Understanding the role of Topoisomerase 2 in chromosome associations

Amber Marie Hohl
University of Iowa

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UNDERSTANDING THE ROLE OF TOPOISOMERASE 2 IN CHROMOSOME ASSOCIATIONS

by

Amber Marie Hohl

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Genetics in the Graduate College of The University of Iowa

July 2012

Thesis Supervisor: Professor Pamela K. Geyer
ABSTRACT

Homologous chromosomes display associations in many organisms. *Drosophila melanogaster* (hereafter, Drosophila) serves as an excellent model to study pairing interactions since chromosomes are paired in all somatic cells throughout development. For many genes, the degree of homolog association influences gene expression. These effects, collectively referred to as transvection, can promote gene activation or silencing. Requirements for transvection are poorly understood. Chapter One reviews what is known about transvection in Drosophila and chromosome interactions in mammals. Recent cell culture studies implicated a requirement for Topoisomerase 2 (*Top2*) in chromosome pairing. *Top2* encodes an ATP dependent homodimeric enzyme that generates double stranded breaks to change DNA topology. This enzyme is a common target of anticancer drugs due to its role in DNA metabolism. To understand the *in vivo* role of Drosophila *Top2*, an EMS screen was completed. Chapter Two describes the identification and characterization of fifteen new EMS generated *Top2* mutations. Fifteen null and hypomorphic alleles were obtained, including one that displays temperature sensitivity. Molecular analyses of these alleles uncovered single or multiple base pair substitutions within the coding region of each mutant gene. Even though flies carrying individual missense alleles in *trans* to a deficiency are inviable, heteroallelic combinations of several missense alleles produced viable flies, including two lines carrying mutations that display resistance to anti-cancer drugs. These data indicate that *Top2* activity can be restored by dimerization of defective subunits. Our new *Top2* alleles establish a novel allelic series and provide a platform for understanding drug resistance. In Chapter Three, the role of *Top2* in chromosome associations was tested to determine whether mutations in *Top2* disrupted transvection. Viable heteroallelic combinations of *Top2* mutations were used to test transvection at three classically studied loci. For each gene, homologous interactions were analyzed by screening for alterations
in pairing-dependent changes in phenotype involving transvecting alleles. Only one of the three genes tested displayed phenotypic changes in Top2 complementing adults that were consistent with an alteration in pairing dependent changes in expression. Transcript levels were assessed at the three genes studied that display transvection. Our studies indicate that changes in the phenotype, due to altered Top2, are likely gene specific transcriptional changes. Further investigation of gene associations in Top2 mutants employed fluorescence in situ hybridization (FISH). These studies showed that all loci examined were paired near wild type levels, suggesting that Top2 does not globally disrupt homolog associations in vivo. The differences observed in Top2 function in vivo and in vitro may be explained by two possibilities. First, the probes studied differ from those used in vitro, indicating that different genetic loci may have different sensitivities to unpairing. Second, Top2 plays a role in the segregation of sister chromatids during anaphase and loss of Top2 causes improper resolution of chromosomes resulting in aneuploidy. In cell culture, cells were allowed to go through one division and then were subsequently fixed, permitting analyses on all cells. It is possible that nuclei exhibiting aneuploidy have undergone cell death in vivo, explaining why we do not see increased amounts of unpairing. In conclusion, Top2 contributions to nuclear functions are complex. Loss of Top2 may result in subtle changes in pairing that may affect transcription and transvection.

Abstract Approved: ____________________________________

Thesis Supervisor

____________________________________

Title and Department

____________________________________

Date
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July 2012

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CERTIFICATE OF APPROVAL

_________________________________

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Amber Marie Hohl

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Genetics at the July 2012 graduation.

Thesis Committee:  ___________________________________

Pamela K. Geyer, Thesis Supervisor

___________________________________

Josep Comeron

___________________________________

John Logsdon

___________________________________

Sarit Smolikov

___________________________________

Marc Wold
To Jacob and Addison
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ABSTRACT

Homologous chromosomes display associations in many organisms. *Drosophila melanogaster* (hereafter, Drosophila) serves as an excellent model to study pairing interactions since chromosomes are paired in all somatic cells throughout development. For many genes, the degree of homolog association influences gene expression. These effects, collectively referred to as transvection, can promote gene activation or silencing. Requirements for transvection are poorly understood. Chapter One reviews what is known about transvection in Drosophila and chromosome interactions in mammals. Recent cell culture studies implicated a requirement for *Topoisomerase 2 (Top2)* in chromosome pairing. *Top2* encodes an ATP dependent homodimeric enzyme that generates double stranded breaks to change DNA topology. This enzyme is a common target of anticancer drugs due to its role in DNA metabolism. To understand the *in vivo* role of Drosophila *Top2*, an EMS screen was completed. Chapter Two describes the identification and characterization of fifteen new EMS generated *Top2* mutations. Fifteen null and hypomorphic alleles were obtained, including one that displays temperature sensitivity. Molecular analyses of these alleles uncovered single or multiple base pair substitutions within the coding region of each mutant gene. Even though flies carrying individual missense alleles in trans to a deficiency are inviable, heteroallelic combinations of several missense alleles produced viable flies, including two lines carrying mutations that display resistance to anti-cancer drugs. These data indicate that Top2 activity can be restored by dimerization of defective subunits. Our new *Top2* alleles establish a novel allelic series and provide a platform for understanding drug resistance. In Chapter Three, the role of *Top2* in chromosome associations was tested to determine whether mutations in *Top2* disrupted transvection. Viable heteroallelic combinations of *Top2* mutations were used to test transvection at three classically studied loci. For each gene, homologous interactions were analyzed by screening for alterations
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LIST OF ABBREVIATIONS

Angelman Syndrome      AS
Bithorax Complex      BX-C
Chromosome Conformation Capture  3C
Chromosome Territories   CTs
Coiled coil Domain      CCD
C-terminal Domain       CTD
Curly                  Cy
Double stranded breaks DSBs
Ethyl methanesulfonate EMS
Fluorescence in situ hybridization FISH
Green Fluorescent Protein GFP
Germline stem cell     GSC
Histone H1             H1
Histone H3             H3
Human Mammary Epithelial Cells HMECs
Imprinting Control Region ICR
Initiator              Inr
Locus Control Region   LCR
Maternal-to-Zygotic Transition MZT
Mosaic Analysis with a Repressible Cell Marker MARCM
Not Applicable or Assessed NA
Not Determined         ND
Nucleolus Organizing Region NOR
Odorant Receptor       OR
Polycomb Response Elements PREs
Polymerase Chain Reaction PCR
Prader Willi Syndrome   PWS
Quantitative PCR        qPCR
Rearrangement          R
Topoisomerase/Primase   TOPRIM
Tower Domain           TD
Transcription Factories TFs
Transducer Domain       TDD
Scaffold Associated Regions SARs
Structural Maintenance of Chromosome SMC
Winged Helix Domain     WHD
**LIST OF GENES, PROTEINS, AND NON-CODING RNAS**

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CHAPTER 1
INTRODUCTION

Somatic pairing of homologous chromosomes in
Drosophila

Most metazoan cells are diploid. Cells have two copies of every chromosome, termed homologous chromosomes. Homologous chromosomes are inherited from the mother and father. The presence of two copies of each chromosome provides an opportunity for chromosome interactions. While homolog interactions are critical for the reductional division of meiosis, pairing can also occur in non-meiotic cells. Over a century ago, Nettie Stevens and Charles Metz discovered that chromosomes were intimately paired along their length in somatic cells in Drosophila and other dipterans (Metz, 1916; Stevens, 1908). Nettie Steven postulated that homolog pairing might influence gene expression.

“One is tempted to suggest that if homologous maternal and paternal chromosomes in the same cell ever exert any influence on each other, such that it is manifest in the heredity of the offspring, there is more opportunity for such influence in these flies than in cases where pairing of homologous chromosomes occurs but once in a generation. Possibly experiments in cross-breeding of flies may bring out some interesting facts in heredity.” Stevens, 1908.

High levels of pairing are observed in interphase somatic cells in Drosophila. Genomic regions generally pair in 60-100% of nuclei, depending on the locus assayed. The overall level of pairing is influenced by: tissue type, stage of development, region in the genome examined, and stage of the cell cycle. Pairing is not abrogated by changes in nuclear volume or ploidy, as levels of pairing reached 69-86% in cell lines exhibiting a range of ploidies (Joyce et al., 2012; Williams et al., 2007).

How homologs come together and find their partner is unknown. Robust levels of pairing of specific loci are first established early on in development—during interphase of cycle 14 during embryogenesis. During this time, the developmental program
switches from maternal to zygotic gene control, known as the maternal-to-zygotic transition (MZT) (Bateman and Wu, 2008; Fung et al., 1998; Gemkow et al., 1998; Hiraoka et al., 1993). A genome wide deletion screen failed to identify single or clustered genes required for pairing (Bateman and Wu, 2008). These data indicate that multiple genes contribute to pairing or that maternal factors are important in the initiation of pairing. Alternatively, the chromatin state prior to cycle 14 may prohibit pairing. This possibility is consistent with the rapid cell cycles occurring before and after the MZT. Together these findings indicate that homolog pairing may be developmentally regulated.

**Identification of factors important for somatic pairing**

Somatic pairing has been challenging to study. The tissue heterogeneity and requirement for viability of whole organisms precludes many types of studies. High throughput fluorescence in situ hybridization (FISH) analyses has been coupled with RNAi to screen for genes important in pairing in Drosophila cell culture. FISH utilizes fluorescently labeled DNA probes that hybridize to chromosomal DNA and are visualized as a spot within the nucleus. If the chromosomes are paired, one spot will be observed per nucleus. If the chromosomes are unpaired, two spots will be observed. This technique is very powerful as it allows direct examination of pairing in vivo and the design of probes targeting different regions of the genome.

A candidate RNAi screen using FISH tested five classes of genes for unpairing, including proteins with a demonstrated role in pairing dependent expression, chromosome condensation proteins, proteins involved in pairing during meiosis, insulator proteins, and scaffold proteins (B. Williams, Wu lab, unpublished observations). Knock down of only 1/66 genes showed significant alteration in pairing of euchromatic loci. The one gene found to affect pairing was Topoisomerase 2 (Top2) (Williams et al., 2007). Top2 is a type II topoisomerase that is an ATP dependent homodimeric protein that alters DNA topology (Collins et al., 2009; Nitiss, 2009; Schoeffler and Berger,
Top2 is required for transcription, replication, and chromosome segregation (Nitiss, 2009). The enzyme alters DNA topology by generating transient enzyme-linked double strand breaks for passage of one DNA strand through another (Nitiss, 2009). The \textit{in vivo} requirement for this protein was untested. Direct studies of pairing have also demonstrated that the insulator protein, Suppressor of Hairy-wing (Su(Hw)), plays a role in pairing in developing embryos (Fritsch et al., 2006).

A genome wide RNAi FISH screen to identify genes important in pairing was completed in cell culture with probes targeting heterochromatic regions on chromosomes X, II, and III (Joyce et al., 2012). Two classes of pairing genes were identified, including 65 ‘anti-pairing’ genes that when knocked down disrupt pairing, and 40 ‘pairing promoting’ genes that when knocked down enhance pairing (Joyce et al., 2012). This was the first demonstration that two classes of pairing genes exist that have antagonistic roles in the nucleus and that the pairing state in a cell is a result of the balance and interplay between factors (Joyce et al., 2012). Examination of genes identified from the screen revealed that proteins involved in pairing are involved in chromosome alignment and organization, cytokinesis, microtubule organization, transcription, cell cycle, nuclear import, and spindle organization (Joyce et al., 2012). As higher numbers of ‘anti-pairing’ genes were identified relative to ‘pairing promoting’ genes, it is possible that pairing of heterochromatin may be prevented in a normal state as a means to hamper repair or mitotic recombination between heterochromatic sequences (Joyce et al., 2012). A genome wide high-throughput RNAi FISH screen with probes targeted to euchromatic regions of the genome is currently underway (E. Joyce, personal communication). It will be interesting to compare genes involved in heterochromatic and euchromatic pairing to determine whether pairing occurs by similar or different mechanisms.
Transvection in Drosophila

The degree of homolog association influences gene expression. In 1954, Ed Lewis first described the phenomenon of transvection in Drosophila (Lewis, 1954).

“Operationally, transvection is occurring if the phenotype of a given genotype can be altered solely by disruption of somatic pairing. Such disruption can generally be accomplished by introduction of a heterozygous rearrangement that disrupts pairing in the relevant region but has no position effect of its own on the phenotype.” Lewis, 1954.

Lewis studied two mutations in the bithorax complex (BX-C) that regulate the identity of both thoracic and abdominal segments, \( bx^{34e} \) and \( Ubx^l \). Either mutation by itself results in a homeotic transformation, resulting in improper specification of the abdominal segments and/or halteres. Lewis found that \( bx^{34e} \) complements \( Ubx^l \) when in trans (\( bx^{34e}/Ubx^l \)), leading to wild type flies (Figure 1.1). When rearrangements were introduced that disrupt somatic pairing, Lewis found that complementation was also disrupted (\( R(bx^{34e})/Ubx^l \) or \( bx^{34e}/R(Ubx^l) \)). Curiously, a second analogous rearrangement that restored pairing also restored complementation (\( R(bx^{34e})/R(Ubx^l) \)). Lewis coined this phenomenon “transvection,” whereby somatic homolog pairing can influence gene expression.

Transvection has been identified at a number of genes (Table 1.1). These genes are found on different chromosomes and are expressed in distinct tissues. Genes displaying transvection requires special alleles that permit physical manifestation of a phenotype. In general, the genome is permissive for transvection. Studies using ectopically expressed transgenes revealed that transvection can occur at various locations throughout the genome (Bateman et al., 2012; Chen et al., 2002; Mellert and Truman, 2012).

Mechanisms of Transvection

Transvection can result in gene activation or gene silencing. Most cases of transvection result in positive changes of transcription, although some are negative (Table
1.1). Three different mechanisms for transvection have been described. These include enhancer action in trans, bypass of a chromatin insulator in cis, and pairing-dependent gene silencing (Geyer et al., 1990; Morris et al., 1998; Pirrotta, 1999; Wu and Goldberg, 1989). A classical example of each mechanism of transvection is described below.

**Enhancer action in trans**

A classical example of enhancer action in trans comes from studies of the yellow (y) gene. The yellow gene is located near the telomere on the X-chromosome and is responsible for wing and body pigmentation in the fly. Tissue specific enhancers located upstream of the transcription start site and within the single intron are required for proper yellow expression (Geyer and Corces, 1987; Martin et al., 1989). A number of yellow alleles have been identified that reduce or abolish pigmentation in the wing, body, and other cuticular structures. The first transvection studies at yellow involved the y² mutation, associated with reduced wing and body pigmentation (Geyer et al., 1990). This allele is caused by a gypsy transposon insertion between the wing and body enhancers and the promoter (Geyer and Corces, 1987). Since this transposon carriers an insulator, the wing and body enhancers are blocked from acting on the promoter, producing flies with light wings and bodies (Geyer et al., 1986). A second yellow allele, y⁵⁹b, is associated with reduced wing and body pigmentation (Geyer et al., 1990). This allele carries a partial deletion of gypsy and a complete deletion of the yellow gene promoter and first exon (Geyer et al., 1990). When y² is placed in trans to y⁵⁹b, flies have dark wing and body pigmentation (Geyer et al., 1990). Here, transvection involves the enhancers of y⁵⁹b working in trans on the promoter of the y² allele (Figure 1.2A). This was the first demonstration of enhancer action in trans at yellow.

Much work has focused on understanding the requirements for enhancer action in trans. Many enhancers can act trans on generic promoters, suggesting that the ability of enhancers to act across chromosomes may be a general rule in Drosophila (Bateman et
al., 2012; Mellert and Truman, 2012). Recent advances using the phiC31 integrase system have permitted novel transvection studies where trangenes are targeted to the same genomic location, permitting transcription to be monitored from each homolog (Bateman et al., 2012; Mellert and Truman, 2012). Two complementary studies were published assaying enhancer action in *cis* and *trans*. Although these studies used different enhancers and promoters and assayed transvection in different tissues, many of the findings were similar and provide details of promoter choice. An enhancer is able to activate a promoter in *trans* even when a functional, intact promoter is present in *cis* (Bateman et al., 2012; Casares et al., 1997; Goldsborough and Kornberg, 1996; Mellert and Truman, 2012; Sipos et al., 1998). Within the same cell, an enhancer can activate the *cis* and *trans* promoter, resulting in expression of both alleles (Bateman et al., 2012; Goldsborough and Kornberg, 1996; Mellert and Truman, 2012). These findings differ from studies at *yellow* whereby enhancers are committed in *cis*. The integrity of a promoter dictates whether the enhancers act in *trans*. Mutation of core promoter elements, such as the TATA box or initiator (Inr), free enhancers to act in *trans* (Geyer et al., 1990; Morris et al., 1999; Morris et al., 2004). In fact, enhancers prefer to act in *cis* on a heterologous promoter rather than activate an endogenous promoter in *trans* (Morris et al., 1999). In sum, most enhancers have the capacity to act in *cis* and *trans*. The *yellow* gene may be unique in demonstrating *cis*-preference.

**Bypass of an insulator in *cis***

A classical example of insulator bypass comes from studies of the *yellow* gene. Insulator bypass was first described at *yellow* and involves the *y*<sup>3c3</sup> allele (Morris et al., 1998). Female *y*<sup>3c3</sup> flies have light wings and dark bodies due to a deletion of the body enhancer and the *yellow* promoter (Morris et al., 1998). When the *y*<sup>2</sup> allele is paired with *y*<sup>3c3</sup>, the body enhancer of the *y*<sup>2</sup> allele bypasses the chromatin insulator in *cis* and
activates transcription, resulting in females with dark wings and bodies (Figure 1.2B) (Morris et al., 1998).

**Pairing dependent gene silencing**

A classical example of pairing dependent gene silencing comes from studies at the *white* gene. Transvection at *white* results from pairing dependent repression conferred by Zeste1 (Duncan, 2002; Pirrotta, 1999). The X-linked *white* gene is required for red eye pigmentation in the fly, with loss generating white eyes. When *white* genes are paired, as in females, the mutant Z1 protein silences both alleles, producing a yellow/orange eye phenotype (Pirrotta, 1999). When one *white* gene is present, as in males, pairing is not possible, so Z1-mediated repression does not occur, producing a red eye phenotype. In short, Z1 protein recognizes two *white* genes when they are paired but not when they are unpaired.

**Allele proximity is important for transvection**

Studies using ectopically expressed enhancer-less and promoter-less transgenes revealed that transvection only occurred when both transgenes were at the same homologous site in the genome, but not at non-homologous sites (Chen et al., 2002). Molecular details revealed that transgenes >20 kb from one another did not exhibit transvection (S.Ou, Wu lab, unpublished observations). Recent work adds further support to the long standing notion that transvection requires genes proximately at homologous sites. Transvection only occurs between two transgenes inserted at the same integration site but not at other integration sites (Bateman et al., 2012; Mellert and Truman, 2012). Together, these studies indicate that allele proximity is important for transvection.

**Identification of factors important for transvection**

Until recently, the identification of factors that are important for pairing has
largely been completed by examining whether genes alter transvection phenotypes in vivo. A handful of genes that affect pairing mediated phenotypes have been identified to date, and include zeste, Polycomb Group (PcG) genes, and Condensin II (Cap-H2) (Hartl et al., 2008; Kassis, 2002; Pirrotta, 1999). Each factor is described in detail below.

Zeste was the first protein identified that affects transvection (Lewis, 1954). Zeste is a DNA binding protein that multimerizes, suggesting it might bring chromosomes together at the DNA level and mediate long distance interactions between enhancers and promoters (Biggin et al., 1988; Chen et al., 1992; Chen and Pirrotta, 1993; Chen et al., 2002; Kostyuchenko M, 2009). Since its original identification, much attention has focused on understanding whether different alleles of zeste alter transvection. Two well-studied alleles are z¹ and zα. Mutant z¹ is a neomorph that has a known role in pairing dependent repression at white⁺. Mutant zα is a loss of function mutation. Curiously, zeste alleles affect transvection at some loci but not others (Coulthard et al., 2005; Duncan, 2002; Gelbart and Wu, 1982; Gohl et al., 2008; Hopmann et al., 1995; Kennison and Southworth, 2002; Leiserson et al., 1994). Findings also suggest that Zeste is not essential for pairing. Pairing at the Ubx locus, as assayed by FISH, is not disrupted in zeste protein null backgrounds (zα694 or zα), even though these mutations disrupt transvection at Ubx (Gemkow et al., 1998). It will be interesting to assess global Zeste binding by ChIP-Seq to better understand the role of Zeste in chromosome interactions.

Polycomb group (PcG) genes repress Hox genes during development and can influence pairing of cis regulatory elements, known as Polycomb Response Elements (PREs). Binding of PcG proteins to PREs confers pairing-dependent gene silencing (Kassis, 2002; Kavi et al., 2006; Pirrotta, 1999). Pairing-dependent gene silencing was first noticed when PRE containing transgenes marked by a mini-white⁺ gene showed repression when homozygous (Duncan, 2002). Several studies have shown that PREs mediate long distance interactions (Sigrist and Pirrotta, 1997; Vazquez et al., 2006). For
example, the MCP PRE from the BX-C interacts with a second MCP located at ectopic locations throughout the genome (Vazquez et al., 2006). Furthermore, work has demonstrated that loss of function mutations in the \textit{PcG} genes can abolish long-range interactions between different Fab-7 PREs, located on different chromosomes in both imaginal discs and the developing embryo, directly showing that \textit{PcG} genes are important in mediating long-range interactions between PREs (Bantignies et al., 2003). The principles of \textit{trans} silencing differ from the general principles we have learned from enhancers and promoters. In general, \textit{trans} silencing can occur between PREs located on different chromosomes that are far away from one other. This differs from studies of enhancers and promoters where transgenes must be at homologous sites in order for transvection to occur. One way interactions between PREs may be mediated is through the association with different protein partners. For example, Top2 and Barren interact with the \textit{PcG} protein, Polyhomeotic, and Barren is required to maintain pairing dependent silencing of Fab-7 (Lupo et al., 2001).

Loss of the Condensin II subunit, \textit{Cap-H2}, enhances transvection at \textit{yellow} and \textit{Ultrabithorax} (Hartl et al., 2008). Drosophila has two Condensin complexes, I and II, that contain \textbf{S}tructural \textbf{M}aintenance of \textbf{C}hromosome (SMC) proteins, SMC2 and SMC4 (Cobbe et al., 2006; Losada and Hirano, 2005). Both SMC2 and SMC4 are ATPases that form the core of Condensin I and II complexes (Losada and Hirano, 2005). Each SMC subunit complexes with Cap-H, Cap-G, and Cap-D2 to form Condensin I, or alternatively, each SMC subunit complexes with Cap-H2, Cap-G2, and Cap-D3 to form Condensin II. Condensins regulate chromosome structure and segregation during anaphase (Losada and Hirano, 2005). Studies of Drosophila polytene chromosomes revealed that Cap-H2 overexpression promotes unpairing of chromosomes, possibly by introducing positive supercoils in the DNA (Hartl et al., 2008). Cap-H2 may prevent interchromosomal interactions from occurring by altering the chromatin structure (\textit{i.e.} compaction) to favor unpairing.
**Pairing requirements for transvection**

Much work has focused on understanding the chromosomal requirements for transvection. The “critical region” is defined as the region on the chromosome that is needed for transvection, and in turn pairing. Critical regions are typically identified using chromosomal rearrangements that disrupt transvection, and therefore, chromosome pairing. Genes that show transvection effects have critical regions that vary greatly in size (Figure 1.3). In general, genes with large critical regions are easier to disrupt than genes with small critical regions (Ou et al., 2009). For example, *yellow* (~650 kb or less) and *white* (~50 kb) have small critical regions compared to other genes, such as *Ubx* (half of the chromosome arm; 12 Mb), *eya* (one-third of a chromosome arm), and *dpp* (two-thirds of a chromosome arm) (Coulthard et al., 2005; Duncan, 2002; Gelbart, 1982; Leiserson et al., 1994; Ou et al., 2009; Smolik-Utlaut and Gelbart, 1987). While these differences are quite remarkable, it is still unclear why the size of the critical region (i.e. the chromosomal requirements for pairing) varies greatly between genes.

When Ed Lewis first described the phenomenon of transvection he noticed that breakpoints of chromosomal rearrangements proximal (towards the centromere) to the BX-C were more effective at disrupting transvection than those distal (towards the telomere) to the locus (Lewis, 1954). Lewis hypothesized that pairing initiated at the centromere and moved distally towards the telomere, similar to a zipper. Studies using site-specific recombination revealed a similar finding. When recombination was forced in males, that generally have no recombination, recombination was inhibited at sites distal to a rearrangement breakpoint (Golic and Golic, 1996).

Several bodies of work are not consistent with the zipper hypothesis and offer an alternative model. First, not all proximal translocations disrupt transvection or pairing (Garcia-Bellido and Wadosell, 1978; Gemkow et al., 1998; Hinton, 1946; Merriam and Garcia-Bellido, 1972; Smolik-Utlaut and Gelbart, 1987). In fact, *yellow* and *white* both have very small critical regions. Ectopically expressing *yellow* or *white* from transgenes
revealed that only rearrangements with breakpoints very close to the gene disrupt transvection (Ou et al., 2009; Smolik-Utlaut and Gelbart, 1987). These findings suggest that there is not a specific chromosomal requirement for transvection but that critical regions might be gene specific (Ou et al., 2009; Smolik-Utlaut and Gelbart, 1987).

Second, direct analyses of FISH at several loci along different chromosome arms revealed that pairing is established at discrete foci along the length of the chromosome rather than processively from the centromere to the telomere. In sum, chromosomes might “button” rather than zipper (Fung et al., 1998; Gemkow et al., 1998).

Studies directly assaying pairing in vivo indicate somatic pairing is influenced by cell cycle events (Csink and Henikoff, 1998; Fung et al., 1998; Joyce et al., 2012; Williams et al., 2007). It has been demonstrated that the longer the time before ensuing mitoses, the more time homologs have to find one another and pair, the more difficult pairing will be to disrupt. For example, pairing during embryogenesis is not established until cycle 14 when the cell cycle is dramatically slowed, indicating there may not be enough time to intimately pair homologs during the rapid mitotic cycles preceding cycle 14 (Bateman and Wu, 2008; Fung et al., 1998; Gemkow et al., 1998; Hiraoka et al., 1993). A second example involves transvection at Ubx. Transvection has been established at Ubx, one of three genes of the BX-C. These genes regulate the identity of the thoracic and abdominal segments (Castelli-Gair et al., 1990). Cbx1 is a gain-of-function mutation of Ubx that is due to insertion of the Ubx upstream regulatory region into the second intron of the gene, resulting in overexpression of Ubx. Ubx1 is an insertion in the first exon that disrupts the protein-coding region. When Cbx1 Ubx1 is placed in trans to a wild type copy of the Ubx gene, Cbx1 drives ectopic expression of the wild type Ubx gene in trans, causing a homeotic transformation of the wing toward haltere, manifest as a shortened, deformed wing blade (Kennison and Southworth, 2002). Wild type flies have wings with a smooth margin and complete longitudinal cross veins. In contrast, adult Cbx1 Ubx1/+ flies have wings that are more cashew shaped with nicks
and often have incomplete longitudinal cross veins. Chromosomal rearrangements that alter pairing between \( Cbx^I \ Ubx^I \) and the wild type \( Ubx \) gene (\( R(Cbx^I \ Ubx^I )/++ \)) disrupt trans activation of \( Ubx \), resulting in wild type wings (Kennison and Southworth, 2002; Lewis, 1954). Studies revealed that transvection between \( R(Cbx^I Ubx^I )/+ + \) was enhanced in a Minute genetic background, a mutant that slows the rate of cell division, but not a Minute\(^+\) background where transvection was disrupted (Golic and Golic, 1996). These findings indicate that slowing the cell cycle allows the cell to overcome pairing problems (Golic and Golic, 1996).

