

Restoration of Topoisomerase 2 Function by Complementation of Defective Monomers in *Drosophila*

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ABSTRACT Type II topoisomerases are essential ATP-dependent homodimeric enzymes required for transcription, replication, and chromosome segregation. These proteins alter DNA topology by generating transient enzyme-linked double-strand breaks for passage of one DNA strand through another. The central role of type II topoisomerases in DNA metabolism has made these enzymes targets for anticancer drugs. Here, we describe a genetic screen that generated novel alleles of *Drosophila Topoisomerase 2 (Top2)*. Fifteen alleles were obtained, resulting from nonsense and missense mutations. Among these, 14 demonstrated recessive lethality, with one displaying temperature-sensitive lethality. Several newly generated missense alleles carry amino acid substitutions in conserved residues within the ATPase, Topoisomerase/Primase, and Winged helix domains, including four that encode proteins with alterations in residues associated with resistance to cancer chemotherapeutics. Animals lacking zygotic *Top2* function can survive to pupation and display reduced cell division and altered polytene chromosome structure. *Inter se* crosses between six strains carrying *Top2* missense alleles generated morphologically normal *trans*-heterozygous adults, which showed delayed development and were female sterile. Complementation occurred between alleles encoding Top2 proteins with amino acid substitutions in the same functional domain and between alleles encoding proteins with substitutions in different functional domains. Two complementing alleles encode proteins with amino acid substitutions associated with drug resistance. These observations suggest that dimerization of mutant Top2 monomers can restore enzymatic function. 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 type II topoisomerases to development.

TOLOGICAL 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 (Wang 2002; Nitiss 2009a). 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 (Schoeffler and Berger 2008; Collins *et al.* 2009; Nitiss 2009a). Each monomer is composed of distinct domains that cooperate to alter DNA topology (Figure 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, composed of two domains

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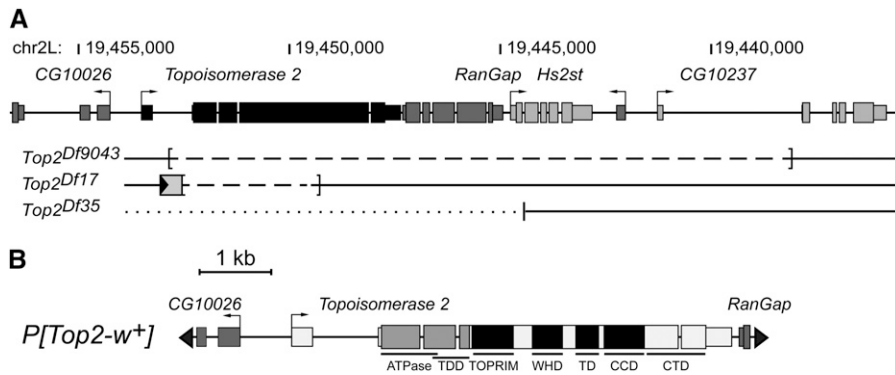


Figure 1 Structure of the *Top2* locus. (A) *Top2* is located on chromosome 2L between the uncharacterized upstream *CG10026* gene and the essential downstream *RanGap* gene. Shown are the structures of three *Top2* deficiency chromosomes used in these studies. *Top2^{Df9043}* is a 14.8-kb deletion allele (dashed line) that removes *Top2*, *RanGap*, *Hs2st* (the gene within the *RanGap* intron), and *CG10237*. *Top2^{Df17}* is an ~3.6-kb deletion allele (dashed line) that removes only *Top2* sequences and retains ~600 bp of the starting *P* element (*Top2^{EP}*). *Top2^{Df35}* is a deletion allele that removes *Top2* sequences, but has unknown limits (dotted line). Promoters are indicated by bent arrows and exons are represented by shaded rectangles. (B) *P[Top2-w⁺]* is a *P* transposon that carries *white⁺* (not shown), a 7.1-kb genomic fragment encompassing the entire *Top2* gene, and the 5' region of *CG10026* and the 3' largely untranslated region of *RanGap*. The coding region of the *Top2* gene is annotated to indicate locations of the ATPase domain, the **Transducer domain** (TDD), the **Topoisomerase/Primase** (TOPRIM) domain, the **Winged helix domain** (WHD), the **Tower domain** (TD), the **Coiled-coiled domain** (CCD), and the **carboxyl-terminal domain** (CTD).

