation of ubiquitin-conjugated proteins. We incubated imaginal discs from mutant Top2\textsuperscript{f} and wild type third instar larvae with and without the MG-132 proteasome inhibitor for 2 hours. Upon addition of the inhibitor, Mod(mdg4)2.2 protein levels were greatly increased in Top2\textsuperscript{f} mutants but not in the control treated imaginal discs as visualized by western blot analysis (Figure 6B). Conversely, in the wild type larva the addition of the inhibitor had little effect on the levels of Mod(mdg4)2.2 when compared to the control treated imaginal discs (Figure 6B). This suggests that the Mod(mdg4)2.2 protein is more susceptible, and possibly actively targeted for degradation, in cells lacking Topo II. To determine the fate of the Mod(mdg4)2.2 protein that accumulates after inhibition of proteasome function, salivary glands from Top2\textsuperscript{f} mutant larvae were incubated with MG-132. As in the case of the imaginal disc cells, this treatment results in increased Mod(mdg4)2.2 accumulation and the protein appears to properly localize to polytene chromosomes and co-localizes with Su(Hw) (Figure 6C). Thus, Topo II seems to be required to prevent the degradation of Mod(mdg4)2.2 and to allow for proper organization of the Su(Hw)/Mod(mdg4)2.2 insulator complex.

Topoisomerase II directly interacts with Mod(mdg4)2.2

To investigate whether the effect of Topo II on Mod(mdg4)2.2 stability is direct or indirect we used co-immunoprecipitation (co-IP) and yeast two-hybrid experiments to determine whether the two proteins interact. Results from the co-IP experiments show that Topo II and Mod(mdg4)2.2 immunoprecipitate one another (Figure 7A). In support of this conclusion, yeast two-hybrid analyses indicate that Topo II and Mod(mdg4)2.2 can directly interact (Figure 7B). The positive controls, Topo II-Ad (activation domain)/Topo II-Bd (binding domain) and Mod(mdg4)2.2-Ad/Mod(mdg4)2.2-Bd, show interaction-dependent phenotypes similar to Topo II-Ad/Mod(mdg4)2.2-Bd while the negative controls do not. These results, combined with the immuno-colocalization studies, suggest that Topo II and Mod(mdg4)2.2 do interact but this interaction may be transient and most likely does not take place on the chromatin. Thus, it appears that the effect of Topo II on the Su(Hw) insulator may be mediated by a direct association between Mod(mdg4)2.2 and Topo II that ultimately leads to modulation of Mod(mdg4)2.2 levels within the cell.
Discussion

Insulators mediate intra- and inter-chromosomal interactions and in doing so they organize the chromatin fiber within the eukaryotic nucleus [10]. Recent evidence suggests that this organization is cell type-specific [17, 21, 44, 45, 46] and that it plays a role in both the control of gene expression [47, 48] and in the epigenetic inheritance of imprinted expression patterns [49]. Topo II is an essential component of a variety of nuclear processes. Reports suggesting the presence of this protein bound to sequences of the insulator present in the gypsy retrotransposon [43] prompted us to examine the possibility of a role for Topo II in insulator function. In support of this possibility, we find that mutations in the Top2 gene interfere with the function of the insulator present in the gypsy retrotransposon. This is confirmed by the presence of Topo II at the yellow locus of y2 flies carrying an insertion of the gypsy retrotransposon in the yellow gene. However, Topo II is absent from both Su(Hw) and dCTCF endogenous insulator sites throughout the Drosophila genome indicating that Topo II is not a stable component of these insulator complexes. Nevertheless, downregulation of the Topo II protein using RNAi results in disruption of insulator bodies formed by the Su(Hw) protein. Furthermore, mutation of Topo II affects the binding of Mod(mdg4)2.2 but not of other insulator proteins to polytenic chromosomes, suggesting that Topo II has a role in regulating Su(Hw) insulator activity by facilitating Mod(mdg4)2.2 interaction.