Studies directly assaying pairing in vivo by FISH indicate that the level of pairing does not always correlate well with the phenotypic outcome (Gemkow et al., 1998). Pairing studies of the \( Ubx \) locus in embryos carrying a \( z^{694} \) or \( z^a \) allele, previously shown to disrupt transvection at \( Ubx \), revealed that the levels of pairing in the mutants was the same as that observed for wild type (Gemkow et al., 2001). This finding was puzzling since zeste mutations disrupt transvection at \( Ubx \), but no changes in pairing were detected. Studies of translocations that disrupt transvection at \( Ubx \) revealed that pairing at \( Ubx \) was only reduced by 50-60\% (Gemkow et al., 1998). Although translocations and zeste mutations result in the same phenotypic outcome at \( Ubx \) (i.e. disruption of transvection), the mechanism in which they affect gene expression differs (Goldsborough and Kornberg, 1996). Specifically, rearrangements that disrupt transvection at \( Cbx^I/+ \) reduce transcription from both homologues whereas mutation of zeste\(^a\) results in transcriptional reduction of the homolog carrying the wild type \( Ubx \) gene (Goldsborough and Kornberg, 1996). In other words, rearrangements do not abolish activation in trans while zeste\(^a\) does. Although the molecular details of this mechanism are not known, it is possible that tracking of polymerase influences expression (P.Geyer, personal communication). Many factors may influence transvection. These include the length of the cell cycle, when during development transvection is established, to what degree chromosomes must pair for a gene to recognize it as paired, how stable chromosome
interactions are over time, and the extent that a gene must be expressed in order to see a physical change in phenotype.

**Chromosome interactions occur in other species**

Chromosome associations have been described outside of Drosophila. In general, chromosomes are not intimately paired along their length throughout development. Eukaryotic homologous chromosomes reside in separate and distinct locations within the nucleus, known as chromosome territories (CTs). It has been speculated that one underlying function of CTs may be to keep the homologs physically separated in space, minimizing associations and cross-talk (Apte and Meller, 2012). In general, gene rich chromosomes and active genes localize to the interior of the nucleus while gene poor chromosomes and silenced genes are found at the periphery (Cremer and Cremer, 2010; Croft et al., 1999; Geyer et al., 2011). Many factors influence chromosome localization, including DNA replication, transcription, and cellular differentiation (Cremer and Cremer, 2010; Kuroda et al., 2004).

A non-random distribution of chromosomes within the nucleus is evolutionarily conserved in higher primates, indicating the functional importance of nuclear position (Tanabe et al., 2002). Although chromosomes occupy specific territories within the nucleus, genes are often still able to interact in trans. Emerging evidence suggests that interchromosomal associations occur between non-homologous chromosomes, suggesting that transcriptional regulatory regions are shared to permit cross-regulation (Lomvardas et al., 2006; Spilianakis and Flavell, 2004; Spilianakis et al., 2005; Xu et al., 2006). Rather than entire chromosomes coming together and pairing, associations are often stochastic and involve coalescence of individual loci (Apte and Meller, 2012). In many cases chromosome interactions lead to changes in gene expression, as exemplified by processes of mammalian X-inactivation and imprinting (Ciaudo et al., 2009; Riesselmann and Haaf, 1999; Xu et al., 2006). Chromosome interactions serve many
functions in the nucleus and are important in regulating several biological processes that are described below. For many examples, conflicting data has been reported in the literature. These observations are discussed below to provide a representative view of chromosome interactions in mammals.

Chromosome pairing is instrumental in mammalian X-inactivation (Augui et al., 2007; Bacher et al., 2006; Masui et al., 2011; Xu et al., 2006). In mammals, females randomly inactivate one of their two X chromosomes to equalize the gene dosage to males. Proper counting and choice are regulated by pairing of the X-inactivation center (Xic) on each X chromosome (Bacher et al., 2006; Xu et al., 2006). Three non-coding genes are important in regulating chromosome choice, including: X (inactive)-specific transcript (Xist), its antisense partner Tsix, and X-inactivation intergenic transcription element (Xite) (Xu et al., 2006). Xite and Tsix are essential for pairing, as loss of either gene abolishes interactions in vivo, resulting in random inactivation of one, none, or both X chromosomes (Augui et al., 2007; Bacher et al., 2006; Xu et al., 2006). Excitingly, ectopically expressing Xite or Tsix on an autosome promotes interactions of the X chromosome and autosome, even though proper X-inactivation was abolished (Augui et al., 2007; Bacher et al., 2006; Xu et al., 2006). Recent work has focused on identifying trans factors that are important for pairing of the X chromosomes; loss of the pluripotency factor, Oct4, or the insulator protein, CTCF (CCCTC-binding factor), was found to disrupt pairing of X chromosomes (Donohoe et al., 2009). Loss of RNA polymerase II mediated transcription also abolishes pairing (Xu et al., 2006). In sum, these findings indicate that in mammals, specific sequences on the X chromosome are important in regulating pairing and that pairing may play a role in counting and choice (Williams et al., 2010). It is possible that pairing regulates transcription (or transcription regulates pairing) and that trans factors (or “pairing factors”) are required for keeping the chromosomes in a paired state.
**Imprinting in Mammals**

Pairing has been implicated in imprinting. Maternal and paternal alleles of the same gene can be differentially expressed. This gene regulating mechanism, known as imprinting or monoallelic expression, is epigenetically regulated. A number of loci in mammals are imprinted, including the 15q11-q13 region of chromosome 15 (Leung et al., 2011). This region is sensitive to lesion, as improper regulation of the imprinting region can lead to the neurodevelopmental disorders, **Angelman syndrome** (AS) and **Prader-Willi syndrome** (PWS).

PWS is caused by a paternal deletion of the 15q11-13 imprinted region. AS is caused by a maternal deletion of UBE3, a ubiquitin protein ligase E3A gene within the same 15q11-13 imprinted region (Horsthemke and Wagstaff, 2008; Leung et al., 2011). Examination of chromosome pairing by FISH in human T lymphocytes revealed that the 15q11-13 imprinted regions of maternal and paternal homologues associated during late S phase of the cell cycle (LaSalle and Lalande, 1996). In cells from patients with AS or PWS, homologous associations were not observed, indicating that pairing is important in establishing or maintaining imprinting at this locus and that lack of pairing may be associated with a diseased state (LaSalle and Lalande, 1996). Furthermore, homologous pairing of the 15q11-13 imprinted domains was perturbed in Rett syndrome, Angelman syndrome, and Autism brain samples compared to controls (Thatcher et al., 2005).

Subsequent studies are incompatible with the idea that homologous associations are important in establishing or maintaining imprinting at the 15q11-13 locus. Examination of human T lymphocytes using different FISH probes revealed no such pairing of 15q11-13 imprinted regions during G1, S, or G2 stages of the cell cycle (Teller et al., 2007). Instead, it was hypothesized that the pairing observed in previous studies may have been due to convergence of the nucleolus organizing region (NOR) that is associated with chromosome 15, and all other acrocentric chromosomes. In fact, direct examination of the centromeres of acrocentric chromosomes with NORs revealed pairing
during late S phase of the cell cycle (Teller et al., 2007). These findings suggest that pairing may not be critical in establishing or maintaining imprinting at the 15q11-13 locus of chromosome 15.

Interchromosomal associations have been described for loci outside of the imprinted 15q11-13 locus. One such interaction is between the insulin-like growth factor 2 (Igf2)/H19 imprinting control region (ICR) on chromosome 7 and the Wsb1/neurofibromin 1 (Nf1) genes on chromosome 11 (Kurukuti et al., 2006; Ling et al., 2006). These interactions were found to be dependent on the maternally provided ICR and CTCF (LING et al. 2006). In addition, loss of CTCF resulted in decreased levels of Nf1 and Wsb1 transcription, consistent with the idea that CTCF or transcription is facilitating these interchromosomal interactions. It is currently unclear the exact role that CTCF plays in this process, although it has been speculated that it may function to bring chromosomes together (Ling et al., 2006).

Cytokine expression

Both intrachromosomal and interchromosomal interactions have been described for cytokine expression in mice. Naïve CD4+ T helper cells differentiate into either T_{H1} or T_{H2} effector T cells that are responsible for building immunity to intracellular microorganisms or parasites, respectively (Amsen et al., 2009; Lee et al., 2006). The T_{H1} cytokine interferon gamma (Ifng-γ) resides on chromosome 10 while the T_{H2} cytokines interleukin (Il)-4, Il-5, and Il-13 are all clustered on chromosome 11 (Williams et al., 2010). Upstream of the T_{H2} cytokine cluster is a locus control region (LCR) that interacts with the promoters of Il-4, Il-5, and Il-13 (Spilianakis and Flavell, 2004). Using a combination of chromosome conformation capture (3C) and DNA FISH, studies revealed that the regulatory regions of the T_{H2} cytokine locus on chromosome 11 interacts with the promoter region of Ifng-γ on chromosome 10 (Spilianakis and Flavell, 2004; Spilianakis et al., 2005). These interchromosomal interactions were observed in
naïve CD4\(^+\) T cells but were lost upon differentiation into Th1 or Th2 effector cells, where intrachromosomal interactions were favored (Spilianakis et al., 2005). It is possible that chromosome associations “poise” chromatin for rapid expression of cytokine genes that occurs upon differentiation into Th1 or Th2 subsets (Spilianakis et al., 2005). This work is supported by data showing that deletion of the Th2 LCR delayed the expression of \(\text{Ifng-}\gamma\) during Th1 differentiation, demonstrating that this interaction alters gene expression (Sexton et al., 2009; Spilianakis et al., 2005).

**Olfactory receptor choice**

*Trans* chromosome interactions have been described for olfactory receptor choice. There are over 1300 different odorant receptor (OR) genes in mice. Specialized cells, known as olfactory sensory neurons, express only one of 1300 OR genes (Chess et al., 1994; Malnic et al., 1999). One of the best-studied clusters of OR genes resides on chromosome 14. Expression of this cluster (including genes \(\text{MOR28, MOR10, and MOR83}\)) requires an upstream enhancer element, known as the \(H\) element, that is 75 kb upstream of \(\text{MOR28}\) (Lomvardas et al., 2006; Serizawa et al., 2003). Given that the \(H\) element regulates OR choice of the \(\text{MOR28}\) cluster in *cis* (Fuss et al., 2007; Serizawa et al., 2003; Serizawa et al., 2006), it was hypothesized that the \(H\) element might be instrumental in directing genome wide OR choice through *trans* interactions. Using a combination of 3C and DNA or RNA FISH, studies revealed that the \(H\) element associates with the promoters of different OR genes on the same and different chromosomes and that this interaction leads to expression of only one OR allele per olfactory sensory neuron in epithelial tissues (Lomvardas et al., 2006). These findings indicated that *trans* interactions between the H element and promoters of different OR genes govern olfactory receptor choice in mice. Curiously, though, subsequent studies found that deletion of the \(H\) element had no effect on expression of OR genes on different chromosomes but did abolish *cis* activation of \(\text{MOR28, MOR10, and MOR83}\) (Fuss et al.,
2007). It is currently unclear what, if any, role the H element plays in dictating OR choice. Most recently another putative regulatory element was identified, known as the P element (Khan et al., 2011). High-throughput expression analyses of ΔP and ΔH mice revealed that both elements may specifically regulate OR choice, but not transcript levels. Further analyses are needed to fully understand the chromosomal interactions associated with olfactory receptor choice and the mechanisms governing this process.

**Estrogen responsive genes**

Intra and interchromosomal interactions have been identified in response to estrogen in mammalian cells (Hu et al., 2008). Using a high throughput approach known as deconvolution of DNA interaction by DSL (3D assay), addition of 17β-estradiol (E2) to human mammary epithelial cells (HMECs) resulted in rapid interactions between the breast cancer protein (GREB1) gene located on chromosome 2 and the trefoil factor 1 (TFF1) gene located on chromosome 21. These interactions resulted in changes in nuclear reorganization and association with interchromatin granules (Hu et al., 2008). It was hypothesized that the interchromatin granules (also known as nuclear speckles) may serve as “enhancer hubs” that bring the chromatin to concentrated areas of transcription factors, chromatin remodeling complexes, and factors necessary for pre-mRNA splicing (Hu et al., 2008; Lamond and Spector, 2003; Saitoh et al., 2004). Chromosome interactions were dependent on both actin and myosin machinery, while association with the interchromatin granules was dependent on the histone lysine demethylase, LSD1, suggesting that associations might be dependent on global nuclear re-organization in response to E2 estradiol treatment (Hu et al., 2008). Subsequent reports, however, including a retraction, do not support these findings (Kocanova et al., 2010; Nunez et al., 2008). In fact, studies using the same HMEC cell line revealed no colocalization of chromosome 2 and 21 upon treatment with E2 estradiol (Kocanova et al., 2010). Furthermore, genome wide analyses of chromosome interactions in a breast cancer cell
line (MCF-7) treated with E2 estradiol revealed hundreds of intrachromosomal interactions, but no interchromosomal interactions that could be validated (Fullwood et al., 2009). It is unclear at this time whether interchromosomal interactions routinely occur in response to estrogen in mammalian cells.

**Pairing in Humans**

Chromosome associations have been described in humans. As in other mammals, human chromosomes are not intimately paired along their length in somatic cells. In fact, extensive somatic pairing in humans may be pathological. Pairing of homologous and/or non-homologous chromosomes has been observed in a number of different tissues and is associated with different cancers, including renal oncocytoma and lymphomas associated with the hematopoietic and lymphoid system (Koeman et al., 2008; Lewis et al., 1993), and follicular lymphoma and prostate cancer, respectively (Atkin and Jackson, 1996; Koeman et al., 2008; Williams et al., 1995). Overall, it is not clear that there are any hard and fast rules when it comes to pairing in humans. In some situations pairing results in deleterious consequences, but in others it does not (Arnoldus et al., 1989). Some chromosomal regions pair, others do not. Some chromosomes are associated with disease, others are not associated with disease. Overall, all studies have pointed to the idea that somatic pairing is likely cell type specific. For example, the pericentromeric region of chromosome 1 was paired in wild type cerebellum nuclei but unpaired in wild type cerebral nuclei (Arnoldus et al., 1989). More studies are needed to understand the mechanism of somatic pairing in humans, whether heterochromatic regions of the chromosomes pair differently than euchromatic regions, whether chromosome specificity is important in the pathological outcome, and what factors are potentially bringing the chromosomes together or driving them apart.
**Bridging pairing in Drosophila and pairing in mammals**

What can pairing in Drosophila reveal about pairing in mammals? It is possible that Drosophila and mammals have overlapping mechanisms that maintain or abolish somatic pairing. The findings that Drosophila has proteins that antagonize somatic pairing suggest that mammals may have similar mechanisms that keep the homologs separated in a normal state (Hartl et al., 2008; Joyce and McKim, 2007; Joyce et al., 2012; Williams et al., 2007). High throughput FISH analyses are needed in mammalian cell lines to determine whether mechanisms of somatic pairing are similar across species. Until then, Drosophila serves as an excellent model to study pairing interactions because homologous chromosomes are fully aligned throughout development.

**Thesis Outline**

This dissertation focuses on understanding the role of Top2 in chromosome associations using *Drosophila melanogaster* as a model system. Recently, disruption of Top2 was found to affect somatic pairing in Drosophila cell culture (Williams et al., 2007). To date, published Top2 studies in Drosophila have used RNAi or chemical inhibitors to understand the function of the enzyme *in vivo*. To determine whether Top2 is required for homolog interactions *in vivo*, germline mutations were needed. In Chapter 2, I discuss a forward F1 and F2 genetic screen used to generate novel mutations in Top2 and defined phenotypes associated with loss of the enzyme. Fifteen novel Top2 mutations were identified and characterized. Molecular analyses of these alleles uncovered single or multiple base pair substitutions within the coding region of each mutant gene. These included changes that generated premature stop codons or missense mutations within previously identified functional domains. Even though flies carrying each missense allele in *trans* to a deficiency are inviable, I found that heteroallelic combinations of several missense alleles produced viable flies. Our studies establish the
first series of Top2 alleles in a multicellular organism. Future analyses of these alleles will enhance our knowledge about the contributions made by Top2 to development.

In Chapter 3, I use the viable combinations of Top2 mutants defined in Chapter 2 to study homolog pairing in vivo. I examine whether mutations in Top2 affect pairing as assayed by the pairing-sensitive phenomenon of transvection. For each gene, homologous interactions between transvecting alleles were analyzed for alterations in pairing-dependent changes in phenotype. Top2 complementing mutants displayed phenotypic changes at one of three genes, consistent with altered pairing dependent changes in expression. Direct tests of chromosome pairing via fluorescent in situ hybrization (FISH) revealed wild type levels of pairing at all loci examined in Top2 mutants. I discuss my findings and offer an explanation of Top2 function. In chapter 4, I summarize my work and offer future perspectives.
Table 1.1 Transvection phenotypes at different genomic loci

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genomic Location</th>
<th>Chr</th>
<th>WT Phenotype</th>
<th>LOF Phenotype</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>yellow</td>
<td>1A</td>
<td>X</td>
<td>Dark wing, body</td>
<td>Yellow wing, body</td>
<td>Enhancer action in cis and trans</td>
<td>Geyer, Green et al. 1990; Morris, Chen et al. 1998</td>
</tr>
<tr>
<td>white</td>
<td>3A</td>
<td>X</td>
<td>Orange/yellow eye color</td>
<td>WT eye color</td>
<td>Pairing dependent repression</td>
<td>Babi and Bhat 1980</td>
</tr>
<tr>
<td>locsce</td>
<td>8D-8D6</td>
<td>X</td>
<td>WT eyes</td>
<td>Rough, shiny eyes</td>
<td>Unknown</td>
<td>Canon and Banerjee 2003</td>
</tr>
<tr>
<td>compensatory response</td>
<td>NA</td>
<td>X</td>
<td>Disproportionate replication of rRNA genes</td>
<td>No disproportionate replication of rRNA genes</td>
<td>Enhancer action in cis and trans</td>
<td>Prowant and Tartof 1978</td>
</tr>
<tr>
<td>decapentaplegic</td>
<td>22F1-22F3</td>
<td>II</td>
<td>WT wings</td>
<td>Held out wings</td>
<td>Enhancer action in trans</td>
<td>Smolik-Ulaut and Gelbart 1987</td>
</tr>
<tr>
<td>eyes absent</td>
<td>26E1-26E2</td>
<td>II</td>
<td>WT eyes</td>
<td>Absence of eyes</td>
<td>Enhancer action in trans</td>
<td>Leiseron, Bonini et al. 1994</td>
</tr>
<tr>
<td>Gpdh</td>
<td>26A3</td>
<td>II</td>
<td>WT levels of Gpdh</td>
<td>Reduced levels of Gpdh</td>
<td>Enhancer action in cis and trans</td>
<td>Gibson, Reed et al. 1999</td>
</tr>
<tr>
<td>wingless</td>
<td>27F1</td>
<td>II</td>
<td>WT wings</td>
<td>Loss of wing structures</td>
<td>Enhancer action in trans</td>
<td>Neumann and Cohen 1996</td>
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<tr>
<td>light</td>
<td>NA</td>
<td>II</td>
<td>WT eyes</td>
<td>Variegated eyes</td>
<td>NA</td>
<td>Hessler 1958</td>
</tr>
<tr>
<td>apterous</td>
<td>41F8</td>
<td>II</td>
<td>WT halter and wings</td>
<td>Reduction in halter, wings</td>
<td>Enhancer action in trans</td>
<td>Gohl, Muller et al. 2008</td>
</tr>
<tr>
<td>vestigial</td>
<td>49E1</td>
<td>II</td>
<td>WT halter and wings</td>
<td>Absence of halter, stumpy wings</td>
<td>Enhancer action in trans</td>
<td>Coulthard, Nolan et al. 2005</td>
</tr>
<tr>
<td>pointed</td>
<td>94E10-94E13</td>
<td>III</td>
<td>WT eyes, wings, and halteres</td>
<td>Rough eyes, abnormal wings and halteres</td>
<td>Enhancer action in trans</td>
<td>Scholz, Deatricken et al. 1993</td>
</tr>
<tr>
<td>Sex combs reduced</td>
<td>84A5</td>
<td>III</td>
<td>WT numbers of sex comb teeth</td>
<td>Reduction in number of sex comb teeth per leg</td>
<td>Pairing dependent repression; enhancer action in cis</td>
<td>Southworth and Kennison 2002</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>87C6-87C7</td>
<td>III</td>
<td>WT levels of malic enzyme activity</td>
<td>Reduced levels of malic enzyme activity</td>
<td>Enhancer action in trans</td>
<td>Lum and Merritt 2011</td>
</tr>
<tr>
<td>Ultrathorax</td>
<td>89D6-89D9</td>
<td>III</td>
<td>Transformation of wing to halter</td>
<td>WT wings</td>
<td>Enhancer action in trans</td>
<td>Lewis 1954</td>
</tr>
<tr>
<td>Abd-B</td>
<td>89E4-89E5</td>
<td>III</td>
<td>Normal A7 tergite</td>
<td>Increased size of A7 tergite</td>
<td>Enhancer action in trans</td>
<td>Lewis 1995</td>
</tr>
<tr>
<td>hedgehog</td>
<td>94E1</td>
<td>III</td>
<td>WT levels of hedgehog</td>
<td>Reduced levels of hedgehog</td>
<td>Enhancer action in trans</td>
<td>Lee, von Kessler et al. 1992</td>
</tr>
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Chr: Chromosome, WT: wild type
Figure 1.1 Chromosomal rearrangements demonstrate transvection is a pairing dependent process within the bithorax complex (BX-C). Alleles of $bx^{34e}$ and $Ubx^I$ display complementation when in trans to one another (left panel). A chromosomal rearrangement disrupts the complementing phenotype (middle panel). A second analogous rearrangement restores complementation between $bx^{34e}$ and $Ubx^I$ (right panel). (Williams, 2008).
Figure 1.2 **Modes of transvection at the yellow locus.** A) Complementation occurs between \( y^{59b} \), an allele that lacks the *yellow* promoter, and \( y^{2} \), an allele that has wing (W) and body (B) enhancer activity blocked by the *gypsy* insulator (inverted black triangle). B) The blocked body enhancer of \( y^{2} \) is able to bypass the chromatin insulator in *cis* when paired with \( y^{3c3} \). The wing enhancer of \( y^{2} \) may be able to act in *cis*, or the wing enhancer of \( y^{3c3} \) can activate transcription in *trans*. Wing and body pigmentation is scored on a scale of 1 to 4. 1 represents complete loss of pigmentation whereas 4 represents wild type levels of pigmentation. Bristle enhancer (Br) and tarsal claw enhancer (T). The *yellow* alleles are drawn approximately to scale.
Figure 1.3 **Critical Regions for transvection**. The “critical region” is defined as the region on the chromosome that is needed for transvection, and in turn homolog pairing. Critical regions are typically identified using chromosomal rearrangements that disrupt transvection. Critical regions are estimated for yellow (y), white (w), decapentaplegic (dpp), eyes absent (eya), and Ultrabithorax (Ubx). (Williams, 2008).
CHAPTER 2
A GENETIC SCREEN UNCOVERS IN VIVO COMPLEMENTATION BETWEEN DEFECTIVE MONOMERS OF DROSOPHILA TOPOISOMERASE 2

Introduction

Topological changes in chromosome structure are resolved through the action of a wide variety of enzymes known as topoisomerases. Among these, type II topoisomerases are conserved enzymes that alter DNA structure by introducing a transient double-strand break (DSB) in one DNA strand and passing a second DNA strand through the cleaved strand (Nitiss, 2009a; Wang, 2002). This catalytic cycle involves the covalent attachment of topoisomerase II to DNA, a reversible reaction that ends by ligation of the DSB. Formation of an enzyme-DNA intermediate protects the DNA ends and prevents activation of a DNA damage checkpoint.

Type II topoisomerases are homodimeric proteins (Collins et al., 2009; Nitiss, 2009a; Schoeffler and Berger, 2008). Each monomer is comprised of distinct regions that cooperate to alter DNA topology (Figure 2.1, Table 2.1). The amino terminal ATPase domain is responsible for ATP binding and hydrolysis, which promotes dimer formation and regulates DNA opening and closing. Flanking the ATPase domain is the Transducer domain (TDD), which signals ATP binding to the catalytic core. Following the TDD is the catalytic core, comprised of two domains required for DNA breakage and religation. Of these, the Topoisomerase/Primase (TOPRIM) domain contains a triad of acidic amino acids that are required for the DNA cleavage reaction and the Winged helix domain (WHD) that contains the active site tyrosine that forms the covalent linkage with DNA. The Tower domain (TD) and Coiled-coiled domains (CCD) follow the catalytic core, and together regulate the passage of one DNA strand. The last domain is the carboxyl-terminal domain (CTD). While the CTD is dispensable for catalytic activity in vitro, it
regulates nuclear accumulation and interactions with partner proteins in vivo (Collins and Hsieh, 2009). Among the topoisomerase domains, the CTD is the least conserved among eukaryotes, differing both in length and sequence (Austin et al., 1993). Structural domains within dimeric type II topoisomerases are formed by contributions from both monomers (Classen et al., 2003; Liu and Wang, 1999), which facilitates coupling of ATP hydrolysis to conformational changes that are necessary for altering DNA structure.

In light of the function of type II topoisomerases, it is not surprising that these enzymes are structurally conserved and encoded by essential genes (Nitiss, 2009a). The yeast and Drosophila genomes each contain a single gene, called Topoisomerase 2 (Top2). Mammalian genomes contain two Top2 genes, Top2A and Top2B, which encode differentially expressed paralogs, Top2α and Top2β, respectively (Austin and Marsh, 1998). Top2A is expressed primarily in proliferating cells, while Top2B is expressed in all cells, with elevated levels in terminally differentiated cells (Capranico et al., 1992; Watanabe et al., 1994). Interestingly, yeast Top2 mutants are rescued by expression of the Drosophila or human Top2 protein (Jensen et al., 1996; Wyckoff and Hsieh, 1988), illustrating the strong functional conservation among eukaryotic type II topoisomerases.

Eukaryotic type II topoisomerases resolve entwined DNA strands and relax supercoiled structures that arise from the action of DNA polymerases. Genetic knockdown and chemical inhibitor studies have revealed that loss of Top2 causes chromosome missegregation and DNA damage during mitosis due to a failure to resolve sister chromatids and centromeres (Baxter and Diffley, 2008; Chang et al., 2003; Coelho et al., 2008; Gonzalez et al., 2011). Some of these defects may result from altered chromosome architecture (Buchenau et al., 1993; Chang et al., 2003; Coelho et al., 2008; Stanvitch and Moore, 2008; Uemura et al., 1987), as Top2α is a major structural component of mitotic chromosomes (Earnshaw et al., 1985; Maeshima and Laemmli, 2003).
Aside from its enzymatic role, Top2 plays a structural role in the nucleus. Top2 was isolated as a component of the “chromosome scaffold” from interphase and mitotic cells (Berrios et al., 1985; Earnshaw et al., 1985; Earnshaw and Heck, 1985; Hirano and Mitchison, 1993). *In vitro* analyses of the scaffold revealed specific AT-rich regions of the DNA, termed scaffold associated regions (SARs), that may form the anchor of chromatin loops (Adachi et al., 1989; Mirkovitch et al., 1987). Interestingly, Top2 localizes to an axial region at the base of loops (Earnshaw and Heck, 1985) and binds/aggregates DNA containing SARs (Adachi et al., 1989; Sander et al., 1987). SARs contain DNA sequences that are homologous to Top2 cleavage sites, indicating Top2 might alter compaction and topology of chromatin through cleavage of SARs (Adachi et al., 1989). Demonstration that Top2 preferentially binds to junctions of bent and linear DNA (Howard et al., 1991) added further validation to these studies.

