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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 Top2 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 Top2 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 chromosomes 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 position 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,
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<sup>we6</sup> 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 insertion affects the communication between upstream retrotransposon inserted into the tissues. The various transgenic fly expressing a black (Figure 1A). Loss of Su(Hw) provides an example of the function manifested by a change in body color from yellow to ct<sub>6</sub>Su(Hw)RNAi line, which results in a decrease in Topo II levels of in the prospective wing margin region of the developing wing (Figure 1A). This allowed us to examine the effect of downregulation of this protein on the phenotype of y<sup>we6</sup> 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 insertion affects the communication between upstream retrotransposon inserted into the tissues. The various transgenic fly expressing a black (Figure 1A). Loss of Su(Hw) provides an example of the function manifested by a change in body color from yellow to ct<sub>6</sub>Su(Hw)RNAi line, which results in a decrease in Topo II levels of in the prospective wing margin region of the developing wing (Figure 1A). This allowed us to examine the effect of downregulation of this protein on the phenotype of y<sup>we6</sup> 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 insertion affects the communication between upstream retrotransposon inserted into the tissues. The various transgenic fly expressing a black (Figure 1A). Loss of Su(Hw) provides an example of the function manifested by a change in body color from yellow to ct<sub>6</sub>Su(Hw)RNAi line, which results in a decrease in Topo II levels of in the prospective wing margin region of the developing wing (Figure 1A).
P-element insertions cause 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 (<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 progress 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

Figure 1. Top2 RNAi knockdown affects gypsy phenotypes. (A) Transgenic UAS-Su(Hw)RNAi and UAS-Top2RNAi lines were used to knockdown Su(Hw) and Topo II in a y<sup>2</sup>c<sup>6</sup> mutant background. The transgenic RNAi lines alone do not affect the y<sup>2</sup> phenotype. In the control, Su(Hw) was knocked down using 2 copies of the Arm-Gal4 driver, causing a reversion in coloration of the abdomen to black and suggesting that the gypsy insulator is no longer functional. Reduction of Top2 also results in decreased function of the gypsy insulator, changing the coloration of the abdomen from yellow to black. (B) The same UAS-Su(Hw)RNAi and UAS-Top2RNAi lines were used to test the effect of loss of Su(Hw) and Topo II on the gypsy induced cut phenotype. The RNAi lines alone have no effect on the ct<sup>6</sup> phenotype. However, reduction of Su(Hw) or Topo II using the wing driver C96-Gal4 cause a reversion from a cut to a more wild type wing.

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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 Topo2 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 larvae 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 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>
<thead>
<tr>
<th>Alleles</th>
<th>Top2c</th>
<th>Top2f</th>
<th>Top2d</th>
<th>Top2LA</th>
<th>Top2MB</th>
<th>Df(2L)</th>
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</thead>
<tbody>
<tr>
<td>Top2c</td>
<td>2nd instar*</td>
<td>2nd instar*</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>2nd instar*</td>
</tr>
<tr>
<td>Top2f</td>
<td>3rd instar*</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>2nd instar *</td>
</tr>
<tr>
<td>Top2d</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td></td>
</tr>
<tr>
<td>Top2LA</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td></td>
</tr>
<tr>
<td>Top2MB</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td>Viable</td>
<td></td>
</tr>
<tr>
<td>Df(2L)</td>
<td>2nd instar*</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*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.

doi:10.1371/journal.pone.0016562.t001
Topo II (Figure 3A). However, unlike the S2 cell knockdown, results indicating that loss of Top2 inhibits the formation of Su(Hw) insulator bodies, Su(Hw) binding is not affected on polytene chromosomes from Top2 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 Top2f 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 Top2f mutant, whereas CP190 is still detectable in punctate bodies within the nucleus of Top2f mutant cells (Figure S4C). It is known that in flies carrying the null mod(mdg4)2.2276 allele, or the hypomorphic mod(mdg4)2.2276 and mod(mdg4)2.2276 alleles, the arrangement of Su(Hw) in 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 Top2 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.

Figure 3. Insulator protein localization on polytene chromosomes from Top2 mutants. (A) CP190 (red) and dCTCF (green) localize normally to polytene chromosomes from Top2f mutant larvae. Overlap of CP190 and dCTCF is indicated by the yellow coloration in the merge panel. (B) Mod(mdg4)2.2 (red) and Su(Hw) (green) localization on wild type polytene chromosomes; Mod(mdg4)2.2 and Su(Hw) co-localize extensively as indicated by the overlap in the merge panel (yellow). (C) Only Su(Hw) is present (green) whereas Mod(mdg4)2.2 is absent (no red) in Top2f mutant polytene chromosomes. In all the panels DAPI is blue.
doi:10.1371/journal.pone.0016562.g003
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 protein 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). 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 y' 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.
doi:10.1371/journal.pone.0016562.g004
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 Top2f larvae. As expected, we see a loss of Top2 in the Top2f 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 Top2f 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) 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 Top2f 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 Top2f 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 Top2f 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 y^2 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 polytene 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 Top2^f larvae. Actin5c and RPL32 were used as controls for mRNA levels. Top2 mRNA is greatly reduced in the Top2^f 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 Top2^f 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 Top2^f 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) [25,50]. 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) 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