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) contains the active-site tyrosine, which forms the covalent linkage with DNA. The **Tower domain** (TD) and **Coiled-coiled domain** (CCD) follow the catalytic core and together regulate the passage of one DNA strand. Finally, the **carboxyl-terminal domain** (CTD) is dispensable for catalytic activity *in vitro*, but regulates nuclear accumulation and interactions with partner proteins *in vivo* (Collins and Hsieh 2009). Among these domains, the CTD is the least conserved among eukaryotes, differing both in length and in sequence (Austin *et al.* 1993). Structural domains within dimeric type II topoisomerases are formed by contributions of both monomers (Liu and Wang 1999; Classen *et al.* 2003), which facilitates coupling of ATP hydrolysis to conformational changes involved in 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 the differentially expressed paralogs *Top2 α* and *Top2 β* , respectively (Austin and Marsh 1998). *Top2 α* is found in proliferating cells, while *Top2 β* is found ubiquitously, 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 (Wyckoff and Hsieh 1988; Jensen *et al.* 1996), 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 (Chang *et al.* 2003; Baxter and Diffley 2008; Coelho *et al.* 2008; Gonzalez *et al.* 2011). Some of these defects may result from altered chromosome architecture (Uemura *et al.* 1987; Buchenau *et al.* 1993; Chang *et al.* 2003; Coelho *et al.* 2008; Stanvitch and Moore 2008), as *Top2 α* is a major structural component of mitotic chromosomes (Earnshaw *et al.* 1985; Adachi *et al.* 1991; Maeshima and Laemmli 2003). A role in global chromosome architecture is further suggested by studies in *Drosophila*, where *Top2* may be involved in somatic homolog pairing (Williams *et al.* 2007) and insulator function (Ramos *et al.* 2011). The essential mitotic requirement for type II topoisomerases has made these enzymes important targets for chemotherapy against a number of cancers (Nitiss 2009b; Chikamori *et al.* 2010).

Our interest in *Drosophila* *Top2* began with an earlier analysis demonstrating that compromising the function of this protein perturbs homolog pairing in cell culture (Williams *et al.* 2007). Here, we describe a genetic screen to generate a series of ethyl methanesulfonate (EMS)-induced *Top2* alleles, as we reasoned that hypomorphic and null mutations would provide a useful resource that would complement the extant *Top2* *P*-element insertion alleles (Bloomington Stock Center). In total, we identified 15 new alleles. Among these, 14 demonstrated recessive lethality, with one of these mutations displaying temperature sensitivity. Lethal alleles resulted from nonsense and missense substitutions in the protein-coding region, representing the first non-deletion, non-insertional *Top2* alleles generated in *Drosophila*. Missense substitutions were in key functional domains of *Top2* and included changes in residues associated with resistance to chemotherapeutic drugs (Wu *et al.* 2011). *Inter se* crosses between lethal missense alleles uncovered interallelic complementation, wherein *trans*-heterozygous *Top2* mutant adults were generated. These adults were morphologically normal, although these flies showed delayed development and were female 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 the *in vivo* activity of Top2. In brief, we have generated a new resource for investigating Top2 function.

Materials and Methods

Drosophila stocks and culture conditions

Flies were maintained at 25° 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°, 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 1A). We refer to this deletion as *Top2^{Df9043}*. *Top2* mutant alleles were carried over the balancer chromosome *CyO-Df(2R)B80*, *y⁺* balancer (BL 4542), referred to as *CyO, y⁺*. The *Top2^{Df17}* and *Top2^{Df35}* deficiencies were generated by excision of an existing *P* element (*Top2^{EP}*). *Top2^{Df17}* contains a 3580-bp deletion within the *Top2* coding region, with 590 bp remaining of the *P* element (Figure 1A). The breakpoints of *Top2^{Df35}* have not been mapped.