*Top2* is an enzyme that plays a central role in maintaining and establishing chromosome topology, including a recently discovered role in homolog pairing (Williams et al., 2007). The requirement of pairing-mediated gene regulation is especially alluring, as the capacity of homologous sequences to influence each other occurs in many organisms. Homolog associations are needed during meiosis, where the maternal and paternal homologs pair and synapse prior to recombination. Pairing can also occur in non-meiotic cells. Drosophila serves as an excellent model to study pairing interactions because homologous chromosomes are fully aligned in all somatic cells and this has been shown to alter the activity of genes through processes known as transvection (Lewis, 1954). Recently, depletion of Top2 by RNAi or chemical inhibitors demonstrated that Top2 is important for homolog pairing (Williams et al., 2007). In spite of the growing recognition of its importance, somatic pairing has been challenging to study as the tissue heterogeneity and requirement for viability of whole organisms precludes many types of studies.
Our studies build from our interest in understanding the role of Top2 in homolog pairing in Drosophila. Here, we describe a genetic screen to generate Top2 alleles, as an allelic series of hypomorphic mutations would provide a useful resource for in vivo analyses. In total, we identified fifteen new alleles. Among these, fourteen demonstrated recessive lethality, with one of these displaying temperature-sensitivity. These lethal mutations represent nonsense and missense mutations, with this latter class including changes in residues associated with resistance to chemical inhibitors of Top2 (Wu et al., 2011). Interse crosses between lethal missense alleles uncovered interallelic complementation, wherein adults were generated. These adults were morphologically normal, although these adults showed delayed development and females were sterile. Interallelic complementation extended to crosses of strains carrying alleles encoding drug resistant analogs of Top2. Taken together, these findings suggest that dimerization of some defective subunits can restore in vivo Top2 function. In brief, we have generated a new resource for investigating the in vivo function of Top2.

Materials and Methods

**Drosophila stocks and culture conditions**

Flies were maintained at 25 ± 1° at 70% humidity on standard Drosophila cornmeal, yeast, sugar, and agar medium with p-hydroxybenzoic acid methyl ester as a mold inhibitor. All crosses were performed at 25°, unless otherwise specified.

*Df(2L)Exel9043* (Bloomington Stock Center, BL 7913) is a 14.8 kb deletion on Chromosome 2 that removes several genes including Top2, RanGap, Hs2st and a portion of CG10237 (Flybase, http://flybase.bio.indiana.edu/; Figure 2.1A). We refer to this deletion as Top2Df9043. Top2 mutant alleles were carried over the balancer chromosome CyO-Df(2R)B80, y+ balancer (BL 4542), referred to as CyO, y+. The Top2Df17 and Top2Df35 deficiencies were generated by excision of an existing P-element (Top2EP). Top2Df17 contains a 3,580 bp deletion within the Top2 coding region, with 590 bp
remaining of the P-element (Figure 2.1A). The breakpoints of $\text{Top2}^{Df35}$ have not been mapped. A genomic rescue transposon was generated called $P[\text{Top2-w}^+]$, which carries the mini-white gene and a 7.1 kb HindIII to XbaI genomic fragment that includes the wild type Top2 gene and part of the $\text{CG10026}$ gene (Figure 2.1B). Transgenic $P[\text{Top2-w}^+]$ flies were generated that carry the transposon on the third chromosome. $P[\text{Top2-w}^+]$ rescues the recessive lethality associated with $\text{Top2}^{Df17}$ and partially rescues the recessive lethality associated with $\text{Top2}^{Df35}$, wherein $\text{Top2}^{Df35}/\text{Top2}^{Df35}; P[\text{Top2-w}^+]$ individuals were produced at 63% of the $\text{Top2}^{Df35}/\text{CyO}\times\text{y}^+$ class. This partial rescue suggests that the $\text{Top2}^{Df35}$ chromosome may have second site mutations that are affecting viability. See Table 2.2 for a list of abbreviated gene names.

**Mutagenic screen for the identification of Top2 mutant alleles**

The strategy for isolating Top2 alleles is shown in Figure 2. Two to four day old $y^1\#8/Y; dp\ cn\ bw/ dp\ cn\ bw$ males were desiccated for twelve to fourteen hours and then fed 25 mM ethyl methanesulphonate (EMS) in 10% sucrose (w/v). This mutagen was chosen as it largely produces nucleotide substitutions resulting from guanine alkylation (Bentley et al., 2000). After 24 hours, mutagenized males were transferred to bottles with standard food, allowed to recover for ten to twelve hours, and then mated for ~24 hours with $y^2/Y; (dp\ cn\ bw)*/\text{CyO}$ females. Mated females were transferred to fresh bottles daily for three to four days. F1 $y^1\#8/y^2; (dp\ cn\ bw)*/\text{Top2}^{Df17}$ or $\text{Df35}/\text{CyO}$ females were scored for wing and body pigmentation. F1 $y^2/Y; (dp\ cn\ bw)*/\text{CyO}$ males were singly mated in vials to $y^2/y^2; \text{Top2}^{Df17}$ or $\text{Df35}/\text{CyO}$ females. The resulting F2 progeny were screened, vial by vial, for decreased viability, as determined by an absence or reduced numbers of straight-winged flies. The $y^2/Y; (dp\ cn\ bw)*/\text{CyO}$ males from such vials were backcrossed to $y^2/y^2; \text{Top2}^{Df17}$ or $\text{Df35}/\text{CyO}$ females to confirm the decreased viability of straight-winged flies and to generate stocks carrying the putative Top2 mutations ($\text{Top2}^m$). Stocks of
putative $Top2^m$ mutations were established by balancing the $Top2^m$ chromosome with the $CyO, y^+$ chromosome. All putative mutations were rescreened by crossing the $y^{1w67c23};\ Top2^m/CyO, y^+$ males or virgin females to flies carrying an independent $Top2$ deficiency chromosome ($y^{1w67c23}; Top2^{Df9043}/CyO, y^+$). Crosses that produced no straight-winged, $y^+$ flies were considered to contain new $Top2$ mutations.

Characterization of newly generated $Top2$ mutant alleles

Once $Top2^m$ alleles were defined, several studies were conducted. First, we determined whether alleles displayed parent of origin effects by crossing $y^{1w67c23};\ Top2^m/CyO, y^+$ to $y^{1w67c23}; Top2^{Df9043}/CyO, y^+$ in both directions. Second, we assessed whether $Top2^m$ alleles were temperature sensitive by crossing females of the genotype $y^{1w67c23}; Top2^m/CyO, y^+$ to males ($y^{1w67c23}/Y; Top2^{Df9043}/CyO, y^+$). Parents were mated in bottles at room temperature for two days, after which time bottles were placed at $18^\circ$C. Progeny from these crosses were scored daily. Third, we assessed complementation between the $Top2$ mutants was performed by interse crosses between $y^{1w67c23};\ Top2^m/CyO, y^+$ flies carrying different $Top2$ alleles were crossed and progeny were scored every 2-3 days. These studies revealed interallelic complementation, wherein viable adults were generated that produced only mutant $Top2$ proteins. These adults are referred to as $Top2$ complementing adults. Fourth, we tested whether any of the $Top2$ mutations represented hypomorphic alleles. In these studies, we assessed partial $Top2$ function by examining viability of $Top2^m/Top2^{Df9043}$ larvae. Flies from each lethal $Top2$ mutant strain ($y^{1w67c23}/y^{1w67c23};\ Top2^m/CyO, y^+$) were crossed to $y^{1w67c23}/Y; Top2^{Df9043}/CyO, y^+$ flies. Resulting $y^{1w67c23};\ Top2^m/Top2^{Df9043}$ second and third instar larvae were collected, recognized by the absence of pigmentation in the mouth hooks and denticle belts. For each genotype studied, at least 100 larvae were selected and placed in vial with no more
than twenty-five larvae to allow development at 25°C under uncrowded conditions. The total numbers of individuals advancing to later stages were scored, with the percent survival at each stage determined by dividing the number of individuals obtained divided by the total number of larvae placed in each vial, multiplied by 100. Of note, no y^I w^67c23; Top2^m/Top2^Dy9043 adults were obtained from these crosses. Top2^m alleles were considered hypomorphic if Top2^m/Top2^Dy9043 larvae showed greater pupal survival than control crosses with confirmed null alleles. Fifth, the fertility of Top2 complementing males and females was assessed. In these studies, eight to ten y^I w^67c23; Top2^m1/Top2^m2 males or females were crossed to ten to fifteen y^I w^67c23; Sco/CyO flies of the opposite sex. Crosses were transferred every two to three days. Top2 complementing males were considered fertile if adults were produced from the cross. Top2 complementing females were considered fertile if they produced eggs that hatched. Crosses of flies from the parental line (y^I88; dp cn bw) were studied in parallel, as a control.

**Molecular characterization of Top2 alleles**

Genomic DNA was isolated from heterozygous y^I w^67c23; Top2^m/Top2^Dy9043 larvae and PCR amplified using primers covering nine overlapping regions encompassing the Top2 protein-coding region. PCR products were purified using QIAquick PCR purification kit (Qiagen, no. 28104) and sequenced at the University of Iowa DNA Core Facility. Primers are listed in Table 2.3. Levels of each Top2 mutant were determined by western analysis of protein extracts obtained from Top2^m/Top2^Dy9043 mutant third instar larvae. Proteins were extracted from a collection of brains, imaginal discs, and salivary gland tissues dissected from 10 larvae, boiled for 5 minutes in 3X sample buffer, separated on a 4-20% Tris-HCl gradient gel [BIO-RAD, no. 161-1159], and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk in PBST (PBS + 1% Tween-20), incubated in primary antibody overnight at 4°, and washed 3X10 minutes with PBST. Blots were incubated independently with two different primary antibodies,
rabbit anti-Top2 (D. Arndt-Jovin) at 1:4000 and rabbit anti-Top2 (T. Hsieh) at 1:10,000.
Blots were then incubated with secondary antibody HRP-conjugated goat anti-rabbit IgG [BIO-RAD, no. 172-1019] at 1:20,000 for 2-3 hours, washed 3X10 minutes in PBST, and detected using chemiluminiscent substrate [BIO-RAD, no. 170-5040 or Millipore, no. WBLUR0100]. To control for amounts of protein loaded, blots were incubated with mouse anti-\(\alpha\)-tubulin IgG primary antibody [Sigma, no. T5168] and detected with the HRP-conjugated rabbit anti-mouse IgG secondary antibody [Sigma, no. A9044].

**Polytene chromosome and immunohistochemical analyses**

Salivary gland polytene chromosomes were studied to define effects of loss of Top2 on chromosome structure. Females of the genotype \(y^{1} w^67c^{23}/y^{1} w^67c^{23}, Top2^{17-5} or 35-15/CyO\), \(y^{+}\) were crossed to males \((y^{1} w^67c^{23}/Y; Top2^{Df9043}/CyO, y^{+})\) at room temperature and transferred to new bottles every two days. Following two days at 25°C, bottles were placed at 18°C. Squashes were performed according to previously described methods (Johansen et al., 2009). Studies using antibodies against the dosage compensation components MSL1, MLE, and Jil1 were stained using the conventional squash protocol, while H4K16ac staining was performed using the acid free method. For each genotype studies, squashes were done on at least ten salivary gland pairs, dissected from at least two independent crosses. Over 100 nuclei were examined for each genotype.

Effects of Top2 loss on imaginal disc and ovary development were studied. Tissues were dissected from third instar larvae in 1X phosphate buffered saline solution (PBS) and kept on ice. Tissues were fixed in 3% paraformaldehyde (Electron Microscopy Sciences, no. 15710) for 20 minutes and subsequently washed 3x10 minutes in PBT (PBS + 0.3% TritonX-100). Samples were blocked for a minimum of four hours in PBT + 5% normal goat serum (Vector laboratories, no. S-1000), and incubated overnight with primary antibodies at 4°C. Next, samples were washed 3X10 minutes in
PBT, incubated with secondary antibodies for 2-3 hours at room temperature, washed 3X10 minutes in PBT, and DAPI stained (1 ug/mL in PBT) for 5-10 minutes. Following an additional PBT wash for 5 minutes, tissues were mounted on slides in Vectashield (Vector Laboratories, H1000). Images were acquired using a Zeiss 710 confocal microscope and processed using ImageJ.

Multiple primary antibodies were used for immunohistochemical analyses including: mouse anti-fibrillarin (Abcam, no. ab4566 ) at 1:500, mouse anti-HP1 (DSHB; C1A9) at 1:200, rabbit anti-H3 phospho-Ser10 (Epitomics, no. 1173-1) at 1:500, rabbit anti-H4K16ac at 1:50 (Active Motif, no. 39929), chicken anti-Jil1 at 1:100 (K. Johansen), mouse anti-lamin Dm0 (DSHB, ADL84.12) at 1:500, and rabbit anti-lamin Dm0 (P. Fisher). All Alexa Fluor (AF) secondary antibodies (Invitrogen, Molecular Probes) were used at a 1:500 dilution, including: AF488 goat anti-chicken IgG (A11039), AF568 goat anti-chicken IgG (A11041), AF488 goat anti-rabbit (A11008), AF568 goat anti-rabbit (A11011), AF488 goat anti-mouse (A11001), and AF568 goat anti-mouse (A11004).

Quantitative PCR (qPCR) analysis

RNA isolation and qPCR analysis were performed according to previously described methods (Soshnev et al. 2008). RNA samples were prepared from approximately 10 male and female third instar larvae. All analyses were performed in two replicates. Average fold change and standard deviation were calculated using Microsoft Excel. All samples were normalized to the housekeeping gene, RpL32.

Results and Discussion

An EMS screen identified new Top2 alleles

Our screen was designed to recover Top2 alleles in the F1 and F2 generations (Figure 2.2). In the F1 generation, we sought putative Top2 alleles by screening for
alteration of a phenotype that depends on somatic homolog pairing, while in the F2 generation, putative Top2 alleles were identified by their failure to complement a chromosome carrying a deletion of Top2. EMS was fed to males that were isogenic for a second chromosome carrying the wild type Top2 gene and the dp cn and bw markers. Following EMS treatment, males were crossed to females bearing one of two deletion alleles of Top2, Top2Df17 or Top2Df35, in trans to the CyO balancer chromosome carrying a wild type Top2 gene and the dominant Curly wing marker.

In the F1 generation, we looked for loss or enhancement of pairing-dependent gene expression of the X-linked yellow (y) cuticle pigmentation gene. In particular, F1 Cy+ females were examined for changes in complementation between a paternally contributed y1#8 allele, which lacks the y promoter and is a complete null, and a maternally contributed y2 allele, which has the y wing and body enhancers blocked by a chromatin insulator (Geyer et al., 1990; Morris et al., 1999). Whereas a Top2+ background, y2/y1#8; (dp cn bw)*/Top2Df17 or Df35 females were expected to show dark, nearly wild type pigmentation because the wing and body enhancers of y1#8 act in trans on the promoter of y2 when the alleles are paired, the presence of a Top2 mutation that affected pairing would be expected to reduce or increase wing and body pigmentation. Importantly, females carrying y2 have darkly pigmented bristles regardless of pairing status, which enabled us to distinguish any exceptional y2/y1#8; (dp cn bw)*/Top2Df17 or Df35 females with reduced pigmentation from y1#8/y1#8; (dp cn bw)*/Top2Df17 or Df35 females arising from nondisjunction of the X chromosome.

From 2,653 F1 females, we identified twelve Cy+ females that had altered pigmentation. At least four of these females showed unambiguously and uniformly lighter cuticle color, resembling females in which y pairing had been disrupted (Chen et al., 2002; Ou et al., 2009). Seven of these exceptional females were successfully mated and all produced males that were presumed to have inherited y1#8 from their mother, based on the level of cuticle pigmentation. Unfortunately, all subsequent crosses with the
progeny of these exceptional females failed to recapitulate the reduced pigmentation of the original putative mutant females. Most likely non-complementation was due to a mutation or a non-disjunction event that was not represented in the germline.

In the F2 generation, we looked for recessive lethal or semi-lethal alleles of Top2. Here, F1 y²/Y; (dp cn bw)*/CyO males, each representing a single mutagenized second chromosome, were singly crossed in vials to y²/y²; Top2^{Df17} or ^{Df35}/CyO females, carrying different tester Top2 deficiency chromosomes. Next, each vial was scored for the absence or reduced numbers of straight-winged progeny, consistent with the presence of a de novo lethal or semi-lethal allele of Top2. Putative mutations were named based on the tester deficiency chromosome used in the identification, with those derived from the Top2^{Df17} tester named Top2^{17-x}, while those derived from the Top2^{Df35} tester named Top2^{35-x}. Of 3,000 crosses, thirty putative Top2 mutations (Top2^m) were identified and we attempted to generate stocks for each. Nineteen y¹ w^{67c23}; Top2^m/CyO, y+ stocks were established (Table 2.4), as eleven putatives were either sterile or unhealthy and were subsequently lost. Each putative mutation was tested in trans to Top2^{Df9043}, a Top2 deficiency that was generated independently of Top2^{Df17} and Top2^{Df35}. A total of fifteen were confirmed (Table 2.4). Fourteen demonstrated complete lethality in trans to Top2^{Df9043}, while the remaining mutation, Top2^{35-4}, showed 25% of the expected class when transmitted maternally and 87% when transmitted paternally. To ensure that lethality of the newly generated mutations was due to mutation of Top2, we tested whether the P[Top2-w⁺] transgene could rescue viability to Top2^m/Top2^{Df35} flies. Of note, Top2^{35-4} was not tested in this study, due to the high viability observed upon transmission. In all cases, viable Top2^m/Top2^{Df35}; P[Top2-w⁺]/+ adults were obtained (Table 2.5), confirming that lethality was due to mutations in Top2. Each allele was crossed to flies with a wild type Top2 gene, to determine whether the Top2 mutation was haplo-insufficient (Table 2.6). These studies showed that all Top2 mutations did not display dosage defects associated
with haplo-insufficiency. In total, fifteen lethal or semi-lethal Top2 alleles were generated.

Several parameters can contribute to gene mutability, including the length of the transcription unit, the size of the protein and different tolerances among proteins for altered amino acid sequence. Among these, a recent screen using Drosophila demonstrated that the best predictors of mutability were length and conservation of the protein (Cooper et al., 2010). Based on the number of lethal mutations identified after screening ~3,000 chromosomes, these investigators predicted that EMS generates one mutation for every 73 evolutionarily conserved amino acids. Based on this information, we estimated that our screen should have generated approximately thirteen new Top2 alleles, as Drosophila Top2 is 167 kDa protein that displays ~67% similarity with human Top2. Our recovery of fourteen lethal mutations is in remarkable agreement with the predicted value, arguing that our identification of a large number of new Top2 alleles reflects the size and high conservation of functional domains throughout the protein.

**Top2 missense alleles carry amino acid substitutions in critical functional domains**

To define the molecular lesions associated with the Top2 mutations, genomic sequences encompassing the coding region were amplified by PCR and sequenced. As a reference, we sequenced the coding region of the Top2 gene amplified from the parental strain, identifying two base pair changes relative to the sequence of the Top2 gene curated at NCBI; one is a silent change (+3040, E565E) and one produces a conservative amino acid substitution (+5628, A1401V) in a less conserved region of Top2 (Crenshaw and Hsieh, 1993b). These data indicate that the sequence of the Top2 coding region is largely unchanged between strains. As an additional control, we sequenced the Top2 coding region from four putative Top2 mutations that had been identified in our original screen, but had been found to complement Top2*D9043 (Table 2.4), because we predicted
that these alleles would be unlikely to harbor changes in Top2. As expected, the coding region in all four alleles was identical to that in the parental strain (data not shown). Sequence analyses of the Top2 coding region in the fifteen lethal and semi-lethal Top2 mutations uncovered that three alleles carried nonsense mutations, one allele carried a missense and a nonsense mutation, one allele carried three missense mutations, and ten alleles carried single missense mutations (Table 2.7). In all cases, missense alleles encoded a Top2 protein with at least one amino acid substitution in a residue conserved with human Top2α, with six alleles that showed alterations in residues invariant among all sequenced eukaryotic type II topoisomerases (Table 2.8; Figure 2.3). We found that the missense mutations were clustered in three functional domains, including the ATPase, WHD and TOPRIM domains (Figure 2.4A). Based on the similarity of our mutants with Top2 mutants characterized in other studies (Wu et al., 2011), we predict that Top217-2 may alter the ATP binding pocket, Top217-3 may alter DNA binding, and Top235-1, Top235-6, Top235-12 might display altered binding to the Top2 inhibitor etoposide.

We determined the levels of Top2 protein produced in the fourteen Top2 strains that carry lethal alleles when raised at 25°C. Our western analyses used two polyclonal antibodies raised against the full-length Top2 protein, which was especially important for optimizing the detection of mutant proteins obtained from strains carrying nonsense mutations. Similar results were obtained using both antibodies (Figure 2.4B, Table 2.7; data not shown). We found that Top2 was low or absent in seven strains, including all four strains carrying nonsense mutations and three strains carrying missense mutations, while the remaining seven strains carrying missense mutations displayed intermediate to high protein levels. In general, Top2 proteins that carried changes in the TOPRIM domain accumulated wild type levels of protein, while those with amino acid substitutions in the ATPase domain or WHD produced varying levels of protein. We detected a full-length sized protein in Top235-14, even though this mutant results in a Q186* nonsense mutation that would result in predicted protein of 20kDa. It is possible
that this full-length protein is a result of readthrough of the stop codon (Jungreis et al., 2011) or remaining wild type maternally contributed Top2 protein. Examination of the stop codons for all alleles carrying nonsense mutations revealed that all result in TAG, indicating the context of the Q186* stop codon may be unique in dictating readthrough.

The low levels of protein detected in the Top235-3 larvae may result from a low frequency of translation initiation at the nearby in-frame ATG codon, which is located twelve codons downstream of the bona fide initiator codon. We were surprised by the low level of protein produced by Top235-2, because a similarly sized truncated Drosophila Top2 protein was functional in vitro and complemented mutations of the yeast Top2 gene (Crenshaw and Hsieh, 1993a). A potential explanation may lie in the observation that the last 60 amino acids of wild type Top2 include a nuclear localization signal (Crenshaw and Hsieh, 1993a); the absence of this signal in Drosophila may prevent nuclear entry and lead to protein degradation. Taken together, our western analyses show that some of the lethal alleles of Top2 accumulate high levels of protein. Previous studies in S. cerevisiae have shown that lethality associated with Top2 depletion and a catalytically inactive Top2 resulted from different mechanisms (Baxter and Diffley, 2008). Here we demonstrate that our collection of Top2 mutations include those that produce no protein and those that produce near wild type levels of a functionally comprised protein. Thus, our mutations have the capacity to gain insight into how Top2 depletion and inactivity cause different mechanisms of lethality.

**Top2 missense alleles include temperature-sensitive and hypomorphic mutations**

Temperature-sensitive alleles are a useful resource for in vivo functional studies. As missense mutations occasionally cause temperature-sensitivity in protein function, we determined whether any of our Top2 mutations were temperature-sensitive. We crossed y1w67c23; Top2m/CyO, y+ females to y1w67c23; Top2D9043/CyO, y+ males, eggs were
collected for two days at 25°C and then transferred to 18°C for the remainder of development. These studies identified one genotype, \( \text{Top2}^{17-2}/\text{Top2}^{\text{Df}9043} \), that produced adults. Based on the number of \( \text{Top2}^m \) heterozygous siblings, we estimate that 29% of \( \text{Top2}^{17-2}/\text{Top2}^{\text{Df}9043} \) flies survived to adulthood (Table 2.4). Although \( \text{Top2}^{17-2}/\text{Top2}^{\text{Df}9043} \) females died shortly after emerging from the pupal case, adult males survived for several days. To determine whether continued growth of \( \text{Top2}^{17-2}/\text{Top2}^{\text{Df}9043} \) adults at 18°C was essential for survival, we shifted larvae from 18°C to 25°C at different developmental times. We found that any growth at 25°C resulted in a loss of adults (Figure 2.5A), suggesting that Top2 function is required throughout development. Considering that \( \text{Top2}^{17-2} \) encodes a full-length protein with a substitution in the highly conserved motif in the ATPase domain (I198N; Table 2.7), it is possible that the temperature sensitivity of \( \text{Top2}^{17-2} \) results from defects associated with ATP binding or an inability of ATP binding or hydrolysis to produce conformational changes needed to alter DNA topology, resulting in an unstable protein (Figure 2.4). Cold and heat sensitive Top2 mutants have been previously isolated in yeast (Thomas et al., 1991). These mutations resulted from missense mutations in conserved residues located (Deuring et al., 2000) outside of the ATPase domain, clustered near the active site. These data suggest that missense mutations in multiple functional Top2 domains can generate a temperature sensitive allele.

We were interested in defining whether our newly generated alleles represent complete or partial loss of Top2 function. To this end, we assessed the viability of \( \text{Top2}^m/\text{Top2}^{\text{Df}9043} \) mutants, predicting that a partial loss of Top2 function would increase survival relative to those with a complete loss. We crossed \( y^Iw^{67c23}; \text{Top2}^m/\text{CyO}, y^+ \) females to \( y^Iw^{67c23}; \text{Top2}^{\text{Df}9043}/\text{CyO}, y^+ \) males and followed development of \( y^Iw^{67c23}; \text{Top2}^m/\text{Top2}^{\text{Df}9043} \) larvae, identified by their y- phenotype. As a control, we tested two \textit{trans}-heterozygous null genotypes (\( \text{Top2}^{\text{Df}17}/\text{Top2}^{\text{Df}9043} \) and \( \text{Top2}^{\text{Df}35}/\text{Top2}^{\text{Df}9043} \)), anticipating that these larvae would show extensive lethality prior to pupation, as
previous studies have shown that Top2 alleles that produced undetectable levels of protein displayed high mortality in second instar larvae, with fewer than 5% escaping into the third instar larval stage (Ramos et al., 2011). Unexpectedly, we found that 49% to 66% of Top2Df17 or Df35/Top2Df9043 null larvae pupated (Table 2.9), implying a later lethal phase than previously reported. Similar results were obtained for Top2m/Top2Df9043 mutant larvae carrying nonsense alleles, wherein 38% to 66% of mutant larvae survived to pupal stages (Tables 2.9). Finally, we studied Top2m/Top2Df9043 larvae that carried Top2 missense alleles. We found that seven Top2m/Top2Df9043 genotypes demonstrated significantly enhanced survival relative to the known null genotypes, with 80% to 92% of mutant larvae becoming pupae (p<0.04, two proportion z-test; Table 2.9). Based on these data, we conclude that newly identified missense mutations include hypomorphic Top2 alleles. One mutant, Top235-3, showed significantly reduced survival relative to the known null genotypes, with 26% of mutant larvae becoming pupae (p<0.04, two proportion z-test; Table 2.9). It is possible that this mutant is dominant negative, as the phenotype is worse than the null alleles, and may result in a protein with altered function.