Figure 6. Topoisomerase II modulates Mod(mdg4)2.2 degradation. (A) Top2 mRNA levels were quantified by qRT-PCR in wild type and mutant Top2f larvae. Actin5c and RPL32 were used as controls for mRNA levels. Top2 mRNA is greatly reduced in the Top2f mutant and mod(mdg4)2.2 mRNA levels are reduced but only to an average of 60% of wild type. (B) Inhibition of the proteasome by addition of the proteasome inhibitor MG-132 can prevent the degradation of Mod(mdg4)2.2 in the Top2f mutants. MG-132 has no effect on Mod(mdg4)2.2 protein levels in wild type larvae. (C) Mod(mdg4)2.2 (red) staining is recovered on polytene chromosomes of Top2f mutant larvae after treatment with the proteasome inhibitor MG-132; Su(Hw) is shown in green and the merge is indicated in yellow.

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with DNA. These results are significant because they highlight a potential regulatory pathway by which Mod(mdg4)2.2 can modulate the activity of Su(Hw) insulators. Previous work has revealed common mechanisms used by different insulators in Drosophila such as the general requirement for the protein CP190. We have recently shown that three previously characterized Drosophila insulators thought to be unrelated, gypsy, Fab8 and scs', actually share the CP190 protein and perhaps contain different isoforms of Mod(mdg4) [23,50]. Based on their distribution with respect to gene features, we have suggested that these different insulators may play distinct roles in gene expression. The fact that loss of Topo II only affects the Su(Hw) insulator subclass supports the conclusion of a distinct and specific role for this insulator. It is possible that dCTCF and BEAF insulators contain other Mod(mdg4) isoforms that are also affected by TopoII or they may be regulated by distinct mechanisms yet to be identified.

It has been proposed that insulators form loops through the interaction of individual insulator sites coming together at specific nuclear locations named insulator bodies. This model is in part supported by recent 3C analyses of intra-chromosomal interactions suggesting that several insulator sites can interact (A. Bushey, K. Van Bortle and V. Corces unpublished data). In Drosophila cells, these insulator bodies are thought to contain both sites of the gypsy retrotransposon as well as endogenous insulators. Thus, their stability would depend on proteins associated with endogenous as well as gypsy retrotransposon sites. We therefore examined the appearance of these insulator bodies as a way to determine how disruption of Topo II may impact the functional state of endogenous insulators along with general chromatin organization. Surprisingly, downregulation of the Topo II protein using RNAi results in disruption of insulator bodies formed by the Su(Hw) protein, suggesting that Mod(mdg4)2.2 is important for the nucleation of insulator bodies. Since mutation of Topo II affects the binding of Mod(mdg4)2.2 but not of other insulator proteins to polytene chromosomes, the results suggest that interaction of Mod(mdg4)2.2 with chromatin is necessary for the formation of insulator bodies.

Providing further insight into the relationship between Topo II and Mod(mdg4)2.2, co-immunoprecipitation and yeast two hybrid experiments suggest that the effect of Topo II on Su(Hw) insulator function is mediated by a direct interaction between Mod(mdg4)2.2 and Topo II. Interestingly, the association between the two proteins cannot be visualized on polytene chromosomes, suggesting that the interaction is transient or that it occurs in the nucleoplasm but not directly on the DNA. The lack of Mod(mdg4)2.2 protein on chromosomes is partly due to its downregulation at the level of RNA synthesis but, more dramatically, at the level of protein degradation, suggesting that the interaction between Topo II and Mod(mdg4)2.2 may bring the latter into contact with proteins that protect it from proteasome targeting or result in the modification of the Mod(mdg4)2.2 protein preventing its degradation. This ability of Topo II to protect Mod(mdg4)2.2 protein from proteasome-dependent degradation is similar to that observed in other systems [51,52]. For example, when Topo II function is inhibited by the Topo II poison R16 in human cells, a reduction of the DNA damage check point protein Chk1 is observed without greatly effecting Chk1 mRNA levels [52]. It is believed that Chk1 reduction contributes to the anticancer effects of R16 thus leading to apoptotic induction and cell death. It is also possible that the targeted reduction of
Topoisomerase II RNAi knockdown in cell culture

RNAi knockdown in cultured Drosophila S2 cells was conducted as per the Drosophila RNAi Screening Center (DRSC) protocol [40]. Primers TopoiIA3’ TAATACGACTCATATAGGTGTTTGCCAGAGCCATATGC, TopoiIA3’ TAATACGACTCATATAGGTGTTTGCCAGAGCCATATGC, and TopoiIB3’ TAATACGACTCATATAGGTGTTTGCCAGAGCCATATGC were used to make ampiclicons targeting the second and fourth exons of Topoi2, and the LacZ gene. Both second and fourth exon amplicons of Topoi2 have no off target sites; the LacZ amplicon does not target any Drosophila gene. Cells were incubated with dsRNAs for 3 days prior to fixation and western analysis.