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 12–14 hr and then fed 25 mM 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 hr of feeding, mutagenized males were transferred to bottles with standard food, allowed to recover for 10–12 hr, and mated for ~24 hr with *y²; Top2^{Df17} or Df35/CyO* females. Mated females were transferred to fresh bottles daily for 3–4 days. F₁ females of the genotype *y²/y^{1#8}; (dp cn bw)^{*}/Top2^{Df17} or Df35* were scored for wing and body pigmentation. F₁ males of the genotype *y²/Y; (dp cn bw)^{*}/CyO* were singly mated in vials to *y²/y²; Top2^{Df17} or Df35/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 *y²/Y; (dp cn bw)^{*}/CyO* males from such vials were backcrossed to *y²/y²; Top2^{Df17} or Df35/CyO* females to confirm the decreased viability of straight-winged flies and to generate stocks carrying the putative *Top2* mutations (*Top2^m*). Stocks of putative *Top2^m* mutations were established by subsequent balancing of the *Top2^m* chromosome with the *CyO, y⁺* chromosome, with 19 stocks established (Supporting Information, Table S2). Most crosses involved *Top2^m* alleles balanced with the *CyO, y⁺* chromosome; exceptional crosses involved *Top2^m* alleles balanced with the *CyO* chromosome. These putative mutations were named based on the tester deficiency chromosome used in their identification, with those using the *Top2^{Df17}* tester named *Top2^{17-x}* and those using the *Top2^{Df35}* tester named *Top2^{35-x}*. The original tester deficiency

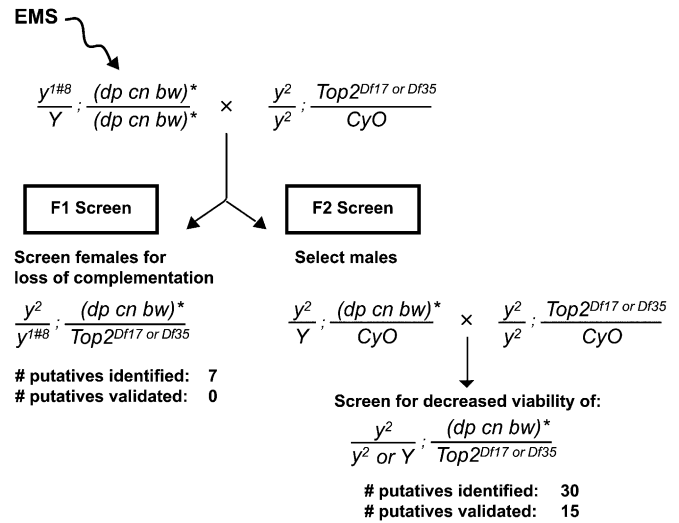


Figure 2 Strategy used in the EMS screen. Males of the genotype *y^{1#8}/Y; dp cn bw/dp cn bw* were fed EMS and mated to *y²/y²; Top2^{Df17} or 35/CyO* virgin females. In the F₁ screen, *y²/y^{1#8}; (dp cn bw)^{*}/Top2^{Df17} or 35* females were screened for altered complementation between *y* alleles. In the F₂ screen, F₁ males carrying a mutagenized second chromosome on *trans* to the *CyO* balancer were crossed to virgin *y²/y²; Top2^{Df17} or 35/CyO* females. Vials were screened for absent or reduced numbers of straight-winged (*Cy⁺*) flies. * indicates the mutagenized chromosome. Of 3000 chromosomes screened, 30 putative *Top2* alleles were identified, with 15 corresponding to *bona fide* alleles.

chromosome for *Top2³⁵⁻¹²* was unknown; this allele was arbitrarily assigned to the *Top2^{Df35}* group.

All putative mutations were rescreened by crossing *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 flies were considered to contain new *Top2* mutations. Next, we tested whether a wild-type *Top2* gene rescued the recessive lethality associated with the *Top2^m* alleles. To this end, we generated transgenic flies that carried an insertion of a genomic rescue transposon, *P[Top2-w⁺]*, on the third chromosome. *P[Top2-w⁺]* carries a *mini-white* gene and a 7.1-kb *HindIII* to *XbaI* genomic fragment that includes the wild-type *Top2* gene and part of the *CG10026* gene (Figure 1B). As a control, we demonstrated that *P[Top2-w⁺]* rescued the recessive lethality associated with *Top2^{Df17}/Top2^{Df35}* (Table S3), wherein *Top2^{Df17}/Top2^{Df35}*; *P[Top2-w⁺]/+* individuals were produced at 86% of the *Top2^{Df17} or Df35/CyO, y⁺* class.