Lethality during pupal stages of development suggested that Top2 mutants might carry defects in imaginal discs, which are ultimately responsible for generating adult tissues. In particular, we surmised that mutations in Top2 might interfere with the growth of imaginal discs, as previous studies in Drosophila cell culture have demonstrated that chemical inhibitors of Top2 decrease mitosis (Coelho et al., 2008). Dissection of tissues from multiple Top2 mutant strains indicated that imaginal discs and brains were larger in strains carrying hypomorphic alleles. For example, Top217-6/Top2Df9043 larvae that produce high levels of a hypomorphic mutant Top2 protein contain nearly wild type sized brains and small discs that display an altered cellular organization (Figures 2.5B, 2.4, data not shown). In contrast, Top217-2/Top2Df9043 larvae that produce very low levels of mutant Top2 contain small brains and no imaginal discs when grown at the non-permissive temperature, but larger brains and discs when grown at the permissive
temperature (Figure 2.5B,C). To access the level of mitosis in imaginal discs obtained from these Top2 mutant strains, dissected tissues were stained with an antibody against phosphorylated serine 10 of histone H3, antibodies that are highly selective markers for mitotic cells (Hendzel et al., 1997). These experiments revealed that the reduced size of the discs correlated with decreased staining of phosphorylated serine 10 of histone H3 (Figure 2.5). Taken together, these data suggest that defects in imaginal disc growth may contribute to pupal lethality.

**Loss of Top2 alters polytene chromosome structure**

Top2 plays a role in chromosome pairing and condensation (Adachi et al., 1991; Buchenau et al., 1993; Chang et al., 2003; Coelho et al., 2008; Stanvitch and Moore, 2008; Uemura et al., 1987; Williams et al., 2007). To examine how loss of Top2 affects chromosome structure, we studied the polytene chromosomes isolated from salivary glands. These chromosomes contain more than 1,000 aligned DNA strands, which provides a powerful system for direct visualization of interphase chromosomes. Two null or nearly null Top2 alleles were studied, Top2^{17-5}/Top2^{Df9043} and Top2^{35-14}/Top2^{Df9043}. Analysis of polytene chromosome squashes from female Top2^{17-5}/Df9043 larvae showed no specific changes to the X chromosome or autosomes, although in general the chromosomes were more fragile and the banding pattern was more diffuse than observed with the control (data not shown).

Polytene chromosomes isolated from these Top2^{17-5}/Top2^{Df9043} mutant female larvae showed no specific changes to the X chromosome of autosomes, although in general the chromosomes were more fragile and the banding pattern was more diffuse than observed with the control (Figure 2.6). In contrast, polytene chromosomes isolated from Top2 mutant male larvae showed a diffuse banding pattern, particularly apparent for the X chromosome, which displayed a “puffy”, decondensed structure, as previously described for Jil-1, ISWI, Nurf-301, SCF, Su(var)3-7, Su(var)2-5, and In(1)BM2 mutants.
(Badenhorst et al., 2002; Deng et al., 2005; Furuhashi et al., 2006; Kulkarni-Shukla et al., 2008; Spierer et al., 2005). These observations suggest that loss of Top2 affects global chromosome structure and condensation, with the male X chromosome showing the greatest sensitivity within the genome.

The sensitivity of the male X chromosome to Top2 loss suggested a connection to the dosage compensation pathway. In particular, we wondered if the Male Specific Lethal (MSL) complex was recruited to the male X in Top2 mutants. In Drosophila, males (XY) up-regulate expression of genes on the X chromosome two-fold to equal the gene dosage in females (XX). This process is regulated by the MSL complex, which is recruited to hundreds of specific sites along the male X chromosome (Gelbart and Kuroda, 2009). The MSL complex consists of: MSL1, MSL2, MSL3, males absent on the first (MOF), maleless (MLE), and two non-coding RNAs (roX1 and roX2). Acetylation of H4K16 by MOF leads to the upregulation of genes on the X (Park and Kuroda, 2001; Smith et al., 2001). Importantly, a puffy X chromosome phenotype has been observed in hypomorphic mutations in JIL-1, a gene encoding a component of the MSL complex kinase that is responsible for interphase histone H3 Ser10 phosphorylation (Deng et al., 2005).

To investigate whether the puffy X phenotype in Top2 mutant males results from an altered function of the dosage compensation pathway, we examined X chromosome localization of components of the MSL complex in Top2^{17-5}/Top2^{Dy9043} and Top2^{35-14}/Top2^{Dy9043} male larvae. We found that loss of Top2 does not affect recruitment of MSL1, MLE and JIL-1 or alter enrichment of H4K16ac mark on male X chromosome (Figure 2.7D-F, G-I; data not shown). Together, these studies imply that Top2 is not required for proper function of the dosage compensation machinery.

ATP-dependent chromatin-remodeling complexes play central roles in transcription and chromatin structure. Previous studies have demonstrated that Imitation Switch (ISWI) regulates higher-order chromatin structure that includes global
decondensation of salivary gland polytene chromosomes, with the male X chromosome displaying increased sensitivity relative to the autosomes (Deuring et al., 2000). These processes were linked to defects in nucleosome assembly due to a reduction in the levels of histone H1 (Corona et al., 2007; Siriaco et al., 2009). Based on the similarity between the Top2 and Iswi mutant phenotypes, coupled with a previous functional interaction between Top2 and H1 (Hsieh and Brutlag, 1980), we reasoned that changes in polytene chromosome structure in Top2 mutants might result from changes in H1 levels. To this end, western analysis of salivary gland proteins was completed, demonstrating H1 accumulates at near wild type levels in Top2 mutants (Figure 2.8). These data suggest that loss of Top2 does not affect H1 production, ruling out that these changes are responsible for altered polytene chromosome structure. We consider that loss of Top2 might alter H1 deposition through other mechanisms, a possibility that we were unable to test due to the inability of H1 antibodies to work in immunohistochemical analyses.

A number of mutants in Drosophila have been identified that result in a puffy X chromosome in males. These include Jil-1, ISWI, Nurf-301, SCF, Su(var)3-7, Su(var)2-5, and In(1)BM2 (Badenhorst et al., 2002; Deng et al., 2005; Furuhashi et al., 2006; Kulkarni-Shukla et al., 2008; Spierer et al., 2005). In all cases tested, the dosage compensation complex is required for the altered morphology of the male X chromosome, as mutations in components of the complex suppress the puffy X phenotype. Our understanding of the structure of the X chromosome was greatly enhanced by studies of ISWI (Corona et al., 2002; Corona et al., 2007; Corona and Tamkun, 2004). Although loss of ISWI resulted in decreased levels of histone H1 (Corona et al., 2007), Top2 mutants did not exhibit the same phenotype.

Why the male X chromosome is so sensitive to pertubation is not well understood. Loss of Top2 might alter the structure of the X chromosome, via its role as a chromosome scaffold protein. Loss of Top2 could inhibit the aggregation of DNA containing SARs, thus, inhibiting the formation of chromatin loops and higher order
chromatin structures (Adachi et al., 1989; Mirkovitch et al., 1987). Similarly, Top2 might alter the structure of the X chromosome via its role in chromatin compaction (Chang et al., 2003). Alternatively, roX RNAs might be misregulated in Top2 mutants, resulting in decondensation of the male X chromosome. In this regard, studies of roX RNAs in a Nurf301 mutant background revealed that a roX transgene targeted to the autosomes could induce decondensation of the chromatin flanking the transgene and that Nurf301 acts as a repressor of roX expression in females (Bai et al., 2007).

Analyses of roX expression in Top2 mutants suggest this hypothesis is unlikely. As the sex of Top2 mutant female larvae is indistinguishable from males, single larvae were used in these assays. Expression analysis of Top2 mutants revealed that roX1 and roX2 levels in males and females are similar to that observed in wild type (Figure 2.9). At this time the mechanism of how Top2 alters the chromatin structure of polytene chromosomes is unclear.

**Top2 mutations display interallelic complementation**

Based on previous *in vitro* studies demonstrating Top2 subunits can undergo cooperative interactions to overcome defective ATP binding by one subunit (Lindsley and Wang, 1993), we predicted that missense alleles encoding Top2 proteins with mutations in distinct functional domains might display interallelic complementation. To this end, we conducted pair-wise complementation tests between all Top2 alleles, assaying each cross for the production of viable adults. Initial crosses determined that six (Top2^{17-1}, Top2^{17-3}, Top2^{17-6}, Top2^{35-1}, Top2^{35-5}, and Top2^{35-13}) of the fourteen lethal alleles supported interallelic complementation, generating Top2^{m1}/Top2^{m2} adults (Table 2.10). We refer to these progeny as Top2 complementing adults. Importantly, mutants that lack protein accumulation, including the premature stop codons, (Top2^{17-7}, Top2^{17-7}, Top2^{35-2}, Top2^{35-3}, and Top2^{35-14}) did not support any interallelic complementation. We noticed that flies carrying heteroallelic Top2 mutations appeared less healthy. To gain a
more accurate picture of complementation, male and female y^1 w^67c^23; Top2^m/CyO, y^+ flies were crossed for the six complementing alleles and progeny scored daily (Table 2.11). Twenty-six crosses produced adults, revealing thirteen heteroallelic genotypes capable of interallelic complementation. Based on the number of Top2^m heterozygous siblings obtained in each complementation crosses, we estimate that the viability of Top2^{m1}/Top2^{m2} adults ranged from ~13 to 98% of Top2^m heterozygotes (Table 2.11). Interestingly, over half of all mutant genotypes displayed greater than 50% viability of Top2^{m1}/Top2^{m2} adults.

Several observations were made from our intersex crosses. First, none of the nonsense alleles demonstrated interallelic complementation, suggesting that complementation requires full-length proteins or a significant level or protein from each isoform. In general, a trend between protein levels and complementation was not strictly observed. Several alleles produced full-length proteins near wild type levels that did not exhibit complementation (For example, see Top2^{35-6} and Top2^{35-12}). Top2^{17-1} and Top2^{35-13} complemented several alleles, even though these alleles produce very low levels of protein (Table 2.11, Figure 2.4). In general, the Top2 complementing alleles expressed high levels of protein. The only situation where we did not observe complementation was between Top2^{17-1} and Top2^{35-13}, indicating this combination does not lead to a stabilized Top2 protein. Second, interallelic complementation occurred between alleles encoding proteins altered in different functional domains. For example, Top2 alleles encoding proteins with defects in the TOPRIM domain complemented alleles encoding proteins with defects in the ATPase domain or WHD (Table 2.11). Third, complementation was observed between alleles encoding proteins with defects in the same domain. However, this property was restricted to alleles encoding proteins mutated in the TOPRIM domain. Remarkably, complementation occurred between Top2 mutants carrying amino acid substitutions separated by only six residues in the TOPRIM domain (Top2^{17-3}/Top2^{35-1}). These studies provide the first demonstration that Top2 mutants can
form a functional heterodimer \textit{in vivo} and suggest that significant cooperation can occur between defective Top2 subunits to restore \textit{in vivo} function.

**Top2 complementing adults show limited developmental defects**

We studied progeny from interse crosses between \textit{Top2} missense alleles to understand the extent of restored Top2 function. In general, we found that \textit{Top2} complementing adults emerged later and died earlier than did their wild type siblings. Nonetheless, these adults were morphologically normal, including having a body size that was similar to Top2\textsuperscript{+} flies. Unexpectedly, the F1 male to female ratio in five of 26 crosses showed significant deviation from the expected 1:1 ratio, wherein a single sex represented 70\% or more of offspring (Table 2.12). Higher numbers of male offspring were observed in all but one case (\textit{Top2}\textsubscript{17-3}/\textit{Top2}\textsubscript{17-6}). Heteroallelic genotypes demonstrating a sex bias were mostly those carrying mutations in the WHD \textit{in trans} to mutations in the TOPRIM domain (Table 2.12). These findings are reminiscent of our observations that \textit{Top2}\textsubscript{17-2}/\textit{Top2}\textsubscript{D\textsuperscript{9043}} males produced by 18\textdegree C development, which were healthier than females (Figure 2.5A). The reason for different viability of \textit{Top2} complementing males and females is unclear.

We assessed the fertility associated with \textit{Top2} complementing adults. In initial studies, fertility was judged by an ability of a \textit{Top2} complementing male or female to generate adult progeny when mated to \textit{y\textsuperscript{l}} \textit{w\textsuperscript{67c23}} \textit{Sco/CyO} flies. This assay established that male fertility was largely not affected, with the one exception representing the heteroallelic genotype \textit{Top2}\textsubscript{17-3}/\textit{Top2}\textsubscript{35-1} (Table 2.13). In contrast, all \textit{Top2} complementing females were sterile. To gain further insights into the cause of female sterility, we examined egg production, finding that \textit{Top2} complementing females produced low number of eggs, which all appeared morphologically normal but did not hatch. As activation of a meiotic checkpoint is linked to defects in egg shell patterning
(Morris and Lehmann, 1999), these findings suggest that oogenesis in $Top2$ complementing females occurs without checkpoint activation. Eggs collected from $Top2^{35-5}/Top2^{35-13}$ and $Top2^{17-6}/Top2^{35-1}$ complementing females were DAPI stained and imaged by confocal microscopy, revealing that the vast majority (95-97%) failed to display evidence of nuclear division or development. We postulate that loss of $Top2$ may lead to defects in transcription during oogenesis that lead to depleted levels of maternal products needed in embryogenesis. Alternatively, $Top2$ may be required for meiosis, resulting in an inability to generate embryo.

Low egg production prompted us to examine the ovary phenotype in $Top2^{17-6}/Top2^{35-1}$ complementing females. While strings of developing egg chambers were observed, the ovaries were smaller, disorganized and contained evidence of egg chamber apoptosis (Figure 2.10A). Occasionally, egg chambers with extra nurse cells were observed (data not shown). Effects of lowered $Top2$ function on the germline stem cell (GSC) niche were examined by staining with antibodies against Vasa, a germline specific RNA helicase (Lasko and Ashburner, 1988), and Spectrin, a structural protein expressed in a cell type specific pattern in all cells of the germarium. In germ cells, Spectrin accumulates in a spherical structure in the GSCs, called the spectrosome, and a branched structure in differentiating cysts, called the fusome, while in somatic cells, Spectrin localizes within the plasma membrane (Lin et al., 1994). We found that $Top2^{17-6}/Top2^{35-1}$ ovaries had complex defects in the germaria, including the presence of germaria showing both GSCs loss and gain (Figure 2.10B). Taken together, these analyses indicate that germ cell differentiation is compromised in $Top2$ complementing females, leading to germ cell loss and defects in egg chamber formation.

In summary, we generated and characterized fifteen new $Top2$ alleles, including fourteen lethal and one semi-lethal allele. These alleles result from nonsense and missense mutations, with all missense mutations altering residues conserved with human $Top2\alpha$. Seven $Top2$ lethal alleles produce no or low levels of stable $Top2$ protein,
suggesting that these are null or nearly null alleles. The remaining seven produce near intermediate to near wild type levels of full-length Top2 protein and represent hypomorphs. These new Top2 alleles represent a novel resource for studying the *in vivo* functions of Top2.

**Concluding Perspectives**

Type II topoisomerases are molecular targets for chemotherapy against several types of cancers (Chikamori et al., 2010; Nitiss, 2009b). However, treatment of cultured cells with Top2 chemotherapeutic agents has been found to produce drug resistant forms of the enzyme, which are caused by altered drug binding, DNA binding or the catalytic function (Nitiss, 2009b; Wu et al., 2011). These drug resistant forms of the protein commonly contain amino acid substitutions in the N-terminal ATPase, TOPRIM and WHD domains. Interestingly, four of our Top2 missense alleles carry changes in amino acid residues previously found to be associated with drug resistance (Nitiss, 2009b; Wu et al., 2011). These include *Top2*<sup>17-3</sup>, *Top2*<sup>35-1</sup>, *Top2*<sup>35-6</sup>, and *Top 35-12*, mutations that have alterations in the TOPRIM and WHD domains (Table 1) and correspond to residues L491, P485, S763, G760 in human TopIIα, respectively. Among these mutations, two (*Top2*<sup>17-3</sup>, *Top2*<sup>35-1</sup>) support interallelic complementation, suggesting that certain heterodimers of drug-resistant proteins may similarly reconstitute enough Top2 function for viability. Based on these data, we speculate that individuals may carry natural Top2 polymorphisms that confer different sensitivities to Top2 inhibitors and may affect outcomes to treatment.
Table 2.1 Comparison of Top2 amino acid sequence between *Drosophila melanogaster* and other species

<table>
<thead>
<tr>
<th>Species</th>
<th>ATPase</th>
<th>TDD</th>
<th>TOPRIM</th>
<th>WHD</th>
<th>TD</th>
<th>CCD</th>
<th>CTD</th>
<th>Entire</th>
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</thead>
<tbody>
<tr>
<td><em>S. pombe</em></td>
<td>53(77)</td>
<td>48</td>
<td>62</td>
<td>64</td>
<td>31</td>
<td>35</td>
<td>17</td>
<td>41</td>
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<tr>
<td><em>S. cerevisiae</em></td>
<td>54 (76)</td>
<td>42</td>
<td>57 (79)</td>
<td>63</td>
<td>36</td>
<td>32</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>66 (84)</td>
<td>46</td>
<td>75 (90)</td>
<td>71</td>
<td>29</td>
<td>36</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td><em>M. musculus</em> Top2α</td>
<td>69 (85)</td>
<td>64</td>
<td>77 (89)</td>
<td>76</td>
<td>49</td>
<td>41</td>
<td>19</td>
<td>52</td>
</tr>
<tr>
<td><em>M. musculus</em> Top2β</td>
<td>70 (87)</td>
<td>67</td>
<td>77 (90)</td>
<td>77</td>
<td>48</td>
<td>42</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td><em>H. sapiens</em> Top2α</td>
<td>69 (86)</td>
<td>63</td>
<td>77 (89)</td>
<td>75</td>
<td>50</td>
<td>40</td>
<td>15</td>
<td>51</td>
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<tr>
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<td>67</td>
<td>77 (90)</td>
<td>77</td>
<td>48</td>
<td>22</td>
<td>15</td>
<td>50</td>
</tr>
</tbody>
</table>

*a* Percent identity.

*b* Percent similarity.

Transducer domain (TDD), winged helix domain (WHD), coiled-coil domain (CCD), C-terminal domain (CTD). 
Table 2.2 **List of alleles used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alleles</th>
<th>Molecular basis</th>
<th>Phenotypic Effect</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>brown</td>
<td>$bw^+$</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$bw^+$</td>
<td>Insertion in transcription unit</td>
<td>Off-white eyes</td>
<td>Phillips et al. (1973); Dreesen et al. (1988)</td>
</tr>
<tr>
<td>cinnabar</td>
<td>$cn^+$</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
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<tr>
<td></td>
<td>$cn^+$</td>
<td>1.5 kb deletion</td>
<td>Dark orange eyes</td>
<td>Bridges and Brehme (1944); Warren et al. (1996)</td>
</tr>
<tr>
<td>dumpy</td>
<td>$dp^+$</td>
<td>NA</td>
<td>Wild type wings</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$dp^+$</td>
<td>NA</td>
<td>Short and dumpy wings</td>
<td>Bridges and Mohr (1918)</td>
</tr>
<tr>
<td>Top2</td>
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<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
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<td>Top2$^{235}$</td>
<td>Deletion</td>
<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
<tr>
<td></td>
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<td>Deletion</td>
<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
<tr>
<td>white</td>
<td>$w^+$</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$w^+$</td>
<td>Doc insertion +3.71</td>
<td>White eyes</td>
<td>Driver et al. (1989)</td>
</tr>
<tr>
<td>yellow</td>
<td>$y^+$</td>
<td>NA</td>
<td>Dark wing, body, bristles</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$y^+$</td>
<td>Gypsy insert at -700</td>
<td>Light wing and body, dark bristles</td>
<td>Geyer et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>$y^{+88}$</td>
<td>-716 to +66 deletion; 17 bp of P-element remains</td>
<td>Light wing and body, light bristles</td>
<td>Geyer et al. (1990)</td>
</tr>
</tbody>
</table>
Table 2.3 Primers sequences used for PCR amplification and genomic sequencing

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top2F + 1030\textsuperscript{a}</td>
<td>TTAACTGGGTGGGTACTGTAGTCTTCTG</td>
</tr>
<tr>
<td>Top2R + 1701</td>
<td>AACGGTGAGCTGGTGGAAGATATG</td>
</tr>
<tr>
<td>Top2F + 1544</td>
<td>TGACCATTGCACAGAGACAGAAG</td>
</tr>
<tr>
<td>Top2R + 2354</td>
<td>ACCTGGACTGCTGATCTTGATGC</td>
</tr>
<tr>
<td>Top2F + 2144</td>
<td>TCCAAAAGGTCTCGTTTGATCAACTC</td>
</tr>
<tr>
<td>Top2R + 2922</td>
<td>GCCATCCTGATCTGATCTGTC</td>
</tr>
<tr>
<td>Top2F + 2772</td>
<td>TAATTTCAAGCAGCTTTCGGAGAATG</td>
</tr>
<tr>
<td>Top2R + 3481</td>
<td>AAGTGAAACATCAACCTCCGTGAC</td>
</tr>
<tr>
<td>Top2F + 3361</td>
<td>ATCACCTATGGACTTTTATCAATCTGG</td>
</tr>
<tr>
<td>Top2R + 4059</td>
<td>TTCACGATTTCCTAAACGGTTTCCG</td>
</tr>
<tr>
<td>Top2F + 3958</td>
<td>TAAAGGAACTTATTTAGGACGATGG</td>
</tr>
<tr>
<td>Top2R + 4655</td>
<td>TTCTTAGCCTTTGAGCTGACACTGG</td>
</tr>
<tr>
<td>Top2F + 4533</td>
<td>ATATCGTCCCGATCCCGTCAAG</td>
</tr>
<tr>
<td>Top2R + 5220</td>
<td>ATCAAATTCGTCACCCGTACCATCG</td>
</tr>
<tr>
<td>Top2F + 5034</td>
<td>ACCCGTCGAGTAAAGATCACCAGAAG</td>
</tr>
<tr>
<td>Top2R + 5377</td>
<td>CTGAAACATTTACCTGCGCTTGCTG</td>
</tr>
<tr>
<td>Top2F + 5156</td>
<td>AACCAGAGGCGCAAGCAGATTAAGC</td>
</tr>
<tr>
<td>Top2R + 6247</td>
<td>CTTTTATTTCCACCTTTCGATTGTC</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Primers are relative to the transcription start site (+1).
Table 2.4 Complementation tests to define Top2 alleles

\( y^{w_{67c23}} \); Top2\(^{m}\)/CyO, \( y^{+} \) x \( y^{w_{67c23}} \); Top2\(^{Df9043}\)/CyO, \( y^{+} \)

<table>
<thead>
<tr>
<th></th>
<th>18°C Maternally transmitted allele</th>
<th>25°C Maternally transmitted allele</th>
<th>25°C Paternally transmitted allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele Name</td>
<td>% viability (Total # CyO)</td>
<td>% viability (Total # CyO)</td>
<td>% viability (Total # CyO)</td>
</tr>
<tr>
<td>Df 17</td>
<td>ND</td>
<td>0 (604)</td>
<td>0 (196)</td>
</tr>
<tr>
<td>Df 35</td>
<td>ND</td>
<td>0 (424)</td>
<td>0 (695)</td>
</tr>
<tr>
<td>17-1</td>
<td>1 (710)</td>
<td>0 (359)</td>
<td>0 (412)</td>
</tr>
<tr>
<td>17-2</td>
<td>29 (906)</td>
<td>0 (193)</td>
<td>0 (170)</td>
</tr>
<tr>
<td>17-3</td>
<td>0 (737)</td>
<td>0 (263)</td>
<td>0 (189)</td>
</tr>
<tr>
<td>17-4</td>
<td>ND</td>
<td>137 (249)</td>
<td>110 (122)</td>
</tr>
<tr>
<td>17-5</td>
<td>0 (895)</td>
<td>0 (430)</td>
<td>0 (287)</td>
</tr>
<tr>
<td>17-6</td>
<td>0 (939)</td>
<td>0 (207)</td>
<td>0 (295)</td>
</tr>
<tr>
<td>17-7</td>
<td>0 (467)</td>
<td>0 (211)</td>
<td>0 (389)</td>
</tr>
<tr>
<td>35-1</td>
<td>0 (555)</td>
<td>0 (300)</td>
<td>0 (99)</td>
</tr>
<tr>
<td>35-2</td>
<td>0 (874)</td>
<td>0 (289)</td>
<td>0 (104)</td>
</tr>
<tr>
<td>35-3</td>
<td>0 (898)</td>
<td>0 (283)</td>
<td>0 (102)</td>
</tr>
<tr>
<td>35-4</td>
<td>ND</td>
<td>25 (438)</td>
<td>87 (453)</td>
</tr>
<tr>
<td>35-5</td>
<td>0 (803)</td>
<td>0 (423)</td>
<td>0 (815)</td>
</tr>
<tr>
<td>35-6</td>
<td>0 (631)</td>
<td>0 (120)</td>
<td>0 (160)</td>
</tr>
<tr>
<td>35-7</td>
<td>ND</td>
<td>127 (229)</td>
<td>159 (208)</td>
</tr>
<tr>
<td>35-8</td>
<td>ND</td>
<td>118 (320)</td>
<td>113 (238)</td>
</tr>
<tr>
<td>35-9</td>
<td>ND</td>
<td>205 (112)</td>
<td>124 (302)</td>
</tr>
<tr>
<td>35-12</td>
<td>0 (703)</td>
<td>0 (69)</td>
<td>0 (207)</td>
</tr>
<tr>
<td>35-13</td>
<td>0 (611)</td>
<td>0 (115)</td>
<td>0 (218)</td>
</tr>
<tr>
<td>35-14</td>
<td>0 (1065)</td>
<td>0 (226)</td>
<td>0 (162)</td>
</tr>
</tbody>
</table>

\(^{a}\) Percent viability is the # of non-CyO flies divided by half the # of CyO flies multiplied by 100.

\(^{b}\) Top2\(^{m}\)/Top2\(^{Df9043}\) flies become stuck in food.

\(^{c}\) Top2\(^{17.2}\)/Top2\(^{Df9043}\) males are sterile; females immediately become stuck in food.

ND: Not determined.