Immunofluorescence analysis of diploid cells and polycene chromosomes

S2 cells were fixed with 3.7% formaldehyde in a solution of 0.1% Triton X-100 and 0.1 M sodium phosphate buffer pH 7.2 (NaP/ TX-buffer) for 30 min and blocked for 30 min in NaP/TX-buffer containing 5% normal goat serum. Cells were then attached to 0.01% poly-L-lysine treated slides for 10 min. The liquid was aspirated and cells were incubated overnight at 4°C in a humidified chamber containing primary antibodies at 1:1000 dilution for α-cDCtCF (1:2000), α-cPI90 (1:1000), α-Su(Hw) (1:1000), α-Topo II (1:2000).

Quantitative real-time PCR analysis

RNA was isolated from wild type and Top2 larvae using the Qiagen RNeasy kit (catalog #74104) with on-column DNA digestion (catalog #79254) and cDNA synthesis was performed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (catalog #4368014). Real-time PCR analysis was then used to quantify levels of Act5c, RPL32, Topo2, and mod(mdg4)2.2 and all mod(mdg4)2.2 RNAs. Primers: Act5c 5’-GTCGCTCAATCCAGAGCACC-3’, 5’-CCAGAGCGGACCTTCCTGG-3’ RPL32 5’-CGG-CTTCTAAGGCGGTATATCT-3’, 5’-GACAATCTCTGCCATTTC-3’, 5’-GGAAGCTCCGTATGCACATATTC-3’, 5’-GGAAGCTCCGTATGCACATATTC-3’. Expression levels were normalized to Act5c and RPL32 with no significant difference; thus, only Act5c is shown.

Western blot and immunoprecipitation analyses

For western analysis, extracts from S2 cells and imaginal tissue from third instar larvae were prepared using standard protocols and run on tricine gels using SDS sample buffer [37]. Co-immunoprecipitation was conducted as described [15]. The Millipore SNAP i.d. protein detection system was used for all the protein immunodetections following the manufacturer’s protocol. Blots were probed with the following primary antibodies α-CP190 (1:6000), α-dCTCF (1:3000), α-Su(Hw) (1:1000), α-Topo II (1:10000), α-β-tubulin (1:1500), α-H3 (1:10000), α-Mod(mdg4)2.2 (1:3000) and appropriate HRP-conjugated secondary antibodies (1:3000). The signal was detected using Thermo Scientific chemiluminescent substrates following the manufacturer’s protocol.

Proteasome inhibition assay

Salivary glands and imaginal discs from wild type and Top2 third instar larvae were dissected and placed into serum free HyClone CCM-3 Insect medium. The salivary glands were then transferred to HyClone medium containing either 50 μM MG-132 proteasome inhibitor dissolved in DMSO or HyClone with DMSO only and incubated for 2 h at 25°C. Western and immunofluorescence analyses were then conducted as described above.

Yeast two-hybrid assay

The yeast two-hybrid assay was conducted using the Invitrogen ProQuest Two-Hybrid System according to the manufacturer’s protocol. Full length Top2 and mod(mdg4)2.2 cDNAs were PCR cloned into the Gateway pENTR vector and shuttled to pDEST22-AD (activation domain) or pDEST32-BD (binding domain) vectors. The Topo II cDNA was a gift of Dr. T. Hsieh. pDest22-AD (activation domain) or pDest32-BD (binding domain) vectors. The Topo II cDNA was a gift of Dr. T. Hsieh. The Topo II cDNA was a gift of Dr. T. Hsieh. The Topo II cDNA was a gift of Dr. T. Hsieh. Controls provided by the kit are negative for mutants mt1-RalGDS-BD/Krev1AD, mt2-RalGDS/Krev1AD and positive for wt-RalGDS-BD/Krev1AD. In addition, the empty activation domain/DNA binding domain (Ad/Bd), empty Ad/Top2-Bd and empty Ad/Mod(mdg4)2.2-Bd plasmids were used as negative controls.