Characterization of newly generated *Top2* mutant alleles

Several studies were conducted to characterize the new *Top2^m* alleles. First, we determined whether alleles displayed parent-of-origin effects by crossing *y^{1w67c23}; Top2^m/CyO, y⁺* males or females to *y^{1w67c23}; Top2^{Df9043}/CyO, y⁺* individuals of the opposite sex and scoring whether *Top2^m/Top2^{Df9043}* adults survived. Second, we assessed whether *Top2^m* alleles were temperature sensitive by crossing *y^{1w67c23}; Top2^m/*

CyO, y^+ females to y^1w^{67c23}/Y ; $Top2^{Df9043}/CyO$, y^+ males at room temperature for 2 days, after which time progeny were placed at 18°. Progeny emerging from these crosses were scored daily to determine whether growth at 18° generated $Top2^m/Top2^{Df9043}$ adults. Third, we assessed complementation between the $Top2^m$ alleles, performing *inter se* crosses between y^1w^{67c23} , $Top2^m/CyO$, y^+ or y^2 ; $Top2^m/CyO$ mutants at 25°, with initial experiments scoring progeny every 2–3 days (data not shown). Complementation was observed between several different pairs of alleles, as $Top2^{m1}/Top2^{m2}$ adults were generated, which we call *Top2*-complementing adults. Crosses producing *Top2*-complementing adults were retested, with progeny screened daily. 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 ($y^1w^{67c23}/y^1w^{67c23}$; $Top2^m/CyO$, y^+) were crossed to y^1w^{67c23}/Y ; $Top2^{Df9043}/CyO$, y^+ flies and the y^1w^{67c23} ; $Top2^m/Top2^{Df9043}$ second- and third-instar larvae were collected, using the absence of pigmentation in the mouth hooks and denticle belts to identify the correct genotype. For each cross, at least 100 larvae were selected and placed in vials, with no more than 25 larvae per vial, and allowed to develop at 25° under uncrowded conditions. The total numbers of individuals advancing to later stages were scored, with the percentage of survival at each stage determined by dividing the number of individuals obtained by the total number of larvae placed in each vial, multiplied by 100. $Top2^m$ alleles were considered hypomorphic if $Top2^m/Top2^{Df9043}$ larvae showed greater survival than did larvae from control crosses performed using confirmed null alleles. Fifth, the fertility of *Top2* complementing males and females was assessed. In these studies, 8–10 y^1w^{67c23} ; $Top2^{m1}/Top2^{m2}$ males or females were crossed to 10–15 y^1w^{67c23} ; Sco/CyO flies of the opposite sex. Crosses were transferred every 2–3 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 ($y^1\#8$; $dp\ cn\ bw$) were studied in parallel, as a control (data not shown).

Molecular characterization of *Top2* alleles

Genomic DNA was isolated from heterozygous y^1w^{67c23} ; $Top2^m/Top2^{Df9043}$ larvae and PCR amplified using primers covering nine overlapping regions encompassing the *Top2* protein-coding region. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA; no. 28104) and sequenced at the University of Iowa DNA Core Facility. Primers are listed in Table S1. Western analysis was used to determine the level of Top2 protein generated in each strain. For these studies, proteins were extracted from a collection of brain, imaginal disc, and salivary gland tissues dissected from 10 $Top2^m/Top2^{Df9043}$ larvae. Two primary antibodies were used to detect Top2: rabbit anti-Top2 (T. Hsieh) at 1:10,000 (Figure 3) and rabbit anti-Top2

(D. Arndt-Jovin) at 1:4000 (data not shown). HRP-conjugated goat anti-rabbit IgG secondaries [Bio-Rad, Hercules, CA; no. 172-1019] were used at 1:20,000.