Bold indicates the fourteen lethal alleles at 25°C.
Table 2.5 Numbers of progeny obtained from rescue crosses using the $P[Top2-w^{+}]$ transgene

\[
y^{1} w^{67c23} ; \ Top2^{Df35}/CyO; \ P[Top2-w^{+}]^{+/+} \times y^{1} w^{67c23}^{Y}; \ Top2^{m}/CyO, \ y^{+}
\]

<table>
<thead>
<tr>
<th>Allele transmitted by male</th>
<th>$Top2^{m}/Top2^{Df35}$; $+/+$</th>
<th>$Top2^{m}/Top2^{Df35}$; $P[Top2-w^{+}]^{+/+}$</th>
<th>$Top2^{m}$ or $Top2^{Df35}/CyO$ or $CyO, \ y^{+}; \ P[Top2-w^{+}]^{+/+}$</th>
<th>% Viability(^{a}) (Total # CyO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF 17</td>
<td>0</td>
<td>32</td>
<td>74</td>
<td>86 (146)</td>
</tr>
<tr>
<td>DF 35</td>
<td>0</td>
<td>26</td>
<td>82</td>
<td>63 (161)</td>
</tr>
<tr>
<td>17-1</td>
<td>0</td>
<td>45</td>
<td>90</td>
<td>100 (185)</td>
</tr>
<tr>
<td>17-2</td>
<td>0</td>
<td>67</td>
<td>101</td>
<td>133 (213)</td>
</tr>
<tr>
<td>17-3</td>
<td>0</td>
<td>30</td>
<td>56</td>
<td>107 (127)</td>
</tr>
<tr>
<td>17-5</td>
<td>0</td>
<td>72</td>
<td>130</td>
<td>111 (256)</td>
</tr>
<tr>
<td>17-6</td>
<td>0</td>
<td>44</td>
<td>105</td>
<td>84 (205)</td>
</tr>
<tr>
<td>17-7</td>
<td>0</td>
<td>79</td>
<td>130</td>
<td>122 (269)</td>
</tr>
<tr>
<td>35-1</td>
<td>0</td>
<td>40</td>
<td>76</td>
<td>105 (157)</td>
</tr>
<tr>
<td>35-2</td>
<td>0</td>
<td>53</td>
<td>76</td>
<td>139 (129)</td>
</tr>
<tr>
<td>35-3</td>
<td>0</td>
<td>48</td>
<td>68</td>
<td>141 (154)</td>
</tr>
<tr>
<td>35-5</td>
<td>0</td>
<td>37</td>
<td>64</td>
<td>116 (131)</td>
</tr>
<tr>
<td>35-6</td>
<td>0</td>
<td>35</td>
<td>64</td>
<td>109 (155)</td>
</tr>
<tr>
<td>35-12</td>
<td>0</td>
<td>38</td>
<td>69</td>
<td>110 (124)</td>
</tr>
<tr>
<td>35-13</td>
<td>0</td>
<td>58</td>
<td>110</td>
<td>105 (210)</td>
</tr>
<tr>
<td>35-14</td>
<td>0</td>
<td>78</td>
<td>127</td>
<td>123 (280)</td>
</tr>
</tbody>
</table>

\(^{a}\) % viability is the # of non-CyO, $P[Top2-w^{+}]$ flies divided by half the # of CyO, $P[Top2, w^{+}]^{+/+}$ flies multiplied by 100.
Table 2.6 Progeny numbers obtained from complementation analyses with wild type

<table>
<thead>
<tr>
<th>Name</th>
<th>Maternally transmitted mutant</th>
<th>Paternally transmitted mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Expected Class&lt;sup&gt;a&lt;/sup&gt; (Total # CyO Scored)</td>
<td>% Expected Class (Total # CyO Scored)</td>
</tr>
<tr>
<td>Df9043</td>
<td>129 (109)</td>
<td>215 (72)</td>
</tr>
<tr>
<td>Df 17</td>
<td>114 (158)</td>
<td>151 (232)</td>
</tr>
<tr>
<td>Df 35</td>
<td>122 (147)</td>
<td>140 (73)</td>
</tr>
<tr>
<td>17-1</td>
<td>133 (194)</td>
<td>206 (92)</td>
</tr>
<tr>
<td>17-2</td>
<td>127 (213)</td>
<td>256 (43)</td>
</tr>
<tr>
<td>17-3</td>
<td>136 (127)</td>
<td>183 (131)</td>
</tr>
<tr>
<td>17-5</td>
<td>134 (90)</td>
<td>125 (155)</td>
</tr>
<tr>
<td>17-6</td>
<td>157 (81)</td>
<td>179 (77)</td>
</tr>
<tr>
<td>17-7</td>
<td>177 (123)</td>
<td>105 (338)</td>
</tr>
<tr>
<td>35-1</td>
<td>103 (150)</td>
<td>153 (77)</td>
</tr>
<tr>
<td>35-2</td>
<td>182 (66)</td>
<td>163 (79)</td>
</tr>
<tr>
<td>35-3</td>
<td>202 (98)</td>
<td>126 (197)</td>
</tr>
<tr>
<td>35-5</td>
<td>120 (155)</td>
<td>162 (173)</td>
</tr>
<tr>
<td>35-6</td>
<td>157 (105)</td>
<td>152 (162)</td>
</tr>
<tr>
<td>35-12</td>
<td>129 (70)</td>
<td>177 (144)</td>
</tr>
<tr>
<td>35-13</td>
<td>138 (138)</td>
<td>141 (68)</td>
</tr>
<tr>
<td>35-14</td>
<td>123 (119)</td>
<td>154 (46)</td>
</tr>
</tbody>
</table>

<sup>a</sup>% expected class is the # of non-CyO flies divided by the # of CyO flies multiplied by 100.
Table 2.7 *Properties of Top2 Alleles*

<table>
<thead>
<tr>
<th>Allele Name</th>
<th>Amino Acid Change</th>
<th>Affected Domain</th>
<th>Protein Accumulation</th>
<th>Interallelic Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-1</td>
<td>S791F</td>
<td>WHD</td>
<td>+/-</td>
<td>Yes</td>
</tr>
<tr>
<td>17-2</td>
<td>I198N</td>
<td>ATPase</td>
<td>+/-</td>
<td>No</td>
</tr>
<tr>
<td>17-3</td>
<td>L471Q</td>
<td>TOPRIM</td>
<td>+++</td>
<td>Yes</td>
</tr>
<tr>
<td>17-5</td>
<td>L318Q, Q1201*</td>
<td>NA</td>
<td>+/-</td>
<td>No</td>
</tr>
<tr>
<td>17-6</td>
<td>Y592N</td>
<td>TOPRIM</td>
<td>+++</td>
<td>Yes</td>
</tr>
<tr>
<td>17-7</td>
<td>L774*</td>
<td>NA</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>35-1</td>
<td>R197C, C347S, P465S</td>
<td>ATPase, TDD, TOPRIM</td>
<td>+++</td>
<td>Yes</td>
</tr>
<tr>
<td>35-2</td>
<td>K1300*</td>
<td>NA</td>
<td>+/-</td>
<td>No</td>
</tr>
<tr>
<td>35-3</td>
<td>M1L</td>
<td>NA</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>35-4</td>
<td>T712I</td>
<td>WHD</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>35-5</td>
<td>D134G</td>
<td>ATPase</td>
<td>+++</td>
<td>Yes</td>
</tr>
<tr>
<td>35-6</td>
<td>S743F</td>
<td>WHD</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td>35-12</td>
<td>G740E</td>
<td>WHD</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td>35-13</td>
<td>R773H</td>
<td>WHD</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>35-14</td>
<td>Q186*</td>
<td>NA</td>
<td>-</td>
<td>No</td>
</tr>
</tbody>
</table>

*a* Bold: invariant between human Top2 α and β, mouse Top2 α and β, yeast Top2.

*b* Topoisomerase/Primase domain (TOPRIM), Transducer domain (TDD), Winged helix domain (WHD).

*c* Protein accumulation as assayed by western (+++ = protein levels equal to wild type, ++ = intermediate, + = low, +/- = very low, - = no protein relative to wild type).

*d* Complementation with other *Top2* alleles.

ND: Not Determined.

NA: Not applicable.

* Denotes nonsense mutation.
<table>
<thead>
<tr>
<th>Allele Name</th>
<th>Amino acid change in Drosophila</th>
<th>Conserved between Top2α?</th>
<th>Conserved between Top2β?</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-1</td>
<td>S791F</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>17-2</td>
<td>I198N</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>17-3</td>
<td><strong>L471Q</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>17-5</td>
<td>L318Q</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Q1201*</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17-6</td>
<td><strong>Y592N</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>17-7</td>
<td>L774*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-1</td>
<td>R197C</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>C347S</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td><strong>P465S</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-2</td>
<td>K1300*</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>35-3</td>
<td>M1L</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-4</td>
<td>T712I</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-5</td>
<td><strong>D134G</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-6</td>
<td>S743F</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>35-12</td>
<td><strong>G740E</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-13</td>
<td>R773H</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>35-14</td>
<td>Q186*</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Bold: invariant between human Top2α and β, mouse Top2α and β, yeast Top2.
Table 2.9 **Analysis of survival of Top2\textsuperscript{m}/Top2\textsuperscript{Df9043} larvae at 25°C**

<table>
<thead>
<tr>
<th>Allele Name(^a)</th>
<th>Allele Classification</th>
<th>% Pupation(^b) (Total # Scored)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Wild-type</td>
<td>98 (100)</td>
</tr>
<tr>
<td>Df 17</td>
<td>Deletion</td>
<td>49 (106)</td>
</tr>
<tr>
<td>Df 35</td>
<td>Deletion</td>
<td>66 (103)</td>
</tr>
<tr>
<td>17-1</td>
<td>Missense</td>
<td>73 (100)</td>
</tr>
<tr>
<td>17-2</td>
<td>Missense</td>
<td>72 (100)</td>
</tr>
<tr>
<td><strong>17-3</strong></td>
<td><strong>Missense</strong></td>
<td><strong>92 (120)</strong></td>
</tr>
<tr>
<td>17-5</td>
<td>Missense, Nonsense</td>
<td>51 (142)</td>
</tr>
<tr>
<td>17-6</td>
<td>Missense</td>
<td>80 (120)</td>
</tr>
<tr>
<td>17-7</td>
<td>Nonsense</td>
<td>49 (135)</td>
</tr>
<tr>
<td><strong>35-1</strong></td>
<td><strong>Missense</strong></td>
<td><strong>82 (100)</strong></td>
</tr>
<tr>
<td>35-2</td>
<td>Nonsense</td>
<td>66 (110)</td>
</tr>
<tr>
<td><strong>35-3</strong></td>
<td><strong>Missense</strong></td>
<td><strong>26 (141)</strong></td>
</tr>
<tr>
<td><strong>35-5</strong></td>
<td><strong>Missense</strong></td>
<td><strong>88 (100)</strong></td>
</tr>
<tr>
<td><strong>35-6</strong></td>
<td><strong>Missense</strong></td>
<td><strong>83 (100)</strong></td>
</tr>
<tr>
<td><strong>35-12</strong></td>
<td><strong>Missense</strong></td>
<td><strong>80 (100)</strong></td>
</tr>
<tr>
<td><strong>35-13</strong></td>
<td><strong>Missense</strong></td>
<td><strong>82 (136)</strong></td>
</tr>
<tr>
<td>35-14</td>
<td>Nonsense</td>
<td>38 (104)</td>
</tr>
</tbody>
</table>

\(^a\) Maternally transmitted mutant Top2\textsuperscript{m}, paternally transmitted mutant Top2\textsuperscript{Df9043}.

\(^b\) Percent Pupation was determined by dividing the total number of observed pupae by the total number of larvae placed in each vial, multiplied by 100.

Bold indicates hypomorphic alleles, strains that showed significant viability (p<0.04, two proportion z-test) relative to both Top2\textsuperscript{Df17} and Top2\textsuperscript{Df35}. 
Table 2.10 Complementation between *Top2* alleles

<table>
<thead>
<tr>
<th>Allele transmitted by female</th>
<th>17-1</th>
<th>17-2</th>
<th>17-3</th>
<th>17-5</th>
<th>17-6</th>
<th>17-7</th>
<th>35-1</th>
<th>35-2</th>
<th>35-3</th>
<th>35-4</th>
<th>35-5</th>
<th>35-6</th>
<th>35-12</th>
<th>35-13</th>
<th>35-14</th>
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<tbody>
<tr>
<td>17-1</td>
<td>0(123)</td>
<td>0(96)</td>
<td>5(115)</td>
<td>0(201)</td>
<td>10(126)</td>
<td>0(69)</td>
<td>16(100)</td>
<td>0(143)</td>
<td>0(129)</td>
<td>11(340)</td>
<td>0(117)</td>
<td>0(128)</td>
<td>0(127)</td>
<td>0(143)</td>
<td></td>
</tr>
<tr>
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<td>0(121)</td>
<td>0(139)</td>
<td>0(160)</td>
<td>0(216)</td>
<td>0(122)</td>
<td>0(121)</td>
<td>0(131)</td>
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<td>1(182)</td>
<td>0(115)</td>
<td>0(119)</td>
<td>0(103)</td>
<td>0(149)</td>
<td></td>
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<tr>
<td>17-3</td>
<td>0(125)</td>
<td>0(198)</td>
<td>3(419)</td>
<td>0(141)</td>
<td>27(190)</td>
<td>0(203)</td>
<td>14(146)</td>
<td>0(165)</td>
<td>0(129)</td>
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<td>9(246)</td>
<td>0(63)</td>
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<td>0(215)</td>
<td>0(176)</td>
<td>0(104)</td>
<td>0(127)</td>
<td>0(135)</td>
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<td>0(92)</td>
<td>0(136)</td>
<td>0(106)</td>
<td>0(219)</td>
<td>0(131)</td>
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<td>17-6</td>
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<td>0(194)</td>
<td>21(444)</td>
<td>0(150)</td>
<td>0(190)</td>
<td>0(114)</td>
<td>58(144)</td>
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<td>0(137)</td>
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<td>0(188)</td>
<td>0(263)</td>
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<td>1(265)</td>
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<td>0(121)</td>
<td>0(121)</td>
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<td>35-1</td>
<td>28(287)</td>
<td>2(267)</td>
<td>24(250)</td>
<td>0(172)</td>
<td>34(82)</td>
<td>0(109)</td>
<td>0(150)</td>
<td>0(142)</td>
<td>0(149)</td>
<td>157(219)</td>
<td>0(100)</td>
<td>0(123)</td>
<td>45(218)</td>
<td>0(230)</td>
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<td>0(108)</td>
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<td>0(97)</td>
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<td>0(333)</td>
<td>0(144)</td>
<td>0(164)</td>
<td>0(231)</td>
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<tr>
<td>35-3</td>
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<td>0(122)</td>
<td>0(247)</td>
<td>0(218)</td>
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<td>0(113)</td>
<td>0(175)</td>
<td>0(234)</td>
<td>0(116)</td>
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<td>0(552)</td>
<td>75(1023)</td>
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<td>6(283)</td>
<td>0(335)</td>
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<td>0(425)</td>
<td>0(241)</td>
<td>0(151)</td>
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<td>4(385)</td>
<td>0(182)</td>
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<td>0(238)</td>
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<td>0(202)</td>
<td>0(144)</td>
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<td>0(187)</td>
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<td>0(125)</td>
<td>0(161)</td>
<td>0(113)</td>
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<td>35-12</td>
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<td>0(119)</td>
<td>76(76)</td>
<td>0(114)</td>
<td>45(277)</td>
<td>0(269)</td>
<td>49(123)</td>
<td>0(173)</td>
<td>0(113)</td>
<td>33(196)</td>
<td>0(232)</td>
<td>0(110)</td>
<td>0(118)</td>
<td>0(119)</td>
<td></td>
</tr>
<tr>
<td>35-13</td>
<td>0(131)</td>
<td>0(139)</td>
<td>0(145)</td>
<td>0(151)</td>
<td>0(120)</td>
<td>0(140)</td>
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<td>0(133)</td>
<td>0(446)</td>
<td>0(148)</td>
<td></td>
</tr>
</tbody>
</table>

*a* % viability is the # of non-CyO flies divided by half the # of CyO flies multiplied by 100.

*b* Total number of CyO flies scored.

**Bold:** 11+ % viability.
Table 2.11 Interallelic complementation between Top2 mutants

\(y^1w^{67c23}/Y; \text{Top}2^m/\text{CyO}, y^+ \times y^1w^{67c23}/y^1w^{67c23}; \text{Top}2^m/\text{CyO}, y^+\)

<table>
<thead>
<tr>
<th>Allele transmitted by female</th>
<th>17-1 WHD</th>
<th>17-3 TOPRIM</th>
<th>17-6 TOPRIM</th>
<th>35-1(^a) TOPRIM</th>
<th>35-5 ATPase</th>
<th>35-13 WHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-1 WHD</td>
<td>0(^b) (165)(^c)</td>
<td>19 (275)</td>
<td>13 (354)</td>
<td>56 (612)</td>
<td>95 (217)</td>
<td>0 (220)</td>
</tr>
<tr>
<td>17-3 TOPRIM</td>
<td>22 (932)</td>
<td>2 (163)</td>
<td>64 (311)</td>
<td>22 (719)</td>
<td>93 (323)</td>
<td>77 (183)</td>
</tr>
<tr>
<td>17-6 TOPRIM</td>
<td>26 (214)</td>
<td>52 (243)</td>
<td>0 (248)</td>
<td>58 (708)</td>
<td>3 (147)</td>
<td>76 (208)</td>
</tr>
<tr>
<td>35-1 TOPRIM</td>
<td>46 (557)</td>
<td>31 (705)</td>
<td>71 (627)</td>
<td>0 (98)</td>
<td>78 (226)</td>
<td>85 (360)</td>
</tr>
<tr>
<td>35-5 ATPase</td>
<td>85 (220)</td>
<td>88 (440)</td>
<td>2 (777)</td>
<td>86 (346)</td>
<td>0 (103)</td>
<td>64 (426)</td>
</tr>
<tr>
<td>35-13 WHD</td>
<td>0 (184)</td>
<td>60 (350)</td>
<td>55 (268)</td>
<td>79 (655)</td>
<td>98 (240)</td>
<td>0 (275)</td>
</tr>
</tbody>
</table>

\(^a\) Top2\(^{35-1}\) has three amino acid changes, however, only one change is in an invariant amino acid (P465S in TOPRIM domain).

\(^b\) Percent viability is the # of non-CyO flies divided by half the # of CyO flies multiplied by 100.

\(^c\) Total number of CyO flies scored.

Bold: 11-100% viability.

Vials were scored daily.
Table 2.12 Percentage of male and female offspring of viable heteroallelic Top2 genotypes

<table>
<thead>
<tr>
<th>Allele transmitted by male</th>
<th>17-1 WHD</th>
<th>17-3 TOPRIM</th>
<th>17-6 TOPRIM</th>
<th>35-1 TOPRIM</th>
<th>35-5 ATPase</th>
<th>35-13 WHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-1 WHD</td>
<td>NA</td>
<td>F: 9 (23)</td>
<td>F: 42 (24)</td>
<td>F: 47 (139)</td>
<td>F: 50 (105)</td>
<td>NA</td>
</tr>
<tr>
<td>35-1 TOPRIM</td>
<td></td>
<td>M: 47 (97)</td>
<td>M: 43 (70)</td>
<td>M: 59 (143)</td>
<td>M: 70 (69)</td>
<td></td>
</tr>
<tr>
<td>35-5 ATPase</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: 59 (205)</td>
<td>NA</td>
<td>F: 45 (154)</td>
<td>NA</td>
<td>F: 38 (125)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M: 55 (145)</td>
<td>NA</td>
<td>F: 45 (154)</td>
<td>NA</td>
<td>M: 62 (125)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: 45 (145)</td>
<td>NA</td>
<td>M: 55 (154)</td>
<td>M: 43 (115)</td>
<td>NA</td>
</tr>
</tbody>
</table>

aTotal number of homozygotes scored.

NA: Not applicable.

Bold: statistically significant (p<0.05).
Table 2.13 **Fertility of complementing Top2 adults**

<table>
<thead>
<tr>
<th>Allele transmitted by male (M)</th>
<th>17-1</th>
<th>17-3</th>
<th>17-6</th>
<th>35-1</th>
<th>35-5</th>
<th>35-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-1</td>
<td>NA</td>
<td>F-NA</td>
<td>F-NA</td>
<td>F-Sterile</td>
<td>F-NA</td>
<td>NA</td>
</tr>
<tr>
<td>17-3</td>
<td>F-NA</td>
<td>M-Fertile</td>
<td>NA</td>
<td>F-Sterile</td>
<td>F-Sterile</td>
<td>F-Sterile</td>
</tr>
<tr>
<td>17-6</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
<td>NA</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
<td>F-Sterile</td>
</tr>
<tr>
<td>35-1</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
<td>F-Sterile</td>
<td>NA</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
</tr>
<tr>
<td>35-5</td>
<td>F-NA</td>
<td>M-Fertile</td>
<td>NA</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
<td>F-Sterile</td>
</tr>
<tr>
<td>35-13</td>
<td>NA</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
<td>F-Sterile</td>
<td>M-Fertile</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: Not assessed.

Males were considered fertile if any progeny eclosed; Females were considered fertile if they produced eggs that hatched.
Figure 2.1 **Structure of the Top2 locus.** A. Top2 is located between the uncharacterized upstream *CG10026* gene and the essential *RanGap* downstream gene. Shown are the structures of three Top2 deficiency chromosomes used in these studies, including *Top2*\(^{\text{Df9043}}\) that is a 14.8 kb deletion allele (dashed line), *Top2*\(^{\text{Df17}}\) that is an ~3.6 kb deletion allele (dashed line) and retains an ~600 bp of the starting *P*-element (*Top2*\(^{\text{EP}}\)), and *Top2*\(^{\text{Df35}}\) that is a deletion allele with unknown limits (dotted line). Promoters are indicated by bent arrows and exons are represented by shaded rectangles. B. *P[Top2-w^+]* is a white\(^+\) marker *P* transposon that carries a 7.1 kb genomic fragment encompassing the entire Top2 gene and parts of the neighboring *CG10026* and *RanGap* genes. Only the structure of the Top2 genomic fragment is shown. *P[Top2-w^+]* rescues *Top2*\(^{\text{Df17}}/\text{Top2}^{\text{Df35}}\) and partially rescues *Top2*\(^{\text{Df35}}/\text{Top2}^{\text{Df35}}\), indicating that the *Top2*\(^{\text{Df35}}\) may have second site mutations that are affecting viability.
Figure 2.2 **Strategy used in the EMS screen.** Males of the genotype $y^{188}/Y;\ dp\ cn\ bw/dp\ cn\ bw$ were fed EMS and mated to $y^2/y^2;\ Top2_{Df17\ or\ Df35}/CyO$ virgin females. In the F1 screen, $y^2/y^{188};\ (dp\ cn\ bw)^*/Top2_{Df17\ or\ Df35}$ females were screened for altered complementation between $y$ alleles. In the F2 screen, F1 males carrying a mutagenized second chromosome in trans to the $CyO$ balancer were crossed to virgin $y^2/y^2;\ Top2_{Df17\ or\ Df35}/CyO$ females. Vials were screened for absence or reduced numbers of straight-winged (non-$CyO$) flies. (*), mutagenized chromosome. Of 3,000 chromosomes screened, thirty putative $Top2$ alleles were identified, with fifteen corresponding to new alleles. The screen was performed with Morgan Thompson and Ting Wu.
Figure 2.3 Amino acid alignment of Drosophila Top2 with Human Top2α and β. Domains are highlighted and color-coded. Newly identified amino acid substitutions in Drosophila are highlighted in black.
Figure 2.3 continued
Figure 2.3 continued
Figure 2.4 Analyses of protein production in Top2 mutations at 25°C. A. The location of the nonsense and missense mutations found in the lethal Top2 alleles are shown relative to the Top2 protein domain structure, which includes an ATPase domain, Transducer domain (TDD), Topoisomerase/ Primase (TOPRIM), Winged helix domain (WHD), Tower domain (TD) and Coiled-coil domain (CCD). B, C. Top2 protein levels were determined by western analysis of proteins extracted from Top2m/Top2Df9043 third instar larve carrying missense (B) and nonsense (C) alleles. Westerns were probed with antibodies generated in the Hsieh laboratory. Antibodies against α-Tubulin serve as a loading control. One larval equivalent was loaded per lane. Black arrowhead indicates the position of the full-length Top2. The white arrowhead marks the position of a full-length protein obtained from Top235-14/Top2Df9043 larvae, even though the Top235-14 nonsense allele is predicted to encode a protein of ~20 kD, suggesting that this band represents stop codon readthrough or remaining wild type maternally contributed Top2 protein. Asterisks mark the position of a cross-reacting band that was routinely observed.
Figure 2.5 *Top2<sup>17-2</sup>* is a temperature-sensitive allele. A. Temperature shift experiments defined that only one *Top2* mutation, *Top2<sup>17-2</sup>* was heat sensitive. *Top2<sup>17-2*/CyO, y<sup>+</sup> flies were crossed to *Top2<sup>Df9043*/CyO, y<sup>+</sup> flies, and eggs were collected for two days before offspring were placed at 18°C for 1, 12, or 27 days. The numbers of *Top2<sup>17-2*/Top2<sup>Df9043* adults were counted, with % survival estimated from the number of *Top2<sup>17-2*/CyO, y<sup>+</sup> or *Df9043*/CyO, y<sup>+</sup> siblings. Only *Top2<sup>17-2*/Top2<sup>Df9043* individuals raised continuously at 18°C survived to adulthood, suggesting that *Top2* is required throughout development. The *Top2<sup>17-2*/Top2<sup>Df9043* females died shortly after eclosion. B, C. *Top2* is required for development of third instar larval brains and imaginal discs. Tissues were isolated from wild type, *Top2<sup>17-2*/Top2<sup>Df9043*, and *Top2<sup>17-6*/Top2<sup>Df9043* mutants, carrying hypomorphic missense alleles. Larvae were raised at a non-permissive (25°C) or permissive (18°C) temperature (B and C, respectively). Tissues were stained with the DNA stain DAPI (grayscale) and antibodies against Phospho-Histone H3 (pS10) (green) to identify dividing cells. Images are shown as a maximum projection of a confocal Z-stack; scale bar represents 100 microns.
Figure 2.6 Loss of Top2 does not affect the structure of the X chromosome in females. Salivary gland polytene chromosomes were prepared from female third instar larvae representing wild type (Top2\textsuperscript{+/Top2\textsuperscript{Df9043}}) or mutant (Top2\textsuperscript{17-5}/Top2\textsuperscript{Df9043}) genotypes. Chromosomes were stained with DAPI.
Figure 2.7 **Loss of Top2 affects chromosome structure in males.** Salivary gland polytene chromosomes were prepared from male third instar larvae representing wild type [Top2\(^{+/+}\) /Top2\(^{Df9043}\) (A,D,G)] or mutant [Top2\(^{17-5}\) /Top2\(^{Df9043}\) (B,E,H) and Top2\(^{35-14}\) /Top2\(^{Df9043}\) (C,F,I)] genotypes. Chromosomes were stained with DAPI alone (A-C), with enlargement of the male X chromosome shown to demonstrate the diffuse DNA banding pattern (A'-C'). Recruitment of the MSL complex was assessed using antibodies specific to MSL-1 (D-F) and H4K16ac (G-I).
Figure 2.8 **Loss of Top2 does not result in loss of histone H1.** Proteins were extracted from wild type, \( \text{Top}^2_{17-5}/\text{Top}^2_{Df9043} \), and \( \text{Top}^2_{235-14}/\text{Top}^2_{Df9043} \) third instar larval salivary glands for western analysis. Representative blots are shown using antibodies against histone H1 and H3 as a loading control.
Figure 2.9 Loss of Top2 does not alter roX expression. Expression of roX1 and roX2 was determined using quantitative real time PCR in wild type, Top2<sup>17-5</sup>/Top2<sup>Top2Df9043</sup>, and Top2<sup>235-14</sup>/Top2<sup>Top2Df9043</sup> third instar female (A) and male (B) single larvae. All genes were normalized to Rpl32. Eight to ten individual larvae were tested per genotype. Each data point represents a single larva. qPCR was performed by Alexey Soshnev.
Figure 2.10 Top2 complementing females show complex ovary phenotypes. A. Wild type and heteroallelic Top2^{17-6}/Top2^{35-1} mutant ovaries were dissected from three-day-old females and stained with DAPI. The Top2 complementing females were smaller than wild type ovaries, but still retained strings of developing egg chambers. Scale bars, 25 microns. B. Wild type and heteroallelic Top2^{17-6}/Top2^{35-1} three-day-old ovaries were stained with DAPI and antibodies against Vasa (red) to mark germ cells, Spectrin (green) to mark spectrosomes (asterisks) present in germline stem cells and fusosomes (arrowheads) present in differentiating germ cells. Ovaries from the Top2 complementing females contained disorganized germaria (G), with a single ovary having germaria filled only with germ cells containing spectrosomes and germaria devoid of germ cells.
CHAPTER 3
UNDERSTANDING THE ROLE OF *TOPOISOMERASE 2* IN CHROMOSOME INTERACTIONS

**Introduction**

Most metazoan cells are diploid with maternally and paternally inherited homologous chromosomes. Since there are two copies of each chromosome, homologs have the opportunity to interact. While homolog interactions are critical for the reductional division of meiosis, pairing can also occur in non-meiotic cells. In some organisms chromosomes are paired throughout their length, whereas in others, interactions occur at the gene level.