Figure 7. Mod(mdg4)2.2 and Topo II directly interact. (A) Topo II and Mod(mdg4)2.2 co-immunoprecipitate. The left panel shows extracts treated with antibodies to Mod(mdg4)2.2 and subjected to western analysis using α-Topo II antibodies. The right panel shows extracts treated with antibodies to Topo II and subjected to western analysis using α-Mod(mdg4)2.2 antibodies. (B) Assay for direct interaction between Topo II and Mod(mdg4)2.2. Yeast expressing Topo II and Mod(mdg4)2.2 fused to either the GAL4 activation domain (AD) or DNA binding domain (BD) were spotted onto plates with nonselective (N/S), lacking uracil (−URA), or medium containing 5FOA (+FOA). Interaction is indicated by growth on −URA and the lack of growth on the FOA plates. doi:10.1371/journal.pone.0016562.g007
Mod(mdg4)2.2 could play a function in cell death, since a splice variant of Mod(mdg4), Doom, has a function in apoptosis [33]. Thus, it is conceivable that loss of Topo II triggers the cell to turn on the proteasome pathway to target a subset of proteins involved in apoptosis or cell survival.

**Materials and Methods**

**Genetics, Drosophila strains and RNAi knockdown in flies**

All stocks were cultured under standard conditions on yeast-agar medium at 25°C. The UAS-Top2RNAi (transformant ID 30625) and the UAS-Su(Hw)RNAi (transformant ID 10724) flies were obtained from the Vienna Drosophila RNAi Center and crossed to the different Gal4 driver lines, The P-element insertions into the Top2 gene, Top2α-G5, Top2α-G3, Top2α-G2, and Top2α-G1 were obtained from the Bloomington Drosophila Stock Center at Indiana University. The following strains were described [37] except that Alexa Fluor secondary antibodies and polytene chromosomes and Drosophila Genetics, 9 were obtained from the Vienna Drosophila RNAi Center and crossed to the different Gal4 driver lines. The P-element insertions into the Top2 gene, Top2α-G5, Top2α-G3, Top2α-G2, and Top2α-G1 were obtained from the Bloomington Drosophila Stock Center at Indiana University. The following strains were used for enhancer blocking assays, RNAi knockdowns and genetic analysis: Actin5c-Gal4, Ptch-Gal4, ey-Gal4, GMR-Gal4, CyO, Actin5c-GFP, Arm-Gal4, C96-Gal4 (a gift from B. Yedvobnick), Df(2L)Exel9043, GFP-AT, TopoII3 and GFP-AT, TopoII5.

**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′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9, TopoIIB3′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9, TopoIIA5′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9, TopoIIB5′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9, TopoIIB3′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9, TopoIIA5′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9, and TopoIIB5′ TAATACGACTCATATAGGGTTCTTGTAACAGCGACAGAAGCATC-9 were raised to the Vienna Drosophila RNAi Screening Center (DRSC) protocol.

**Immunofluorescence analysis of diploid cells and polytene 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 treated with 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-dCTCF (1:20000), α-dCP190 (1:3000), α-Su(Hw) (1:1500), α-Tubulin (1:1500), 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 Top2α 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 Top2α 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. Controls provided by the kit are negative for mutants mt1-RalGDS-BD/Krev1AD, mt2-RalGDS/Krev1-AD and positive controls provided by the kit are negative for mutants mt1-RalGDS-BD/Krev1AD, mt2-RalGDS/Krev1-AD and positive controls provided by the kit are negative for mutants mt1-RalGDS-BD/Krev1AD, mt2-RalGDS/Krev1-AD and positive controls provided by the kit are negative for mutants mt1-RalGDS-BD/Krev1AD, mt2-RalGDS/Krev1-AD and positive controls provided by the kit are negative for mutants mt1-RalGDS-BD/Krev1AD, mt2-RalGDS/Krev1-AD. 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 Top2α in Drosophila using tissue specific Gal4 drivers. (A) Quantification of Top2α transcript using...
qRT-PCR in animals in which Top2 expression was downregulated using RNAi under the control of Arm-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 amplicons (DRSC05349, 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 amplicons. 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, Top2^A4, Top2^LA, Top2^MB, Top2^LA and wild type. (B) Western blot analysis of Topo II levels in wild type, Top2^A4, Top2^LA, Top2^MB, Top2^LA and Top2^LA fly lines.

Figure S4 Immunofluorescence microscopy of polytenic chromosomes fromTop2 mutants and Drosophila cultured cells. (A) Staining of polytenic chromosomes from wild type and Top2^LA flies with antibodies against Topo II (green) and dCTCF (red). dCTCF is unaffected in Top2^LA whereas TopoII is absent. (B) Staining of polytenic chromosomes from wild type and Top2^LA flies with antibodies against JIL-1 (green) and Mod(mdg4)2.2 (red). Mod(mdg4)2.2 is absent in polytenic chromosomes from larvae lacking Topo II. (C) The Mod(mdg4)2.2 (red) foci at insulator bodies are absent in Top2^LA 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 polytenic 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 Top2^LA and Top2^LA alleles prior to their lethal stage. TopoII^LA were collected early in 2nd instar and TopoII^LA 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)2.2 mRNA levels were quantified by qRT-PCR in wild type and mutant Top2^LA larvae using primers for the BTB domain shared by all isoforms; total mod(mdg4)2.2 transcript levels are reduced.

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

References