Polytene chromosome and immunohistochemical analyses

Salivary gland polytene chromosomes were studied to define the impact of loss of Top2 on chromosome structure. Females of the genotype $y^1w^{67c23}/y^1w^{67c23}$; $Top2^{17-5}$ or $35-15/CyO$, y^+ were crossed to y^1w^{67c23}/Y ; $Top2^{Df9043}/CyO$, y^+ males at room temperature for 2 days and then placed at 18°, according to previously described methods (Johansen *et al.* 2009). Polytene chromosomes stained with antibodies against MSL1, MLE, and JIL-1 were prepared using the conventional squash protocol, while those stained with H4K16ac were prepared using the acid-free method. For each genotype, squashes were done on at least 10 salivary gland pairs, dissected from larvae obtained from at least two independent crosses. Over 100 nuclei were examined for each genotype.

The effects of Top2 loss on imaginal disc and ovary development were studied. Tissues were dissected into 1× phosphate-buffered saline (PBS) solution and fixed in 3% paraformaldehyde (Electron Microscopy Sciences; no. 15710) for 20 min. Each immunohistochemical analysis was completed at least twice, with each analysis including tissues obtained from at least 8–10 individuals of each genotype. After tissues were blocked in PBT + 5% normal goat serum (Vector Laboratories, Burlingame, CA; no. S-1000), they were incubated overnight with primary antibodies at 4°. Following washes, tissues were stained with secondary antibodies for 2–3 hr and with DAPI (1 µg/ml in PBT) for 5–10 min. Samples were mounted in Vectashield (Vector Laboratories; H1000), imaged with a Zeiss (Thornwood, NY) 710 confocal microscope, and processed using ImageJ.

Primary antibodies used in the immunohistochemical analyses include rabbit anti-Phospho-Histone H3(pS10) (Epitomics; no. 1173-1) at 1:500, rabbit anti-H4K16ac at 1:50 (Active Motif; no. 39929), chicken anti-JIL-1 at 1:100 (K. Johansen), mouse anti-Lamin Dm0 [Developmental Studies Hybridoma Bank (DSHB), University of Iowa; ADL84.12] at 1:500, rabbit anti-Lamin Dm0 (P. Fisher), rabbit anti-MSL1 at 1:100 (M. Kuroda), rabbit anti-MLE at 1:100 (M. Kuroda), rabbit anti-Vasa (Santa Cruz; no. sc-30210) at 1:500, and mouse anti-Spectrin (DSHB; no. 3A9) at 1:50. The secondary Alexa Fluor (AF) antibodies (Invitrogen; Molecular Probes, Eugene, OR) were used at a 1:500 dilution, including AF488 goat anti-rabbit (A11008), AF568 goat anti-rabbit (A11011), AF488 goat anti-mouse (A11001), AF568 goat anti-mouse (A11004), AF488 goat anti-chicken (A11039), and AF568 goat anti-chicken (A11041).

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). In the F₁ generation, we sought