Chromosome associations have been described in mammals and plants. In general, chromosomes are not intimately paired along their length as in diptera somatic cells. Emerging evidence suggests that associations occur between non-homologous chromosomes such that transcriptional regulatory regions are shared to permit cross-regulation. A number of biological processes involve chromosome interactions, including mammalian X-inactivation, cytokine expression, and olfactory receptor choice (Lomvardas et al., 2006; Spilianakis and Flavell, 2004; Spilianakis et al., 2005; Xu et al., 2006).

*Drosophila* serves as an excellent model to study pairing interactions because chromosomes are fully aligned in all somatic cells throughout development (Metz, 1916; Stevens, 1908). For many genes, the degree of homolog association influences gene expression. These effects, collectively referred to as transvection, can promote transcriptional activation or repression (Lewis, 1954). Three different mechanisms for transvection have been described. These include: enhancer action in *trans*, bypass of a chromatin insulator in *cis*, and pairing-dependent gene silencing (Geyer et al., 1990; Morris et al., 1998; Pirrotta, 1999; Wu and Goldberg, 1989).
The genome is permissive for transvection. Transvection has been identified at a number of genes. These genes are found on different chromosomes and are expressed in distinct tissues. Genes displaying transvection require special alleles that permit physical manifestation of a phenotype. Studies using ectopically expressed transgenes revealed that transvection can occur at various locations throughout the genome (Bateman et al., 2012; Chen et al., 2002; Mellert and Truman, 2012).

Allele proximity is important for transvection. Studies using ectopically expressed enhancer-less and promoter-less transgenes revealed that transvection only occurred when both transgenes were at the same homologous site in the genome, but not at non-homologous sites (Chen et al., 2002). Molecular details revealed that transgenes >20 kb from one another did not exhibit transvection (S.Ou, Wu lab, unpublished observations).

Factors affecting transvection have been identified (Duncan, 2002; Hartl et al., 2008; Pirrotta, 1999). These factors alter transvection at the level of individual genes or on a whole chromosome scale. To date, proteins that affect transvection phenotypes include Zeste, Polycomb Group (PcG) proteins, and Condensin II (Cap-H2) (Hartl et al., 2008; Pirrotta, 1999).

Zeste was the first protein identified that affects transvection (Lewis, 1954). Zeste is a DNA binding protein that multimerizes, suggesting it brings chromosomes together at the DNA level and mediates long distance interactions between enhancers and promoters (Biggin et al., 1988; Chen et al., 1992; Chen and Pirrotta, 1993; Chen et al., 2002; Kostyuchenko M, 2009). A gain of function mutation of zeste causes silencing of paired white\textsuperscript{+} genes. Since its original identification, much attention has focused on understanding whether alleles of zeste alter transvection at various loci. Curiously, zeste effects appear to be gene specific, as zeste affects transvection at some loci but not others. It is currently unclear why loss of Zeste results in changes at some genes but not others. Pairing is not disrupted in Zeste protein null backgrounds, suggesting Zeste is essential
for transvection but is not essential for pairing (Gemkow et al., 1998).

*Polycomb group* (*PcG*) genes repress *Hox* genes during development and can influence pairing of *cis* regulatory elements, known as Polycomb Response Elements (PREs). Binding of PcG proteins to PREs confers pairing-dependent gene silencing (Kassis, 2002; Kavi et al., 2006; Pirrotta, 1999). Several studies have shown that PREs mediate long distance interactions (Sigrist and Pirrotta, 1997; Vazquez et al., 2006). For example, the MCP PRE from the BX-C interacts with a second MCP element located at ectopic locations throughout the genome (Sigrist and Pirrotta, 1997; Vazquez et al., 2006). Furthermore, work has demonstrated that loss of function mutations in the *PcG* genes can abolish long-range interactions between different Fab-7 PREs located on different chromosomes in both imaginal discs and the developing embryo. These studies directly showed that *PcG* proteins are important in mediating long-rang interactions between PREs (Bantignies et al., 2003).

The Condensin II subunit, Cap-H2, influences pairing at the chromosomal level. Condensins regulate chromosome structure and segregation of during anaphase (Losada and Hirano, 2005). *Drosophila* has two Condensin complexes, I and II, that each contains Structural Maintenance of Chromosome (SMC) proteins, SMC2 and SMC4 (Cobbe et al., 2006; Losada and Hirano, 2005). Both SMC2 and SMC4 are ATPases that form the core of Condensin I and II complexes (Losada and Hirano, 2005). Each SMC subunit (SMC2 or SMC4) can complex with Cap-H, Cap-G, and Cap-D2 to form Condensin I, or alternatively, each SMC subunit can complex with Cap-H2, Cap-G2, and Cap-D3 to form Condensin II. Loss of *Cap-H2* enhances transvection at both *yellow* and *Ultrabithorax* (Hartl et al., 2008). Direct examination of polytene chromosomes revealed that Cap-H2 overexpression promotes unpairing of chromosomes, possibly through the introduction of positive supercoils in the DNA (Hartl et al., 2008). Cap-H2 may negatively regulate chromosome interactions by altering the chromatin structure (i.e. compaction) to favor unpairing (Hartl et al., 2008).
RNAi screens using FISH as the readout have been completed in cell culture (Joyce et al., 2012; Williams et al., 2007). A genome wide screen using heterochromatic probes targeted to chromosome X, II, and III identified ‘pairing promoting’ genes and ‘anti-pairing’ genes (Joyce et al., 2012). When pairing promoting genes are knocked down pairing is disrupted, however, when anti-pairing genes are knocked down pairing is enhanced. One ‘pairing promoting’ gene identified through an RNAi FISH screen was Topoisomerase 2 (Williams et al., 2007). Top2 is an ATP dependent homodimeric enzyme required for transcription, replication, and chromosome segregation (Nitiss, 2009). These enzymes alter DNA topology by generating transient enzyme-linked double strand breaks that allow passage of one DNA strand through another (Nitiss, 2009). RNAi and chemical inhibitors demonstrated that Top2 is important for pairing in Drosophila cell culture. Depletion of Top2 by RNAi reduced pairing by ~13-33%, depending on the euchromatic locus assayed. For example, These studies revealed that at euchromatic loci, 8C8, 16E1, 28B1, and 44F1, loss of Top2 led to an 18-33% decrease in pairing of single signal nuclei in tetraploid KC cells (Williams et al., 2007). The levels of unpairing were similar at the 28B1 and 44F1 euchromatic loci in clone 8 diploid (XY) cells (15-31% decrease in pairing relative to wild type). It is possible that loss of Top2 is not globally affecting pairing of all loci, but that some loci are more sensitive to perturbation than others. Although Top2 was identified as a protein important for somatic pairing, studies have not confirmed that Top2 is required for homolog interactions in vivo. Furthermore, it is not clear if loss of Top2 alters pairing-dependent changes in expression.

Here, we investigated requirements for Top2 function in vivo. These studies examined whether reduced Top2 function disrupts pairing dependent gene expression. These experiments took advantage of observations that heteroallelic combinations of Top2 mutants produce viable adults. These viable heteroallelic combinations of Top2 mutants were used to examine transvection at three classically studied loci (yellow, white,
and Ultrabithorax). These genes were chosen as they are expressed in different tissues, are located at different genomic positions, and exhibit different mechanisms of transvection. Transvection at yellow and Ultrabithorax leads to transcriptional activation, while mutant Zeste1 causes pairing-dependent gene silencing of the white+ gene. For each gene, homologous interactions between transvecting alleles were analyzed for alterations in pairing-dependent changes in phenotype. Of the three transvecting systems tested in Top2 complementing adults, only one displayed an altered phenotype consistent with loss of homolog pairing. Our studies indicate that changes in the phenotype, due to altered Top2, are likely gene specific changes. Direct examination of pairing interactions of the classic transvection genes demonstrated wild type levels of pairing in Top2 mutants, even for the hypomorphic Top2 allele that shows reduced activity. These data indicate that loss of Top2 does not result in global unpairing in vivo.

Materials and Methods

Drosophila stocks and culture conditions

Flies were maintained at 25 ± 1°C at 70% humidity on standard Drosophila cornmeal, yeast, sugar, and agar medium with ρ-hydroxybenzoic acid methyl ester as a mold inhibitor. All crosses were performed at 25°C and transferred regularly. The y^1^88, y^2^, y^3c3^, z^w^, z^w^, and Cbx^Ubx^ alleles used in this study have previously been described (Bender et al., 1983; Casanova et al., 1985; Gelbart and Wu, 1982; Geyer et al., 1990; Morris et al., 1999; Wu and Goldberg, 1989). Stocks obtained from the Bloomington Stock Center include: a stock carrying Df(2L)Exel9043 (BL 7913; hereafter referred to as Top2^Df9043^ that removes genes in the cytological region 37E1, including Top2) and a stock carrying the balancer chromosome CyO, y^+^ (BL 4542). Stocks obtained from the Harvard Medical School Exelixis Drosophila stock center include: Top2^c05388^ (hereafter referred to as Top2^c^), Top2^d05357^ (Top2^d^), and Top2^05145^ (Top2^f^).
Western analysis

Proteins were extracted from the brains/imaginal discs/salivary glands of ten larvae, boiled for 5 minutes in 3X sample buffer, separated on a 4-20% Tris-HCl gradient gel [BIO-RAD, no. 161-1159], and transferred to a 0.45 µm nitrocellulose membrane (70V for 90 minutes). Membranes were blocked in 5% milk in PBST (PBS + 1% Tween-20), incubated in primary antibody overnight at 4°, and washed 3X10 minutes with PBST. Blots were incubated with chicken anti-Mod67.2 primary antibody at 1:7,500. Blots were incubated with secondary antibody HRP-conjugated chicken anti-mouse IgG secondary antibody [Sigma, no. A9792] for 2-3 hours, washed 3X10 minutes in PBST, and detected using chemiluminiscent substrate [BIO-RAD, no. 170-5040 or Millipore, no. WBLUR0100]. To control for amounts of protein loaded, blots were incubated with mouse anti-α-tubulin IgG primary antibody [Sigma, no. T5168] and detected with the HRP-conjugated rabbit anti-mouse IgG secondary antibody [Sigma, no. A9044].

Immuno-fluorescent in situ hybridization (FISH)

Chromosome pairing was assayed by FISH hybridization with BACs (Children’s Hospital Oakland Research Institute-CHORI) corresponding to several regions of the chromosome that included yellow (CH322-36F22; 18 kb), white (CH321-85C15; 75 kb), and Ubx (CH321-87G13; 90 kb). BACs were isolated using cesium chloride gradients and labeled using Nick Translation (Vysis no. 32-801300) according to manufacturer’s protocol. Nick translation incorporated fluorescently labeled dUTPs (Red-dUTP) into the DNA using DNase I and DNA polymerase I. Unincorporated nucleotides were removed using ethanol precipitation. An oligonucleotide probe for the 359-bp repeat was synthesized with a 5’ Cy5 fluorescent dye (Integrated DNA Technology; GGG ATC GTT AGC ACT GGT AAT TAG CTG C).

Eye and wing tissues were dissected from female third instar larvae in 1X PBS on ice, rinsed in CSB (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 10 mM PIPES-
pH 6.8), incubated in CSBT (0.5% Triton X-100) pre-fix for 1-2 minutes and rinsed again in CSB. Samples were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, no. 15710) in 1X PBS for 10 minutes at room temperature (RT) with gentle rocking. After fixation, samples were washed in 2xSSCT (0.3 M NaCl, 0.03 M sodium citrate, 0.1% Tween-20) 3X10 minutes, and subsequently washed in 2xSSCT with increasing amounts of formamide (20%, 40%, 50% for 10 minutes each; note 50% formamide was warmed to 37°C) at RT with gentle rocking. Fresh 2xSSCT containing 50% formamide (warmed to 37°C) was added to each sample and tissues were incubated at 37°C for 3 hours. Nick translated probes were diluted to a final concentration of 1 µg/40 µL of hybridization buffer (50% formamide/2xSSCT, 10% dextran sulfate; warmed to 37°C). Six to eight pairs of eye discs or five to six wing discs were added to the hybridization buffer/probe mixture prior to denaturation in a thermocycler for 2 minutes at 91°C and hybridized for 16 hours at 37°C. Tissues were transferred to 50% formamide/2xSSCT (warmed to 37°C; 3X20 minute washes at RT), and subsequently washed in 2xSSCT/formamide (50%, 40%, 20% for 1X10 minutes at RT), 2xSSCT (3X10 minutes at RT), 1X PBS (3X10 minutes at RT), 1X PBST (0.3% Tween-20, 3X10 minutes at RT). Samples were incubated in blocking solution (1XPBST + 0.5% BSA) for 3-4 hours and incubated overnight with primary antibodies (rabbit anti-lamin Dm0 at 1:2,000 or mouse anti-lamin Dm0 at 1:100, P.Fisher and DSHB, respectively) at 4°C. Next, samples were washed in 1X PBST (3X10 minutes at RT), incubated with secondary antibodies for 2-3 hours at RT, washed in 1X PBST (3X10 minutes at RT), and DAPI stained (1 µg/mL in PBT) for 5-10 minutes. Following an additional wash in 1X PBST for 5 minutes, tissues were mounted on slides in Vectashield (Vector Laboratories, H1000). FISH images were acquired using a 63X oil objective with 2.5 digital zoom on a Zeiss 710 confocal microscope. For eye discs, cells were imaged posterior of the morphogenetic furrow. For wing discs, cells were imaged in the anterior portion of the body wall. One image was acquired per disc, and approximately one hundred cells were analyzed per image.
Scoring was done on blinded samples. Distances between the center of FISH signals were measured using Imaris software (Bitplane). Chromosome were considered paired if the center-to-center distance between FISH signals was $\leq 0.5 \ \mu \text{m}$ (Williams et al., 2007).

**Quantitative PCR (qPCR) analysis**

RNA isolation and qPCR analysis were performed according to previously described methods (Soshnev et al. 2008). RNA samples were prepared from 10 adult male or female flies aged 2-3 days or 10 third instar larvae. All analyses were performed in two replicates. Average fold change and standard deviation were calculated using Microsoft Excel. All samples were normalized to the housekeeping gene, *RpL32*. Primers used for qPCR are shown in Tables 3.1-3.4.

**Results**

Requirements for Top2 in transvection were studied. Transvection was assessed at three classically studied genes: *yellow*, *white*, and *Ubx*. These three genes affect cuticle pigmentation, eye color, and wing pattern, respectively. For each gene, homologous interactions between transvecting alleles were analyzed for alterations in pairing-dependent changes in phenotype. These experiments took advantage of observations that heteroallelic combinations of Top2 mutants produce viable adults.

*Top2* alleles were carried on a second chromosome with the recessive markers *dp*, *cn*, and *bw*. In order to study transvection, these mutations were removed by recombination (Figure 3.1). Males of the genotype *y^{l}w_6^{7c23}/Y; dp Top2^{m} cn bw/CyO, y+* were crossed to wild type females. In the F1 generation, virgin females were collected that bore the multiply marked second chromosome over a wild type chromosome (*dp Top2^{m} cn bw/+ + + +*). These virgin females were subsequently crossed to males carrying the dominant marker, *Scutoid (Sco)*, over the balancer chromosome *CyO*. Single males were selected carrying the recombinant chromosome (*dp Top2^{m} cn bw*)/Sco and each male was subsequently crossed to: 1) *Sco/CyO* virgin females to maintain the stock,
2) \( \text{Top2}^{D9043/\text{CyO}}, y^+ \) virgin females to ensure that the \( \text{Top2} \) mutant allele was retained on the recombined chromosome, and 3) \( \text{dp cn bw} \) virgin females to ensure that the \( \text{dp} \) and \( \text{bw} \) alleles were recombined off the starter chromosome. Recombinants were isolated that failed to complement the \( \text{Top2} \) deficiency and those that had wild type wings and red/orange eye pigmentation. Recombinants were generated for three complementing \( \text{Top2} \) alleles, \( \text{Top2}^{17-1R}, \text{Top2}^{17-3R}, \) and \( \text{Top2}^{35-13R} \) (Where “R” denotes recombinant).

Interse crosses were performed between each of the recombinant chromosomes to check viability associated with the heteroallelic mutants. Previous studies indicated that viability for \( \text{Top2}^{35-13}/\text{Top2}^{17-3} \) or \( \text{Top2}^{17-3}/\text{Top2}^{35-13} \) ranged from 60-77% while that for \( \text{Top2}^{17-3}/\text{Top2}^{17-1} \) or \( \text{Top2}^{17-1}/\text{Top2}^{17-3} \) was on the lower end, representing 19-22% viability. Following recombination, complementation between \( \text{Top2} \) alleles changed, suggesting the original levels of viability were influenced by second site mutations on the second chromosome. The viability for \( \text{Top2}^{35-13R}/\text{Top2}^{17-3R} \) or \( \text{Top2}^{17-3R}/\text{Top2}^{35-13R} \) were near wild type (>100%) while the viability for \( \text{Top2}^{17-3R}/\text{Top2}^{17-1R} \) or \( \text{Top2}^{17-1R}/\text{Top2}^{17-3R} \) ranged from 12-56%. All alleles studied showed improved complementation after recombination. For transvection analyses, two heteroallelic combinations were used that yielded high and intermediate numbers of viable adults, including \( \text{Top2}^{17-3R}/\text{Top2}^{17-1R} \) and \( \text{Top2}^{35-13R}/\text{Top2}^{17-3R} \).

**\( \text{Top2} \) mutants do not disrupt insulator function at \( \text{yellow} \)**

The \( \text{yellow} \) gene is responsible for wing and body pigmentation in the fly. Tissue specific enhancers located upstream of the transcription start site and within the single intron are required for proper \( \text{yellow} \) expression (Geyer and Corces, 1987; Martin et al., 1989). To date, a number of \( \text{yellow} \) alleles have been identified that either reduce or abolish pigmentation in the wing, body, and other cuticular structures. Transvection at the \( \text{yellow} \) gene involves the \( y^2 \) mutation. This allele is caused by insertion of a \( \text{gypsy} \)
insulator between the wing and body enhancers and the promoter. Since the wing and body enhancers are blocked from the promoter, flies have light wings and bodies (Geyer et al., 1986). Importantly, the bristle enhancer is not blocked from the promoter, resulting in flies with dark bristles. Enhancer blocking by the gypsy insulator at yellow depends upon three insulator proteins, Su(Hw), Modifier of (mdg4) 67.2 (Mod67.2), and Centrosomal Protein of 190 kDa (CP190) (Georgiev and Gerasimova, 1989; Pai et al., 2004). Recent reports suggest that gypsy insulator function depends on Top2 function, which would preclude the ability to test transvection at yellow (Ramos et al., 2011).

Top2 may contribute to nuclear function. Recent studies suggest that protein knockdown and catalytically inactive Top2 cause lethality in yeast by different mechanisms (Baxter et al., 2011), suggesting that these functions can be genetically separable. We considered that the requirement for Top in insulation might similarly show distinct requirements, wherein loss of Top2 might alter insulator function. The effect of Top2 on gypsy insulator function was proposed to be through interaction with the insulator protein, Mod67.2 (Ramos et al., 2011). Previous studies revealed that the stability of Mod67.2 decreased in a Top2 mutant background (Ramos et al., 2011). To examine effects of loss of Top2 on Mod67.2 accumulation, western analysis on Top2 insertion alleles and EMS-generated point mutations were performed. Studies indicate that Mod67.2 accumulates near wild type levels in larvae carrying either the Top2 insertions or point mutations (Figure 3.2). In fact, Top2 nonsense alleles that do not accumulate a Top2 protein, accumulate near wild type levels of Mod67.2. Ten additional Top2 missense mutations were tested for accumulation of Mod67.2, and also demonstrated accumulation of the Mod67.2 isoform. We also noticed accumulation of a cross reacting polypeptide of approximately 65 kDa that was observed primarily in Top2c, Top217-5, or Top235-2 genetic backgrounds (Figure 3.2 open arrowhead at 65 kDa). It is currently unclear what this band is, however, it is possible that it may be
accumulation of a modified isoform of Mod. Together, these studies indicate that Mod67.2 accumulates near wild type levels in Top2 mutant backgrounds.

We investigated whether loss of Top2 impairs insulator activity at yellow (Figure 3.3). We reasoned that Top2 complementing adults might display differences than RNAi knock down experiments done previously. Females with the genotype $y^2/y^2; Top2^m/CyO$ were crossed to $y^2/Y; Top2^m/CyO$ males and pigmentation was scored in male non-CyO flies. Pigmentation of flies carrying heteroallelic Top2 mutants were compared to pigmentation of flies carrying mutations in genes encoding the insulator proteins, $su(Hw)^v/f$ and $mod(mdg4)u1$. Loss of Su(Hw) disrupts gypsy insulator function to yield $y^2$ flies with dark colored wings, bodies, and bristles. Loss of Mod67.2 partially disrupts gypsy insulator function, altering pigmentation levels to produce darker wings and bodies than $y^2$, but below wild type levels. Examination of pigmentation in heteroallelic Top2 mutant combinations revealed that flies had light wings, bodies, and dark bristles (Figure 3.3). These data suggest that viable heteroallelic Top2 mutants do not alter gypsy insulator function at yellow, permitting transvection studies at this locus.

**Top2 mutants do not affect expression of yellow or Ubx**

Top2 has a demonstrated role in transcription. DNA structures produced by progression of RNA Polymerases are resolved by Top2 (Brill et al., 1987; Gartenberg and Wang, 1992; Sperling et al., 2011). A global transcriptional requirement for Top2 is suggested by the genome-wide enrichment of yeast Top2 at promoters of highly transcribed genes, with evidence that this protein confers optimal recruitment of RNA Polymerase II (Sperling et al., 2011). We wondered whether yellow, white, or Ubx were misexpressed in heteroallelic Top2 mutants. Because of the known role for Top2 in transcriptional elongation (Garcia-Rubio and Aguilera, 2012), we designed qPCR primers to the 5’ and 3’ end of each gene, to determine if loss of Top2 decreased the full length
RNA production. The yellow and Ubx genes are robustly expressed in L2 and L3 larvae, respectively, we assayed expression during this window of development. In contrast, white expression was assayed in adult males, when this gene is robustly expressed (Graveley et al., 2011). For each assay, two heteroallelic Top2 combinations were tested (Top2^{35-13R}/Top2^{17-3R}, Top2^{17-3R}/Top2^{17-1R}), representing strong and weak complementing combinations. We tested expression in one additional genotype, Top2^{17-6}/Top2^{D9043}, that contains a Y592N amino acid substitution in the TOPRIM domain that represents a greater compromised Top2 mutant background than the Top2 complementing adults. As larvae die at the larval to pupal transition, we used this mutant to assess expression levels of yellow and Ubx, since white was assessed in male adults. Overall, we found that expression of yellow or Ubx did not significantly change relative to wild type (p>0.05, t-test, Figure 3.4). Expression of white showed a modest decrease relative to wild type, suggesting that Top2 mutants may affect expression of white (p<0.05, t-test).

**Top2 mutants do not disrupt transvection at yellow**

Having established that Top2 complementing adults retain gypsy insulator function, we tested whether mutations in Top2 disrupted transvection at the yellow locus. Two mechanisms of transvection have been established at yellow. One mechanism involves enhancers working in trans on a promoter, and the other involves enhancer bypass of a chromatin insulator (Geyer et al., 1990; Morris et al., 1998). Transvection involves the enhancers of an allele lacking a promoter (y^{1\#8}) working in trans on the promoter of a paired homolog whose enhancer activity is blocked from its promoter by the gypsy insulator (y^{2}). Female y^{1\#8} flies have light wings, bodies, and bristles while y^{2} female flies have light wings, bodies and dark bristles. Females with the genotype y^{2}/y^{1\#8} have dark wings, bodies, and bristles because of transvection.

Interallelic complementation between y^{2} and y^{1\#8} assessed whether mutations in Top2 disrupt enhancer action in trans. Females with the genotype y^{1\#8}/y^{1\#8}; Top2^{m}/CyO
were crossed to $y^2/Y; Top2^{17-13R}/CyO$ males and pigmentation was scored in female non-$CyO$ flies. Resulting female $Top2^{17-3R}/Top2^{17-3R}$ flies had dark wings, bodies, and bristles, indicating that transvection was not disrupted (Figure 3.5A). The heteroallelic combination $Top2^{17-3R}/Top2^{17-1R}$ could not be tested, as the crosses yielded no viable $Top2^{17-3R}/Top2^{17-1R}$ male or female adult flies. Together, these data indicate that the heteroallelic $Top2$ mutant combination tested does not disrupt enhancer action in trans.

A second mechanism of transvection involves insulator bypass in cis. When the $y^2$ allele is paired with a special yellow allele that lacks the body enhancer and promoter region ($y^{3c3}$), the body enhancer of the $y^2$ allele is able to bypass the chromatin insulator in cis and activate transcription (Figure 3.5B). Female $y^{3c3}$ flies carry a deletion of the bristle enhancer and promoter that extends close to the first exon. Female $y^{3c3}$ flies are similar to $y^{1#8}$ females in that they have light wings, bodies, and bristles. Females with the genotype $y^2/y^{3c3}$ had dark wings, bodies, and bristles suggesting that the blocked body enhancer of $y^2$ bypasses the chromatin insulator when paired with $y^{3c3}$.

Effects of Top2 loss on insulator bypass between $y^2$ and $y^{3c3}$ were tested (Figure 3.5B). Female $Top2^{17-13R}/Top2^{17-3R}$ and $Top2^{17-3R}/Top2^{17-1R}$ flies had dark wings, bodies, and bristles (Figure 3.5B), indicating that $Top2$ mutants do not disrupt insulator bypass. Together, these studies indicate that $Top2$ mutants do not disrupt transvection at yellow.

A phenotypic effect at white is observed in $Top2$ mutants

Transvection at yellow results from pairing dependent interactions that leads to increased transcription. In contrast, transvection at white$^+$ results from pairing dependent repression conferred by Zeste (Duncan, 2002; Pirrotta, 1999). The X-linked white$^+$ gene is required for red eye pigmentation in the fly, with loss generating white eyes. When white$^+$ genes are paired, as in females, the mutant $Z^1$ protein silences both alleles, producing a yellow/orange eye phenotype (Pirrotta, 1999). When one white$^+$ gene is
present, as in males, pairing is not possible, so $Z^1$-mediated repression does not occur, producing a red eye phenotype.

To determine whether mutations in Top2 disrupted pairing-dependent gene silencing, two white alleles were used. First, eye color was assessed in $z^I w^+$ males where there is only one copy of the white$^+$ gene, so pairing dependent silencing does not occur. As expected, eye pigmentation in $z^I w^+/Y; Top2^{35-13R}/Top2^{17-3R}$ males remained red, resembling the sibling controls. Second, transvection was assayed in $z^I w^+$ females where homolog pairing can be assessed in trans. If Top2 mutants disrupt pairing mediated repression then we would expect to see darkening of eye color in $z^I w^+; Top2^{35-13R}/Top2^{17-3R}$ females. Instead, $z^I w^+; Top2^{35-13R}/Top2^{17-3R}$ females had white or very light yellow eyes (Figure 3.6A). These findings suggest that Top2 causes hyper-silencing of white$^+$ and makes Zeste a stronger repressor.