Supporting Information

Figure S1 Downregulation of Top2 in Drosophila using tissue specific Gal4 drivers. (A) Quantification of Top2 transcript using
qRT-PCR in animals in which Top2 expression was downregulated using RNAi under the control of *Ami-Gal4*. Significant reduction of Top2 can be observed compared to wild type. (B) Top2 RNAi under the control of the tissue specific Gal4 driver *C96-Gal4* shows reduction of Topo II at the dorsal-ventral boundary (red arrow) as visualized in wing discs by immunofluorescence microscopy. Topo II is in green and *Drosophila* discs large (Dlg), a marker for wing margin cells, is in red.

**Figure S2** Structure of the Top2 locus and western analysis of dsRNA knockdowns. (A) A schematic diagram of the Top2 locus, detailing the location of the Top2 mRNA, the Top2 RNAi amplons (DRSC03459, DRSC36057) used for dsRNA knockdowns and P-element insertions in intron 2 of the Top2 gene. (B) Western blot analysis of Topo II after a 72 hr incubation of S2 cells with dsRNA made using either exon 2 or exon 4 amplions. β-tubulin is used as a loading control.

**Figure S3** Characterization of Top2 alleles. (A) Quantification of Top2 transcript levels in each P-element-induced allele, *Top2Δ4*, *Top2Δ9*, *Top2ΔMB*, *Top2b*, *Top2d* and wild type. (B) Western blot analysis of Topo II levels in wild type, *Top2Δ4*, *Top2Δ9*, *Top2ΔMB*, *Top2b* and *Top2d* fly lines.

**Figure S4** Immunofluorescence microscopy of polytene chromosomes from Top2 mutants and *Drosophila* cultured cells. (A) Staining of polytene chromosomes from wild type and *Top2b* flies with antibodies against Topo II (green) and dCTCF (red). dCTCF is unaffected in *Top2b* whereas TopoII is absent. (B) Staining of polytene chromosomes from wild type and *Top2d* flies with antibodies against JIL-1 (green) and Mod(mdg4)2.2 (red). Mod(mdg4)2.2 is absent in polytene chromosomes from larvae lacking Topo II. (C) The Mod(mdg4)2.2 (red) foci at insulator bodies are absent in *Top2b* mutant animal tissue. Topo II is labeled in green.

**Figure S5** Localization of Topo II with respect to insulator proteins. (A–D) Magnified regions of polytene chromosomes from Figure 4. Yellow arrows indicate co-localization of Topo II and insulator proteins. (E) Nuclear localization of Topo II and Mod(mdg4)2.2 in S2 cells. In all panels Topo II is green and the corresponding insulator protein is labeled in red.

**Figure S6** Insulator protein levels in Top2 alleles. (A) Western blots of Topo II, Mod(mdg4)2.2, and Su(Hw) using protein extracts from larval imaginal tissue of Top2 alleles. (B) Western analysis of Topo II and Top2 alleles prior to their lethal stage. Topo II larvae were collected early in 2nd instar and Top2d larvae were collected by mid 2nd instar. Mod(mdg4)2.2 and Topo II are still detectable at these stages of development. (C) Western analysis of Topo II, Mod(mdg4)2.2 and Su(Hw) in *Arm-Gal4 UAS-Top2RNAi* larvae knockdowns. (D) *mod(mdg4)* mRNA levels were quantified by qRT-PCR in wild type and mutant *Top2b* larvae using primers for the BTB domain shared by all isoforms; total *mod(mdg4)* transcript levels are reduced.

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**Author Contributions**

Conceived and designed the experiments: ER VC. Performed the experiments: ET AB BG. Analyzed the data: ER VC. Wrote the paper: ER AB VC.

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**References**