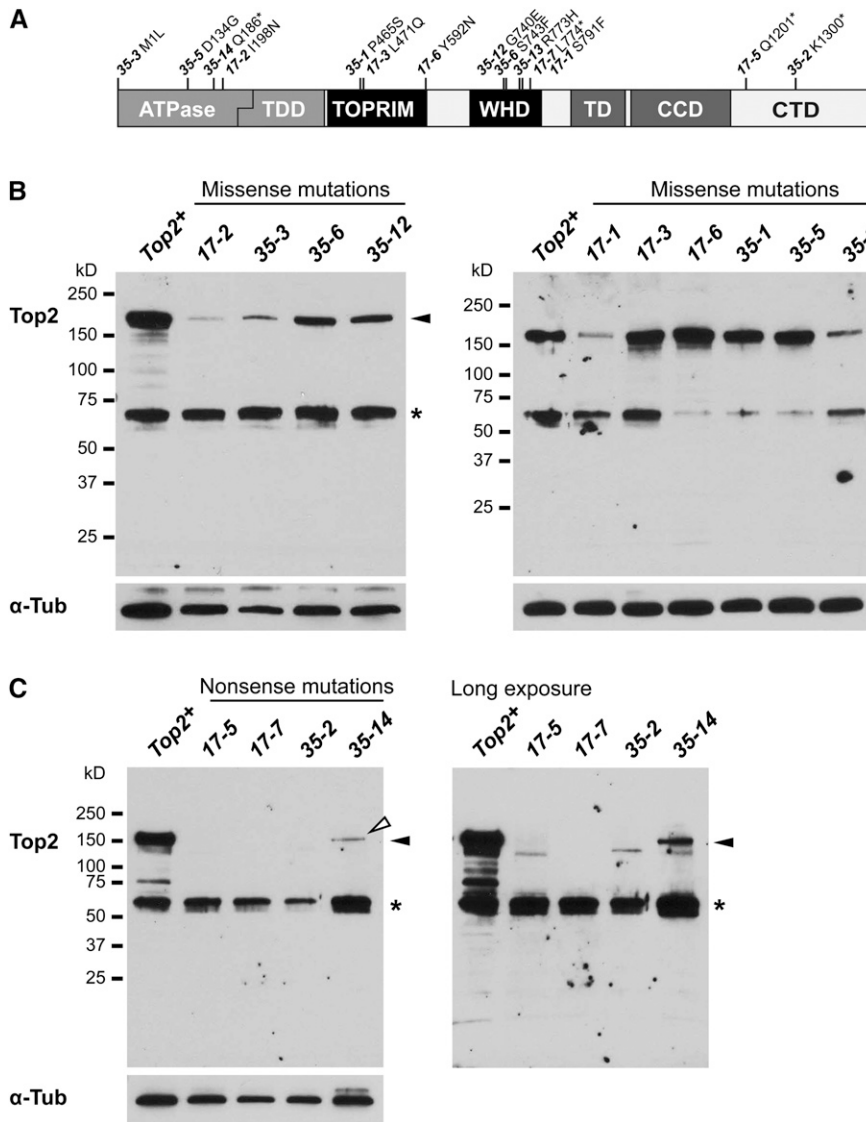


Figure 3 Analyses of protein production in *Top2* mutations. (A) The locations of the nonsense and missense mutations in the lethal *Top2* alleles are shown relative to the *Top2* protein domain structure. Abbreviations are described in Figure 1. (B and C) *Top2* protein levels were determined by Western analysis of proteins extracted from *Top2^m/Top2^{Df9043}* third-instar larvae carrying missense (B) and nonsense (C) alleles. Westerns were probed with antibodies generated in the Hsieh laboratory. Antibodies against α -tubulin served as a loading control. One larval equivalent was loaded per lane. Solid arrowheads indicate the position of the full-length *Top2*. The open arrowhead marks the position of a full-length protein obtained from *Top2³⁵⁻¹⁴/Top2^{Df9043}* larvae, even though the *Top2³⁵⁻¹⁴* nonsense allele is predicted to encode a protein of ~ 20 kDa. The absence of a full-length band in protein extracts obtained from lines carrying other nonsense alleles suggests that this band represents context-specific readthrough of the nonsense codon. Asterisks mark the position of a cross-reacting band that was routinely observed.

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*. For this mutagenesis, we used EMS, which 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*, *Top2^{Df17}* or *Top2^{Df35}*, 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 *y^{1#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). In a *Top2⁺* background, *y²/y^{1#8}*; (*dp cn bw*)*/*Top2^{Df17}* or *Df35* females were expected to show dark, nearly wild-type pigmentation because chromosome pairing permits the wing and body enhancers of *y^{1#8}* to act in trans on the promoter of *y²*. If a *Top2* mutation was recovered that affected pairing, then altered wing and body pigmentation would be expected. Importantly, females carrying *y²* have darkly pigmented bristles regardless of pairing status, which enabled us to distinguish any exceptional *y²/y^{1#8}*; (*dp cn bw*)*/*Top2^{Df17}* or *Df35* females with reduced pigmentation from *y^{1#8}/y^{1#8}*; (*dp cn bw*)*/*Top2^{Df17}* or *Df35* females arising from nondisjunction of the X chromosome.