During these studies, and when testing whether Top2 mutants disrupted yellow transvection, we noticed that no viable $Top2^{17-3R}/Top2^{17-1R}$ flies were obtained. For this reason, we did not study this genetic background, but focused on using $Top2^{35-13R}/Top2^{17-3R}$. We did not see changes in $Top2^{35-13R}/Top2^{17-3R}$ viability when the direction of the cross was changed, therefore, we refer to the genotypic offspring that are a result of either cross.

Transvection was assayed in a different white genetic background, $z^I w^{js}$, where transvection effects can be assessed in cis or trans (Figure 3.6A). The $w^{js}$ allele is a gene duplication allowing $Z^1$ effects to be assessed in cis in males and in trans in females (Gelbart and Wu, 1982; Wu and Goldberg, 1989; Wu et al., 1989). Eye color of $z^I w^{js}; Top2^{35-13R}/Top2^{17-3R}$ males and females was assessed to determine whether Top2 is important in gene silencing. Eye pigmentation was examined in wild type, $z^I w^{js}; Top2^{35-13R}/Top2^{17-3R}$, and $z^I w^{js}; Top2^{35-13R}$ or $17-3R/Top2^+$ males and females. As observed with in the $z^I w^+$ background, $Top2^{35-13R}/Top2^{17-3R}$ females had white or very light yellow eyes (Figure 3.6A). Males had light orange eyes, relative to their sibling and $Top2^+$ controls,
suggesting Top2 may still be hypersilencing Zeste, but to a lesser degree in males.

These data described above indicate that heteroallelic Top2 mutants result in a phenotypic effect at white, but not in the way one would expect if pairing were disrupted. Therefore, we decided to directly test chromosome pairing at the white locus using fluorescent in situ hybridization (Figure 3.6B; FISH). FISH was assayed in wild type, Top2^{35-13R}/Top2^{17-3R}, and Top2^{17-6}/Top2^{Df9043} eye imaginal discs of third instar larvae, where the white gene is robustly expressed. Immuno-FISH was performed using an antibody against lamin Dm0 that recognizes the nuclear envelope. The level of pairing was determined by counting the number of FISH signals present per nucleus. Blinded images were scored and any cells undergoing mitosis were excluded and not scored. Approximately 85% ± 9% of nuclei had one FISH signal in wild type, indicating high levels of pairing (Figure 3.6C; n=1373, where n is the total number of nuclei scored). Upon examination of the mutants, we did not observe a significant change in pairing status in Top2^{35-13R}/Top2^{17-3R} or Top2^{17-6}/Top2^{Df9043} eye disc nuclei relative to the wild type control (Figure 3.6C; 90% ± 4%, n=611; 80% ± 5%, n=747 respectively, p>0.05 t-test). Together, these studies indicate that pairing at the white locus is maintained in Top2 mutant backgrounds.

Since the pairing data did not explain the phenotypic effect at white^+ that results from loss of Top2, we wondered whether lower Top2 function might lead to increased levels of Zeste^1, leading to stronger repression. RNA was also isolated from z^1w^+ and z^1w^is; Top2^+ or Top2^{35-13R}/Top2^{17-3R} male and female whole flies and quantitative real time PCR was performed. In particular, we wondered if zeste expression was upregulated in heteroallelic Top2 mutants, thus explaining the enhanced repression of white^+ or is. Expression of zeste was unchanged in the Top2 mutants relative to wild type in both males and females (Figure 3.7). In sum, the phenotypic effect at white is not due to upregulation of zeste.
A phenotypic effect at Ultrabithorax is observed in Top2 mutants

Transvection was tested at Ubx. The Ubx gene is one of three genes of the bithorax locus. These genes regulate the identity of the thoracic and abdominal segments (Castelli-Gair et al., 1990). Cbx$^l$ is a gain-of-function mutation of Ubx that is due to insertion of the Ubx upstream regulatory region into the second intron of the gene, resulting in overexpression of Ubx. Ubx$^l$ is an insertion in the first exon that disrupts the protein-coding region. When Cbx$^l$ Ubx$^l$ is placed in trans to a wild type copy of the Ubx gene, Cbx$^l$ drives ectopic expression of the wild type Ubx gene in trans, causing a homeotic transformation of the wing toward haltere, manifest as a shortened, deformed wing blade (Kennison and Southworth, 2002). Wild type flies have wings with a smooth margin and complete longitudinal cross veins. In contrast, adult Cbx$^l$ Ubx$^l$/+ flies have wings that are more cashew shaped with nicks and often have incomplete longitudinal cross veins. Chromosomal rearrangements that alter pairing between Cbx$^l$ Ubx$^l$ and the wild type Ubx gene disrupt trans activation of Ubx, resulting in a wild type wing (Kennison and Southworth, 2002; Lewis, 1954).

The Cbx$^l$ Ubx$^l$ chromosome was crossed into Top2 mutant backgrounds. To determine whether mutations in Top2 cause a phenotypic effect at Ubx, wing phenotypes were scored in Top2$^{35-13R}$/Top2$^{17-3R}$; Cbx$^l$ Ubx$^l$/+ adult flies (Figure 3.8A). We found that in Top2$^{35-13R}$/Top2$^{17-3R}$ mutants, the wing phenotype is suppressed in adult flies (1-2% nicked wings as versus 35-55% for Cbx$^l$ Ubx$^l$, Table 3.4). Together, these data indicate that a phenotypic effect at Ubx is observed in Top2 mutants.

We wondered whether these changes in phenotype could be explained by unpairing, as expression analysis indicated that Ubx was not misexpressed in Top2 mutants. Previous studies analyzed pairing at the Ubx locus in developing embryos and found that pairing reached high levels in wild type (Gemkow et al., 1998). Therefore, we directly assayed chromosome pairing at the Ubx locus using FISH (Figure 3.8B).
Approximately 78% ± 10% of nuclei had one FISH signal in wild type, levels comparable to that observed at the white locus (Figure 3.C; n=925). Examination of Top2^{17-6}/Top2^{Df9043} wing disc nuclei did not reveal significant levels of unpairing relative to the wild type control (59% ± 8%, n=506 relative to 78% ± 10%, n=925 observed in wild type, p>0.05 t-test), nor for mutant Top2^{35-13R}/Top2^{17-3R} (90% ± 3, n=412, p>0.05, t-test). We were surprised that pairing was not altered in Top2^{35-13R}/Top2^{17-3R} mutants, since this was the genotype where the phenotypic effect was observed at Ubx.

**Top2 is not required for pairing of the heterochromatic 359-bp repeat**

Previous studies indicate that heterochromatic regions may pair less frequently than euchromatic regions (Williams et al., 2007). To assess pairing of heterochromatic regions in our Top2 mutants, analysis of the X-linked 359-bp repeat was completed in both wing and eye disc (Figure 3.9). Approximately 74% ± 5% of wing disc nuclei had one FISH signal in wild type, indicating the chromosomes are paired (Figure 3.9; n=562). Upon examination of the mutants, we did not observe a change in pairing status in Top2^{35-13R}/Top2^{17-3R} or Top2^{17-6}/Top2^{Df9043} wing disc nuclei relative to the wild type control (67% ± 3%, n=208 and 67% ± 10%, n=463). Approximately 80% ± 9% of eye disc nuclei (n=1105) had one FISH signal in wild type relative to Top2^{35-13R}/Top2^{17-3R} (72% ± 8%, n=511) and Top2^{17-6}/Top2^{Df9043} (88% ± 3%, n=308). Together, these studies indicate that pairing at the X-linked 359-bp heterochromatic locus is maintained in Top2 mutant backgrounds.

**Discussion**

The role of Top2 in chromosome associations was tested *in vivo*. These experiments capitalized on the identification of novel Top2 alleles that display interallelic complementation between lethal alleles, providing an opportunity to obtain adults with compromised Top2 function. We used two different heteroallelic combinations for our...
studies that included, Top2^{17-3R}/Top2^{17-1R} and Top2^{17-3R}/Top2^{17-1R}. We found that heteroallelic combination Top2^{17-3R}/Top2^{17-1R} was very sensitive to genetic background, indicating that compromised Top2 function results in decreased viability. Our studies used classical genes that display transvection.

Recent reports suggested that gypsy insulator function might be dependent on Top2 (Ramos et al., 2011). Results from our studies differ from previous reports. This difference may be due to the nature of the Top2 alleles studied. Our studies took advantage of viable heteroallelic combinations of EMS-generated amino acid substitutions in Top2, rather than an RNAi line that resulted in partial knock-down of Top2, permitting viable adults. The lack of an effect on gypsy insulator function can be explained by the possibility that Top2 complementing adults have higher levels of Top2 activity than the conditionals that were generated using Top2 knock-down. Furthermore, studies in S. cerevisiae have shown that lethality resulting from a Top2 deletion and lethality caused by a catalytically inactive Top2 represented different causal mechanisms (Baxter and Diffley, 2008).

The effect of Top2 on insulator function is proposed to be through direct interaction with Mod67.2, where loss of Top2 results in decreased levels of Mod67.2 (Ramos et al., 2011). Western analysis on the same Top2 insertion alleles previously studied, Top2^{f} and Top2^{c}, did not result in loss of Mod67.2, as previously documented. These differences may be reconciled by the nature of the alleles and the use of different Mod antibodies. Our Western analyses of these Top2 insertion alleles and EMS-generated point mutations indicate that Mod67.2 accumulates near wild type levels (Figure 3.2).

Viable heteroallelic combinations of Top2 mutants were used to examine transvection at three classically studied loci (yellow, white, and Ultrabithorax). Of these three transvecting systems tested, only one displayed an altered phenotype consistent with altered pairing dependent changes in expression. Studies of transvection at yellow
demonstrated Top2 mutants did not disrupt enhancer action in trans or bypass of a chromatin insulator in cis (Figure 3.4). We also assessed yellow expression in three different Top2 mutant backgrounds and showed that expression does not change relative to wild type (Figure 3.4).

Our studies revealed that Top2 mutants result in a phenotypic effect at white, but not in the way one would expect if pairing were disrupted (Figure 3.6). Expression of white did show a modest decrease relative to wild type, suggesting that Top2 mutants may affect expression of this gene (p<0.05, t-test). Approximately a two-fold change in expression of white was observed in Top2 mutants, suggesting that this change in expression level coupled with a zeste mutation may have a significant effect on the phenotype. It is formally possible that loss of Top2 results in greater tissue specific transcriptional changes than were detected in adult flies. We hypothesized that lower Top2 function might increase Zeste\(^1\) levels, leading to stronger repression of white\(^+\). However, expression of zeste was unchanged in the Top2 mutants relative to wild type in males and females, indicating that the effect at white\(^+\) is not due to upregulation of zeste (Figure 3.7). FISH studies directly assaying pairing at white revealed that pairing reaches wild type levels in Top2 mutants.

Studies revealed that Top2 mutants result in a phenotypic effect at Ubx, consistent with altered pairing dependent changes in expression. Curiously, though, we found that expression of Ubx was unchanged relative to wild type (Figure 3.4) and that pairing was maintained in Top2 mutants. As at white, it is formally possible that loss of Top2 results in greater tissue specific transcriptional changes that were not detected in whole larvae, but might be detected in haltere or wing discs if assayed. Many questions remain unresolved. It is currently unknown how stable chromosome interactions are over time, to what degree chromosomes must pair for a gene to recognize it as paired, and the extent that a gene must be expressed in order to see a physical change in phenotype. For example, studies at yellow have shown that transcription can be dramatically reduced
(~65-95%) and still result in a wild type phenotype, suggesting yellow is extremely difficult to disrupt (Geyer et al., 1990; Lee and Wu, 2006). On the other hand, the level of pairing as assayed by FISH does not always correlate well with the phenotypic outcome (Gemkow et al., 1998). Pairing studies of the Ubx locus in embryos carrying a \(z^{a694}\) or \(z^a\) allele, previously shown to disrupt transvection at Ubx, revealed that the levels of pairing in the mutants was the same as that observed for wild type (Gemkow et al., 2001). Our studies are comparable with these results in that pairing levels of Ubx in wing disc were 78% ± 10% in wild type. Furthermore, our studies revealed that Top2 mutants cause a phenotypic effect at Ubx that is consistent with pairing dependent changes in gene expression, however, we never observed significant changes in pairing in Top2 mutants. It is curious that loss of Top2 and loss of zeste result in a phenotypic effect at Ubx, but that neither mutant shows disruption in pairing. This may be due to the mechanism in which these mutants affect gene expression (see below).

Studies of translocations that disrupt transvection at Ubx revealed that pairing at Ubx was only reduced by 50-60% (Gemkow et al., 1998). Although translocations and zeste mutations result in the same phenotypic outcome at Ubx (i.e. disruption of transvection), the mechanism in which they affect gene expression differs (Goldsborough and Kornberg, 1996). Specifically, rearrangements that disrupt transvection at Cbx1/+ reduce transcription from both homologues whereas mutation of zeste\(^a\) results in transcriptional reduction of the homolog carrying the wild type Ubx gene (Goldsborough and Kornberg, 1996). In other words, rearrangements do not abolish activation in trans while zeste\(^a\) does. In sum, many factors may influence pairing dependent changes in expression.

Why do we see a phenotypic effect at Ubx that is consistent with altered pairing dependent changes in expression, but not at white? Why do we not see a change in phenotype at yellow? One explanation might involve the critical region. The “critical region” is defined as the region on the chromosome that is needed for homolog pairing,
and in turn transvection. Critical regions are typically identified using breakpoints of chromosomal rearrangements that disrupt chromosome pairing and therefore, transvection. The *yellow* gene has a very small critical region, ~650 kb or less, compared to other genes that show transvection, especially *Ubx*, which encompasses 12 Mb or half of a chromosome arm (Duncan, 2002; Ou et al., 2009). Although mechanistically different than *yellow*, transvection studies of *white* revealed it also has a very small critical region (only cytogenetic location 3C; ~50 kb) and that only rearrangements with breakpoints very close to the gene disrupted transvection (Ou et al., 2009; Smolik-Utlaut and Gelbart, 1987). As *Ubx* has a critical region that encompasses nearly half of chromosome 3R, it is easier to disrupt (Duncan, 2002; Lewis, 1954). It is possible that the size of the critical region may be influencing the phenotypic outcome at *Ubx*.

A second explanation may involve the length of the cell cycle in different tissues. The *white* gene is expressed in post-mitotic cells of the developing eye imaginal disc while *Ubx* is expressed in the wing disc, where cells actively divide during larval development (Kaufman et al., 1973; Masucci et al., 1990; Steller and Pirrotta, 1985; Zachar et al., 1985). It has been hypothesized that the time for homologs to find each other and pair may be dependent on the length of the cell cycle (Golic and Golic, 1996). The longer the cell cycle before ensuing mitoses, the more time homologs have to find one another and pair, and the more difficult it will be to disrupt. *Ubx* may be easier to disrupt because homologs have a very short window of time to find each other and pair, prior to the next division. It is also possible that Top2 is altering the length of the cell cycle in different tissues *in vivo* and this in turn is influencing pairing.

FISH studies demonstrated that loss of Top2 did not result in global unpairing (Figure 3.7 and 3.8). These results were quite surprising, as previous studies demonstrated loss of Top2 perturbed somatic pairing in cell culture (Williams et al., 2007). These studies revealed that at euchromatic loci, 8C8, 16E1, 28B1, and 44F1, loss of Top2 led to an 18-33% decrease in pairing of single signal nuclei in tetraploid KC cells.
The levels of unpairing were similar at the 28B1 and 44F1 euchromatic loci in clone 8 diploid (XY) cells (15-31% decrease in pairing relative to wild type). It is possible that loss of Top2 is not globally affecting pairing of all loci, but that some loci are more sensitive to perturbation than others. This may explain why we do not see unpairing at white or Ubx. We also demonstrated that levels of heterochromatic pairing, as assayed by the 359-bp repeat, were comparable to those previously reported for wild type (Joyce et al., 2012). Previous studies also demonstrated that knockdown of Top2 via RNAi did not affect pairing of the heterochromatic regions, as assayed by 359-bp repeat, AACAC, and dodeca probes (Joyce et al., 2012). Our findings are consistent with these studies indicating that pairing is not disrupted at the 359-bp repeat in Top2 mutants (Figure 3.9).

Aneuploidy may account for the differences in homolog pairing we observe. As Top2 plays a role in the segregation of sister chromatids during anaphase of the cell cycle (DiNardo et al., 1984; Holm et al., 1985; Uemura et al., 1987), loss of Top2 causes improper resolution of chromosomes resulting in aneuploidy. Indeed, cell culture studies using dsRNAs or inhibitors targeting Top2 revealed an increase in aneuploidy, as demonstrated by an increase in the number of nuclei with no FISH signal (Williams et al., 2007). In cell culture, cells were allowed to go through one division and then were subsequently fixed, permitting analyses on all cells. It is possible that nuclei exhibiting aneuploidy have undergone cell death in vivo, thus explaining why we do not see increased amounts of unpairing. As a note of support, apoptotic nuclei were observed in imaginal discs from Top2\(^{17-6}/\text{Top2}^{D9043}\) larvae (data not shown). Furthermore, Lamin Dm0 was not used as a marker to recognize nuclear envelope breakdown, suggesting that all cells were scored. In our in vivo system, nuclei that exhibited nuclear envelope breakdown were not scored. It is also formally possible that the heteroallelic and hypomorphic mutants tested all had “too much” Top2 function, revealing why we did not
see global unpairing. As RNAi experiments did not confirm level of Top2 knock-down, it is not clear how much Top2 activity might be necessary for pairing.

In sum, we investigated requirements for Top2 function in vivo. These studies examined whether reduced Top2 function disrupts pairing dependent gene expression. For each gene, homologous interactions between transvecting alleles were analyzed for alterations in pairing-dependent changes in phenotype. Top2 complementing mutants displayed phenotypic changes at one of three genes, consistent with altered pairing dependent changes in expression. Further investigation using FISH demonstrated wild type levels of pairing at all loci examined.
### Table 3.1 Alleles used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alleles</th>
<th>Molecular basis</th>
<th>Phenotypic Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>brown</td>
<td>bw^+</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>bw^t</td>
<td>Insertion that disrupts transcription unit</td>
<td>White eyes</td>
<td>Phillips et al. (1973); Dreessen et al. (1988)</td>
</tr>
<tr>
<td>cinnabar</td>
<td>cn^+</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>cn^t</td>
<td>1.5 kb deletion</td>
<td>Dark orange eyes</td>
<td>Bridges and Brehme (1944); Warren et al. (1996)</td>
</tr>
<tr>
<td>dumpy</td>
<td>dp^+</td>
<td>NA</td>
<td>Wild type wings</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>dp^x</td>
<td>NA</td>
<td>Short and dumpy wings</td>
<td>Bridges and Mohr (1918)</td>
</tr>
<tr>
<td>modifier of (mdg4)</td>
<td>mod(mdg4)^*</td>
<td>NA</td>
<td>Wild type</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>mod(mdg4)^t</td>
<td>Stalker element insertion</td>
<td>Partial loss of enhancer blocking at y^2; Disruption of Mode7.2 isoform</td>
<td>Baxley et al. (2011)</td>
</tr>
<tr>
<td>suppressor of Hairy wing</td>
<td>su(Hw)^+</td>
<td>NA</td>
<td>Wild type</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>su(Hw)^*/^t</td>
<td>1 kb deletion, mutation in zinc finger 10</td>
<td>Loss of enhancer blocking at y^2</td>
<td>Baxley et al. (2011)</td>
</tr>
<tr>
<td>Top2</td>
<td>Top2^17/18</td>
<td>S791F</td>
<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Top2^17/39</td>
<td>L471Q</td>
<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Top2^17/39</td>
<td>R773H</td>
<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Top2^17/39</td>
<td>Y592N</td>
<td>Lethal</td>
<td>Hohl et al. (2012)</td>
</tr>
<tr>
<td>Ubx</td>
<td>Ubx^+</td>
<td>NA</td>
<td>Wild-type wings and halters</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Ubx^t</td>
<td>Insertion of Doc element into the first exon of Ubx, disrupting the protein coding region</td>
<td>Increased size of haltere</td>
<td>Lewis (1951), Frayne and Sato (1991), Bender et al. (1993), Ragab (2006)</td>
</tr>
<tr>
<td></td>
<td>Chx^t</td>
<td>17-kb insertion of upstream Ubx regulatory region into the second intron</td>
<td>Small wings, wing to haltere transformation</td>
<td>Lewis (1954), Bender et al. (1993), Casanova et al. (1985), Castelli-Gair et al. (1992), Lewis (1998)</td>
</tr>
<tr>
<td>white</td>
<td>w^+</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>w^*/^t</td>
<td>Duplication of w^+</td>
<td>Red eyes</td>
<td>Lindsley and Zimm (1992), Wu and Howe (1995)</td>
</tr>
<tr>
<td>yellow</td>
<td>y^+</td>
<td>NA</td>
<td>Dark wing, body, bristles</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>y^t</td>
<td>Gypsy insert at -700</td>
<td>Light wing and body, dark bristles</td>
<td>Geyer et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>y^*/^t</td>
<td>-716 to +66 deletion; 17 bp of P-element remains</td>
<td>Light wing and body, light bristles</td>
<td>Geyer et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>y^225</td>
<td>5060 to -950 deletion</td>
<td>Light wing and body, dark bristles</td>
<td>Morris et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>y^245</td>
<td>-1425 to +2162 deletion</td>
<td>Light wing and body, light bristles</td>
<td>Morris et al. (1998)</td>
</tr>
<tr>
<td>zeste</td>
<td>z^+</td>
<td>NA</td>
<td>Red eyes</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>z^*</td>
<td>K425M</td>
<td>Orange/yellow eyes in females (z^<em>/w^</em>); red eyes in males (z^*/w^+)</td>
<td>M. Gans (1946), Jack and Judd (1979), Bickel and Pirrotta (1990), Rosen et al. (1998)</td>
</tr>
</tbody>
</table>

*^w^t (w^soxanthopterless*)

NA: Not applicable.
Table 3.2 **Primer sequences of yellow, white, and Ubx genes used for qPCR Analysis**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Size of gene (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>white 5’ F</td>
<td>CTCCAAGCCGGTTTACGCCATCAAT</td>
<td>59</td>
</tr>
<tr>
<td>white 5’ R</td>
<td>TGGCTCCGCGAATTAATAGCTCT</td>
<td></td>
</tr>
<tr>
<td>white 3’ F</td>
<td>TTAGCTGCACATCGTCCGAAACACCA</td>
<td></td>
</tr>
<tr>
<td>white 3’ R</td>
<td>AGCTTCAGATTGAGAATGCTGCCAGA</td>
<td></td>
</tr>
<tr>
<td>yellow 5’ F</td>
<td>ATCACACAAGCGAAAGCTAGAGAA</td>
<td>4.7</td>
</tr>
<tr>
<td>yellow 5’ R</td>
<td>ATCCACCTTGTCTGGAACAGT</td>
<td></td>
</tr>
<tr>
<td>yellow 3’ F</td>
<td>TCGCCAAATACCCACTAATCGGT</td>
<td></td>
</tr>
<tr>
<td>yellow 3’ R</td>
<td>ATTTGGAATTGTGTCACGCGAGG</td>
<td></td>
</tr>
<tr>
<td>Ubx 5’ F</td>
<td>ATGAGTCCCTATGCCCAACCACAT</td>
<td>783</td>
</tr>
<tr>
<td>Ubx 5’ R</td>
<td>TTCACGCCGTCCTGTTGTAGCT</td>
<td></td>
</tr>
<tr>
<td>Ubx 3’ F</td>
<td>TTAGTGATCCAAACGACGGCTGCT</td>
<td></td>
</tr>
<tr>
<td>Ubx 3’ R</td>
<td>ACTGCGACACCGCATGCCGAACACTCA</td>
<td></td>
</tr>
<tr>
<td>zeste 5’ F</td>
<td>GTAAAACCACGGCGAAGGCCCTCA</td>
<td>28</td>
</tr>
<tr>
<td>zeste 5’ R</td>
<td>ATACACACACAGTGCGCGCTGGA</td>
<td></td>
</tr>
<tr>
<td>zeste 3’ F</td>
<td>ATGGAAGCCAATGCGTCTGCTA</td>
<td></td>
</tr>
<tr>
<td>zeste 3’ R</td>
<td>TGACTTGAATTCTGCCTGGCTC</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3 Viability of recombinant heteroallelic Top2 mutants

<table>
<thead>
<tr>
<th>Allele transmitted by male</th>
<th>17-1R</th>
<th>17-3R</th>
<th>35-13R</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHD</td>
<td>0(^a) (173(^b))</td>
<td>12 (432)</td>
<td>0 (170)</td>
</tr>
<tr>
<td>TOPRIM</td>
<td>56 (368)</td>
<td>0 (180)</td>
<td>107 (365)</td>
</tr>
<tr>
<td>WHD</td>
<td>0 (422)</td>
<td>106 (512)</td>
<td>0 (169)</td>
</tr>
</tbody>
</table>

\(^a\) % expected class is the # of non-CyO flies divided by half the # of CyO flies multiplied by 100

\(^b\) Total number of CyO flies scored

Black: 0-10% expected class, white: 11-100%


Winged helix domain (WHD)

Scored daily
Table 3.4 Transvection studies at \textit{Chx}^I \textit{Ubx}^I

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Nicked wings</th>
<th>% Wings with incomplete longitudinal veins</th>
<th>% Crumpled wings</th>
<th>Total # scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Top}^2 +; \textit{Ubx}^+ Female (CS)</td>
<td>0.0</td>
<td>0.8</td>
<td>0.0</td>
<td>131</td>
</tr>
<tr>
<td>\textit{Top}^2 ; \textit{Ubx}^+ Male (CS)</td>
<td>0.0</td>
<td>0.8</td>
<td>0.0</td>
<td>123</td>
</tr>
<tr>
<td>\textit{Top}^2 +; \textit{Chx}^1 \textit{Ubx}^+/+ + Female</td>
<td>55.3</td>
<td>63.1</td>
<td>0.0</td>
<td>141</td>
</tr>
<tr>
<td>\textit{Top}^2 ; \textit{Chx}^1 \textit{Ubx}^+/+ + Male</td>
<td>35.3</td>
<td>44.9</td>
<td>2.6</td>
<td>157</td>
</tr>
<tr>
<td>\textit{Top}^2 \textit{13R/17-3R}; \textit{Chx}^1 \textit{Ubx}^+/+ + Female</td>
<td>1.1</td>
<td>12.1</td>
<td>0.0</td>
<td>91</td>
</tr>
<tr>
<td>\textit{Top}^2 \textit{13R/17-3R}; \textit{Chx}^1 \textit{Ubx}^+/+ + Male</td>
<td>2.4</td>
<td>7.1</td>
<td>0.0</td>
<td>84</td>
</tr>
</tbody>
</table>
Figure 3.1 Recombination scheme. A). Recombination crosses were performed to remove dp, bw, and/or cn from the starter chromosome. One crossover can occur between dp and Top2 and a second between Top2 and cn. This double recombination event would yield a dp Top2 cn bw recombinant chromosome. B) Similar to part A, one crossover can occur between dp and Top2 and a second between cn and bw. This double recombination event would yield a dp Top2 cn bw recombinant chromosome. C) Males of the genotype y^1 w^67c23 / Y; dp Top2m cn bw / CyO, y^+ were crossed to wild type females. In the F1 generation, virgin females were collected that bore the multiply marked second chromosome over a wild type chromosome (dp Top2m cn bw / + + + +). These virgin females were subsequently crossed to males carrying the dominant marker, Scutoid (Sco), over the balancer chromosome CyO. Single males were selected carrying the recombinant chromosome (dp Top2m cn bw) / Sco and each male was subsequently crossed to: 1) Sco / CyO virgin females to maintain the stock, 2) Top2Df9043 / CyO, y^+ virgin females to ensure that the Top2 mutant allele was retained on the recombinant chromosome, and 3) dp cn bw virgin females to ensure the dp and bw alleles were recombined off the starter chromosome. Recombinants were isolated that failed to complement the Top2 deficiency and those that had wild type wings and red/orange eye pigmentation, revealing that dp and bw were recombined off the starter chromosome.
Figure 3.2 Effects of loss of Top2 on Mod67.2 stability. Western analyses of Top2 insertion alleles and EMS-generated point mutations using an antibody against the Mod67.2 isoform. Proteins were extracted from brains/imaginal disc/salivary glands of Top2<sup>+/Top2<sup>mod9043</sup></sup> third instar larvae. As a control, proteins were extracted from homozygous mod(mdg4)<sup>u1</sup> larvae that lack the Mod67.2 isoform. Representative blots are shown using antibodies against Mod67.2 and α-Tubulin. Top2 controls include Canton S (1), y<sup>2</sup> (2), Xα/CyO; MKRS (3), and su(Hw)<sup>Y<sup>of</sup></sup> (4), otefin<sup>β2<sup>79<sup>α</sup></sup></sup>/CyO, y<sup>+</sup> (5). Asterisk marks bands of unknown origin, closed arrowhead marks Mod67.2 isoform, open arrowhead marks lower molecular weight band observed in some genetic backgrounds.
Figure 3.3 *Top2 mutants do not disrupt insulator function at yellow*. The $y^2$ allele has wing (W) and body (B) enhancer activity blocked by the *gypsy* insulator (inverted black triangle). *Canton S (CS)* flies have dark wings, bodies, and bristles; $y^2$ flies have light wings and bodies with dark bristles; *mod(mdg4)* flies do not completely revert the $y^2$ phenotype, but instead alters pigmentation levels to produce lighter wings, bodies, and bristles than wild type; and *su(Hw)* flies have dark wings, bodies and bristles. Wing and body pigmentation was scored in heteroallelic *Top2* males ($y^2$; *Top2* and *Top2*). *Gypsy* insulator function is indicated by: Lost (dark wings, bodies, and bristles), partial loss, or active (light wings and bodies with dark bristles). Bristle enhancer (Br) and tarsal claw enhancer (T).
Figure 3.4 Effects of Top2 mutants on yellow, white, and Ubx expression. Expression of yellow and Ubx was determined using quantitative real time PCR in wild type, Top2^{17-3R}/Top2^{17-1R}, Top2^{235-13R}/Top2^{17-3R}, and Top2^{17-6}/Top2^{Df9043} L2 and L3 larvae, respectively. Expression of white was determined using quantitative real time PCR in wild type, Top2^{17-3R}/Top2^{17-1R}, and Top2^{235-13R}/Top2^{17-3R} adult male flies. All genes were normalized to RpL32. Error bars indicate standard deviation (n=2, n=3 for yellow). Asterisk indicates p<0.05, t-test. Significance between 5’ and 3’ primer sets was determined for each gene (Top2^{17-3R}/Top2^{17-1R} and Top2^{235-13R}/Top2^{17-3R} for Ubx and wild type for yellow were significant, p<0.05, t-test).
Figure 3.5 *Top2* mutants do not disrupt transvection at yellow. A) *Top2* mutants do not disrupt enhancer action in *trans* at yellow. Intra-allelic complementation occurs between \( y^{188} \), an allele that lacks the yellow promoter, and \( y^2 \), an allele that has wing (W) and body (B) enhancer activity blocked by the gypsy insulator (inverted black triangle), resulting in females that have dark wings, bodies, and bristles. Br (bristle enhancer), T (tarsal claw enhancer). Wing and body pigmentation is shown for \( y^{188}/y^2 \), \( Top2^{35-13R}/Top2^{17-3R} \) and \( Top2^{17-3R}/Top2^{17-1R} \) females. B) *Top2* mutants do not disrupt insulator bypass at yellow. Females with the genotype \( y^2/y^{3c3} \) have dark wings, bodies, and bristles because the blocked body enhancer of \( y^2 \) is able to bypass the chromatin insulator in *cis* when paired with \( y^{3c3} \). Wing and body pigmentation is shown for \( Top2^{35-13R}/Top2^{17-3R} \), \( Top2^{35-13R}/Top2^{17-3R} \), \( Top2^{17-5R/17-1R} \), and \( Top2^{17-3R}/Top2^{17-1R} \) females all in \( y^2/y^{3c3} \) background.
Figure 3.6 **A phenotypic effect at white is observed in Top2 mutants.** A) Zeste\(^1\) causes pairing-dependent gene silencing of the \(w^+\) gene in *trans* (left panel). When \(w^+\) genes are paired (females), the mutant protein encoded by \(Z^1\) silences both alleles, producing a yellow/orange eye phenotype. When there is one \(w^+\) gene present (males), pairing is not possible, so \(Z^1\)-mediated repression does not occur, resulting in a red eye phenotype. Eyes from wild type, \(Top2^{35-13R}\) or \(17^-3R/Top2^+\), and \(Top2^{35-13R}/Top2^{17-3R}\) males and females in a \(z^1w^+\) background. Zeste\(^1\) causes pairing-dependent gene silencing of the \(w^+\) gene in *cis* and *trans* (right panel). The \(w^a\) allele is a gene duplication of the \(w^+\) gene allowing zeste\(^1\) effects to be assessed in males. In females, pairing of the \(w^+\) genes can occur in *cis* and/or *trans*. In males, pairing can occur in *cis*. Eyes from wild type, \(Top2^{35-13R}\) or \(17^-3R/Top2^+\), and \(Top2^{35-13R}/Top2^{17-3R}\) males and females in a \(z^1w^a\) background. B) Representative images of FISH targeting *white*. Scale bars represent 10 microns. C) Average percentage of nuclei with indicated number of FISH signals per nucleus targeting *white*. \(N = \) total number of nuclei scored.
Figure 3.7 Effects of Top2 mutants on zeste expression. Expression of zeste was determined using quantitative real time PCR in wild type and Top2^{35-13R/17-3R} male and female whole flies in a z^{1}w^{+} and z^{1}w^{is} genetic background. The fold change in expression of wild type was set to 1. All genes were normalized to RpL32. Error bars indicate standard deviation (n=2). Dotted lines indicate a 0.5-fold change. Black lines indicate a 1-fold change.
Figure 3.8 **A phenotypic effect at Ultrabithorax is observed in Top2 mutants.**

A) When Cbx\(^1\) \(Ubx^1\) is placed in *trans* to a wild type copy of the *Ubx* gene, Cbx\(^1\) drives ectopic expression of the wild type *Ubx* gene, causing a homeotic transformation of the wing toward haltere. Wings from wild type, Cbx\(^1\) \(Ubx^1/+\) +, and Top2\(^{55-13R}/Top2^{17-3R}\), Cbx\(^1\) \(Ubx^1/+\) + males and females. Note nicks in wings and incomplete longitudinal cross veins, black and white arrowhead, respectively. B) Representative images of FISH targeting *Ubx*. Scale bars represent 10 microns. C) Average percentage of nuclei with indicated number of FISH signals per nucleus targeting *Ubx*. N = total number of nuclei scored.
Figure 3.9 **FISH analysis of the 359-bp repeat in Top2 mutants.** A) Average percentage of wing and eye disc nuclei with indicated number of FISH signals per nucleus targeting heterochromatic 359-bp repeat. $N$ = total number of nuclei scored.
CHAPTER 4
SUMMARY AND PERSPECTIVES

Top2 is required for chromosome pairing in Drosophila cell culture (Williams et al., 2007). Top2 was originally identified as a gene important for pairing through an RNAi screen using FISH as the readout (B. Williams, Wu lab, unpublished observations). Five classes of genes were examined, including: proteins with a demonstrated role in transvection, chromosome condensation proteins, proteins involved in pairing during meiosis, insulator proteins, and scaffold proteins. Only 1/66 genes showed significant alteration in pairing, that being Top2.

When I started my work, decreasing Top2 function using RNAi or chemical inhibitors demonstrated the function of the enzyme in vivo, as no extant alleles were available. To determine whether Top2 is required for homolog interactions in vivo, germline mutations were needed. In Chapter 2, I report data obtained from a forward genetic screen used to generate novel mutations in Top2. Fifteen recessive alleles were identified from a screen of 3,000 chromosomes. Molecular analyses of these alleles uncovered single or multiple base pair substitutions within the coding region of each mutant gene. These included changes that generated premature stop codons or missense mutations within previously identified functional domains. Among the missense alleles obtained, one displayed temperature sensitivity. Four missense mutations altered residues previously associated with drug resistance to cancer chemotherapeutics. Null mutants die during the larval to pupal transition, showing small or missing imaginal discs. Even though flies carrying each missense allele in trans to a deficiency are inviable, I found that heteroallelic combinations of several missense alleles produced viable flies, including two lines carrying mutations that display resistance to anti-cancer drugs. Complementation occurred between alleles encoding mutant proteins with amino acid substitutions in the same and different functional domains. These observations
suggest that Top2 activity can be restored by dimerization of defective subunits. Top2 mutant adults resulting from interallelic complementation were morphologically normal, but displayed delayed development and defects in oogenesis. These studies establish the first series of Top2 alleles in a multi-cellular organism and provide a platform for understanding drug resistance.

In Chapter 3, viable heteroallelic combinations of Top2 mutants were used to study homolog pairing in vivo. I determined whether mutations of Top2 affect pairing as assayed by the pairing-sensitive phenomenon of transvection. Transvection was assessed at three classically studied genes (yellow, white, and Ubx) that are expressed in different tissues and are located at different genomic positions. For each gene, homologous interactions between transvecting alleles were analyzed for alterations in pairing-dependent changes in phenotype. Top2 complementing mutants displayed phenotypic changes at one of three genes, consistent with altered pairing dependent changes in expression. Further investigation using FISH demonstrated wild type levels of pairing at all loci examined. In the sections below I discuss how my studies relate to the field of somatic pairing and entertain experiments that might be performed using my newly generated Top2 alleles.

The role of Top2 in homolog pairing

It is currently unclear why Top2 complementing mutants displayed a phenotypic change at only one of the three genes studied that is consistent with altered pairing dependent changes in expression and why loss of Top2 does not result in global unpairing (See Chapter 3 for detailed discussion of results). To date, many questions remain unresolved. It is unclear how stable chromosome interactions are over time, to what degree chromosomes must pair for a gene to recognize it as paired, and the extent that a gene must be expressed in order to see a physical change in phenotype. Studies have even revealed that FISH does not always correlate well with the phenotypic outcome
One way that we can start to address the answers to these questions is to better understand the process of somatic pairing as a whole. We currently know of only a few proteins that are involved in this process. Identifying key players involved in somatic pairing will help move the field forward and potentially reveal mechanisms that are involved in the establishment and maintenance of pairing.

Recently a genome wide high-throughput RNAi FISH screen assessed pairing of heterochromatic loci in cell culture (Joyce et al., 2012). This approach proved quite fruitful in identifying 40 ‘pairing promoting’ genes and 65 ‘anti-pairing’ genes (Joyce et al., 2012). This was the first demonstration that two classes of pairing genes exist. When pairing promoting genes are knocked down, pairing is disrupted; however, when anti-pairing genes are knocked down, pairing is enhanced. Studies revealed proteins involved in cell division promote and antagonize pairing, highlighting that pairing and the cell cycle may be linked (Joyce et al., 2012). A genome wide high-throughput RNAi FISH screen with probes targeted to euchromatic regions of the genome is currently underway (E. Joyce, personal communication). It will be interesting to compare genes involved in heterochromatic and euchromatic pairing to determine whether pairing of these genomic regions occurs by similar mechanisms.

Genome wide RNAi FISH studies can also be extended to mammalian cells, where intimate pairing is not the norm. It is possible that Drosophila and mammals may use overlapping mechanisms that maintain or abolish somatic pairing. The findings that proteins in Drosophila antagonize somatic pairing suggest that mammals may have similar mechanisms that keep the homologs separated in a normal state (Joyce et al., 2012). High throughput FISH analyses are needed in mammalian cell lines to determine whether mechanisms of somatic pairing are similar across species.
A role for Top2 in maintaining the structure of the X chromosome in males

One line of future investigation might include determining the role of Top2 in maintaining chromosome structure. Analysis of polytene chromosome squashes from male $Top2^{17-5}$/Top2$^{D9043}$ and $Top2^{35-14}$/Top2$^{D9043}$ larvae revealed that the appearance of the X chromosome was “puffy” and had a bloated appearance relative to the autosomes. As this phenotype was specific to the X chromosome in males, we wondered if the Male Specific Lethal (MSL) complex was recruited to the male X in a Top2 mutant background. Therefore, we turned our attention to the mechanism of dosage compensation. In Drosophila, males (XY) up-regulate expression of genes on the X chromosome two-fold to equal the gene dosage in females (XX). This process is regulated by the MSL complex, which is recruited to hundreds of specific sites along the male X chromosome (Gelbart and Kuroda, 2009). The MSL complex consists of: MSL1, MSL2, MSL3, males absent on the first (MOF), maleless (MLE), and two non-coding RNAs ($roX1$ and $roX2$). Acetylation of H4K16 by MOF leads to the upregulation of genes on the X (Park and Kuroda, 2001; Smith et al., 2001).

We found that Top2 is not required for proper recruitment of the dosage compensation machinery and that loss of Top2 does not affect H1 production, ruling out that these changes are responsible for altered polytene chromosome structure. We considered that loss of Top2 might alter H1 deposition through other mechanisms, a possibility that we were unable to test due to the inability of H1 antibodies to work in immunohistochemical analyses. It would be interesting to assess histone H1 deposition on polytenes in Top2 mutants using a GFP tagged histone H1 (H1-GFP) construct (J. Tamkun, personal communication).

A number of mutants in Drosophila have been identified that result in a puffy X chromosome in males. These include $Jil-1$, $ISWI$, $Nurf-301$, $SCF$, $Su(var)3-7$, $Su(var)2-5$, and $In(1)BM2$ (Badenhorst et al., 2002; Deng et al., 2005; Furuhashi et al., 2006;
Kulkarni-Shukla et al., 2008; Spierer et al., 2005). In all cases tested, the dosage compensation complex is required for the altered morphology of the male X chromosome, as mutations in components of the complex suppress the puffy X phenotype. Our understanding of the structure of the X chromosome was greatly enhanced by studies of ISWI (Corona et al., 2002; Corona et al., 2007; Corona and Tamkun, 2004). Although loss of ISWI resulted in decreased levels of histone H1 (Corona et al., 2007), Top2 mutants did not exhibit the same phenotype.

Why the male X chromosome is so sensitive to perturbation is not well understood. Loss of Top2 might alter the structure of the X chromosome via its role as a chromosome scaffold protein. Loss of Top2 could inhibit the formation of chromatin loops and higher order chromatin structures predicted to involve coalescence of SARs (Adachi et al., 1989; Mirkovitch et al., 1987). Alternatively, loss of Top2 may result in decondensation of the male X, due to misregulation of roX RNAs. In this regard, studies of roX RNAs in a Nurf301 mutant background revealed that a roX transgene targeted to the autosomes could induce decondensation of the chromatin flanking the transgene and that Nurf301 acts as a repressor of roX expression in females (Bai et al., 2007).

Preliminary studies of roX expression in Top2 mutants suggest this hypothesis is unlikely. As the sex of Top2 mutant female larvae is indistinguishable from males, single larvae were used in these assays. Expression analysis of Top2 mutants revealed that roX1 and roX2 levels in males and females are similar to that observed in wild type. One area of future investigation might involve performing microarray analyses on male and female Top2 mutants to determine if specific genes are upregulated or downregulated relative to wild type. A GFP tagged sex-lethal (Sxl) protein could be used to distinguish males from females since Sxl is repressed in males (M. Kuroda, personal communication). Microarray analyses may be a powerful tool in uncovering the mechanism of how Top2 is altering the structure of the male X chromosome.
A role for Top2 in the female germline

A second line of future investigation includes determining the role of Top2 in the female germline. All viable heteroallelic Top2 mutant combinations are female sterile, laying low numbers of eggs that do not hatch, with the majority being male fertile. This finding suggests that female germline development is sensitive to decreased Top2 activity. Low egg production prompted us to examine the ovary phenotype in Top2<sup>17-6</sup>/Top2<sup>35-1</sup> complementing females. While strings of developing egg chambers were observed, the ovaries were smaller, disorganized and contained evidence of egg chamber apoptosis. Occasionally, egg chambers with extra nurse cells were observed. We found that Top2<sup>17-6</sup>/Top2<sup>35-1</sup> ovaries had complex defects in the germaria, including the presence of germaria showing both GSCs loss and gain. Taken together, these analyses indicate that germ cell differentiation is compromised in Top2 complementing females, leading to germ cell loss and defects in egg chamber formation.

Clonal analysis is a powerful tool that is useful for studying stem cell function in Drosophila (Fox et al., 2008). Clones can be generated in the ovaries of Top2-deficient females to ensure that the phenotype associated with loss of Top2 is strictly a germline defect, and not somatic. Germline clones can be generated using the mosaic analysis with a repressible cell marker (MARCM) technique (Figure 4.1). As complete loss of Top2 is lethal, the MARCM technique will be used to generate clones lacking Top2 in an otherwise heterozygous, viable background.

Fly RNAi lines that target Top2 are also available through the Transgenic RNAi Project (TRIP) at Harvard Medical School. Currently two lines are available (JF01300 and GL00338) that are expressed in the soma and germline, respectively. These fly lines may be useful in understanding the role of Top2 in female germline development.

Homolog associations are needed during meiosis, where the maternal and paternal homologs pair and synapse prior to recombination. Recombination is initiated by double-strand breaks (DSBs) during prophase I of meiosis (Joyce and McKim, 2010; McKee,
Preliminary data suggests that heteroallelic Top2 mutants may have increased levels of DSBs in the female germline. This finding would indicate that Top2 might promote pairing and not prevent it. It would be interesting to characterize this phenotype and cross the Top2 mutants into a mei-P22 background (Spo11 homolog in flies) to see if mei-P22 suppresses the DSB phenotype. It is possible that Top2 plays a role in DNA damage or repair.

**A role for Top2 mutants in studying resistance or hypersensitivity to anti-cancer drugs**

The central role of Top2 in DNA metabolism has made this enzyme a target for anticancer drugs. Anti-cancer drugs targeting Top2 are currently being used to treat small cell lung cancer, testicular cancer, and lymphomas. Although widely used, many anti-cancer drugs that target Top2 cause secondary malignancies, such as acute myelogenous leukemia (Anderson and Berger, 1994; Azarova et al., 2007; Haffner et al., 2010). Much attention is focused on identifying novel anticancer drugs that would minimize secondary side effects to treatment.

A number of key residues in eukaryotic Top2 have been identified that confer resistance to anti-cancer drugs (Mao et al., 1999; Nitiss, 2009; Wu et al., 2011). Four missense mutations identified in our mutagenesis altered residues previously associated with drug resistance to cancer chemotherapeutics. Even though flies carrying each missense allele in trans to a deficiency are inviable, heteroallelic combinations of several missense alleles produced viable flies, including two lines carrying mutations that display resistance to anti-cancer drugs. Humans may carry polymorphisms that confer resistance, as exemplified in a human leukemic cell line (Danks et al., 1987). Much attention is focused on identifying novel drugs that would minimize secondary side effects to treatment. Our newly generated Top2 allelic series can be used to: 1) Identify mutations that are specific for different classes of Top2 poisons and inhibitors; and 2) Allow us to
study mutations that confer resistance or hypersensitivity to different Top2 targeting drugs. This idea is supported by studies using “Drosophila-based anticancer pharmacology” to identify drugs that might be of clinical interest in humans (Chelouah et al., 2011).
**Figure 4.1 Mosaic analysis with a repressible cell marker (MARCM) strategy.** The MARCM technique will be used to generate clones in the female germline that lack Top2. This strategy utilizes the well-established Gal4/UAS system and Gal4 repressor, Gal80. Heat shock induced FLP-mediated recombination between FRT sites during G2 of the cell cycle will result in two types of clones. Homozygous mutant clones will be GFP positive due to loss of the Gal80 repressor and activation of a UAS-GFP transgene by Tub-Gal4. GFP negative clones will go uncovered because genotypically they are wild type for Top2 and the presence of Gal80 causes repression of the UAS-GFP transgene. Adapted from (Fox et al., 2008).
Table A.1 **Progeny numbers obtained from complementation analyses with Top2<sup>35-4</sup>**

<table>
<thead>
<tr>
<th>Name</th>
<th>Maternally transmitted</th>
<th>Paternally transmitted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top2&lt;sup&gt;35-4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Expected Class&lt;sup&gt;a&lt;/sup&gt; (Total # CyO Scored)</td>
<td>% Expected Class (Total # CyO Scored)</td>
</tr>
<tr>
<td>17-1</td>
<td>71 (156)</td>
<td>21 (141)</td>
</tr>
<tr>
<td>17-2</td>
<td>9 (93)</td>
<td>63 (197)</td>
</tr>
<tr>
<td>17-3</td>
<td>10 (98)</td>
<td>63 (276)</td>
</tr>
<tr>
<td>17-5</td>
<td>65 (190)</td>
<td>121 (81)</td>
</tr>
<tr>
<td>17-6</td>
<td>50 (117)</td>
<td>53 (163)</td>
</tr>
<tr>
<td>17-7</td>
<td>11 (239)</td>
<td>31 (90)</td>
</tr>
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<td>78 (184)</td>
<td>69 (166)</td>
</tr>
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<td>36 (242)</td>
<td>40 (196)</td>
</tr>
<tr>
<td>35-3</td>
<td>1 (148)</td>
<td>0 (118)</td>
</tr>
<tr>
<td>35-5</td>
<td>76 (220)</td>
<td>75 (159)</td>
</tr>
<tr>
<td>35-6</td>
<td>112 (157)</td>
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<tr>
<td>35-12</td>
<td>100 (279)</td>
<td>150 (52)</td>
</tr>
<tr>
<td>35-13</td>
<td>79 (402)</td>
<td>69 (221)</td>
</tr>
<tr>
<td>35-14</td>
<td>4 (140)</td>
<td>14 (156)</td>
</tr>
</tbody>
</table>

<sup>a</sup>% expected class is the # of non-CyO flies divided by the # of CyO flies multiplied by 100.

<sup>b</sup>Top2<sup>35-4</sup> X Top2<sup>35-4</sup> yields 56 (182).
Table A.2 Polymorphisms in Top2α found in cancer

<table>
<thead>
<tr>
<th>Database</th>
<th>Human Top2α amino acid substitution</th>
<th>Conserved Drosophila amino acid substitution</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalogue of somatic mutations in cancer (COSMIC)</td>
<td>V255I</td>
<td>V236</td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td>G307G</td>
<td>G288</td>
<td>Ovary</td>
</tr>
<tr>
<td></td>
<td>Q355E</td>
<td>Q336</td>
<td>Ovary</td>
</tr>
<tr>
<td></td>
<td>G389G</td>
<td>G370</td>
<td>CNS</td>
</tr>
<tr>
<td></td>
<td>Q422Q</td>
<td>D403</td>
<td>Large intestine</td>
</tr>
<tr>
<td></td>
<td>V913I</td>
<td>I893</td>
<td>Ovary</td>
</tr>
<tr>
<td></td>
<td>E1109K</td>
<td>P1096</td>
<td>Digestive tract</td>
</tr>
<tr>
<td></td>
<td>L1166F</td>
<td>L1164</td>
<td>Ovary</td>
</tr>
<tr>
<td></td>
<td>D1473Y</td>
<td>NA</td>
<td>Ovary</td>
</tr>
<tr>
<td></td>
<td>S1483S</td>
<td>K1416</td>
<td>Digestive tract</td>
</tr>
</tbody>
</table>

NA: Not alignable (gap).
Figure A.1 **Loss of Top2 does not affect overall nuclear organization in whole-mount salivary glands or diploid eye tissue.** A) Shown are individual nuclei from Top2<sup>+/Df9043</sup> Top2<sup>+/Top2<sup>Df9043</sup></sup> third instar larval salivary glands immunostained with Fibrillarin (red, Top panel), Heterochromatin Protein 1 (HP1; green, Middle panel), Lamin Dm0 (red, Bottom panel), and DAPI (grayscale, Top panel). All images are shown as a single slice of a confocal Z-stack; scale bar represents 10 microns. B) Shown are eye discs from Top2<sup>+/Df9043</sup> Top2<sup>+/Top2<sup>Df9043</sup></sup> third instar larvae immunostained with Fibrillarin (red, left panel), HP1 (red, right panel), Lamin Dm0 (green, left and right panels), and DAPI (blue, left and right panels). Fields-of-view are representative to show nuclear organization. Images are shown as a single slice of a confocal Z-stack; scale bar represents 5 microns. Arrowheads denote apoptosis frequently observed in the mutant background.
Figure A.2 **Effects of Top2 mutants on gene expression.** Expression of short and long genes were determined using quantitative real time PCR in wild-type, Top2<sup>35-13R/35-17-3R</sup>, Top2<sup>17-3R/17-1R</sup>, and Top2<sup>17-6/17-Df9043</sup> third instar larvae. Genes are organized according to increasing size from left to right. All genes were normalized to *RpL32*. Error bars indicate standard deviation (n=2). Significance determined by paired t-test for wild type short genes vs mutant short genes (Top2<sup>35-13R/35-17-3R</sup> (P=0.0065*), Top2<sup>17-3R/17-1R</sup> (P=0.0391*), and Top2<sup>17-6/17-Df9043</sup> (P=0.0572)) and wild type long genes vs mutant long genes (Top2<sup>35-13R/35-17-3R</sup> (P=0.0972), Top2<sup>17-3R/17-1R</sup> (P=0.0083*). Asterisk (*) indicates significance.
Figure A.3 **Effects of heteroallelic Top2 mutants on expression of genes on the X and autosomes.** Expression of X-linked and autosomal genes were determined using quantitative real time PCR in wild type, \( \text{Top2}^{35-13R/17-3R} \) and \( \text{Top2}^{17-3R/17-1R} \) male and female adult flies. All genes were normalized to \( \text{RpL32} \). Error bars indicate standard deviation (n=2). Significance determined by paired t-test for autosomal genes in females (\( \text{Top2}^{35-13R/17-3R} (P=0.3594) \), \( \text{Top2}^{17-3R/17-1R} (P=0.0014^*) \)) and X-linked genes in females \( \text{Top2}^{35-13R/17-3R} (P=0.7239) \), \( \text{Top2}^{17-3R/17-1R} (P=0.0023^*) \)). Significance was also determined for autosomal genes in males (\( \text{Top2}^{35-13R/17-3R} (P=0.5765) \), \( \text{Top2}^{17-3R/17-1R} (P=0.0028^*) \)) and X-linked genes in males \( \text{Top2}^{35-13R/17-3R} (P=0.9094) \), \( \text{Top2}^{17-3R/17-1R} (P=0.0990^*) \)). Asterisk (*) indicates significance.
Figure A.4 Western Analyses of Top2 mutants. Proteins were extracted from heterozygous Top2<sup>m</sup>/Top2<sup>Df1043</sup> third instar larval brains/imaginal discs/salivary glands. One larval equivalent was loaded per lane. Blots are shown using antibodies against Top2 (T. Hsieh) and α-Tubulin as a loading control. Black, closed arrowhead indicates full-length Top2. Asterisk marks cross-reacting band consistently observed.
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