From 2653 F₁ females, we identified 12 straight-winged females that had altered cuticle pigmentation. At least 4 of these females showed an unambiguous, uniform light 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 with the light cuticle pigmentation characteristic

of the $y^{1\#8}$ allele, which was presumed to have been inherited from their mother. The recovery of such males is consistent with the parental exceptional females being $y^2/y^{1\#8}$; $(dp\ cn\ bw)^*/Top2^{Df17\ or\ Df35}$, with such females carrying a mutant *Top2* allele that encoded a protein that was unable to promote chromosome pairing but provided adult viability. Alternatively, it may be that these exceptional females arose from X chromosome nondisjunction, wherein nondisjunction of y^2 in conjunction with loss of $y^{1\#8}$ generated homozygous y^2 tissues except in the germline, which remained heterozygous for $y^{1\#8}$ and y^2 . Unfortunately, all subsequent crosses with the progeny of these exceptional females failed to recapitulate the reduced pigmentation of the original females.

In the F₂ generation, we looked for recessive lethal or semilethal alleles of *Top2*. Here, F₁ y^2/Y ; $(dp\ cn\ bw)^*/CyO$ males, each representing a single mutagenized second chromosome, were singly crossed in vials to y^2/y^2 ; $Top2^{Df17\ or\ Df35}/CyO$ females, representing two 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 semilethal *Top2* allele. Of 3000 crosses, 30 putative *Top2^m* alleles were identified. From these putatives, we successfully established 19 y^1w^{67c23} ; *Top2^m/CyO*, y^+ stocks (Table S2), while 11 putatives either were sterile or produced unhealthy stocks that were subsequently lost. Each of the 19 putative alleles was tested in *trans* to *Top2^{Df9043}*, a *Top2* deficiency that was generated independently of *Top2^{Df17}* and *Top2^{Df35}*, and a total of 15 were confirmed (Table S2). Fourteen demonstrated complete lethality in *trans* to *Top2^{Df9043}*, while the remaining mutation, *Top2³⁵⁻⁴*, showed 25% of the expected class when transmitted maternally and 87% when transmitted paternally. To ensure that the lethality of the newly generated mutations was due to mutation of *Top2*, we tested whether the *P[Top2-w⁺]* transgene that carried a wild-type *Top2* gene rescued the viability of *Top2^m/Top2^{Df35}* flies. Of note, *Top2³⁵⁻⁴* was not tested in this study, due to the high viability observed upon paternal transmission. In all cases, viable *Top2^m/Top2^{Df35}*; *P[Top2-w⁺]/+* adults were obtained (Table S3), confirming that lethality was due to mutations in *Top2*. In total, 15 lethal or semilethal *Top2* alleles were generated.

Several parameters 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 are length and conservation of the protein (Cooper *et al.* 2010). Based on the number of recessive lethal mutations identified after screening ~3000 chromosomes, these investigators predicted that EMS generates one lethal mutation for every 73 evolutionarily conserved amino acids. Based on this information, we estimated that our screen should have generated ~13 new *Top2* alleles, as *Drosophila* *Top2* is a 167-kDa protein that displays ~67% similarity to

human *Top2*. Our recovery of 14 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 PCR amplified and sequenced. As a reference, we sequenced the coding region of the *Top2* gene 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 (+G3040A, E565E) and one produces a conservative amino acid substitution (+C5628T, A1401V) in a less conserved region of *Top2* (Crenshaw and Hsieh 1993). 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 were found to complement *Top2^{Df9043}* in secondary analyses (Table S2). We predicted that these alleles would be unlikely to harbor changes in *Top2*. As expected, the coding region in all four was identical to that in the parental strain (data not shown). Sequence analyses of the *Top2* coding region in the 15 lethal or semilethal *Top2* mutations revealed that 3 alleles carried nonsense mutations, 1 allele carried a missense and a nonsense mutation, 1 allele carried 3 missense mutations, and 10 alleles carried single missense mutations (Table 1). In all cases, missense alleles encoded *Top2* proteins with one amino acid substitution in a residue conserved with human *Top2 α* , with 6 alleles that showed alterations in residues found to be invariant among all sequenced eukaryotic type II topoisomerases. The missense mutations in our alleles were clustered in three functional domains, including the ATPase, WHD, and TOPRIM domains (Figure 3A). Based on structural information provided from studies of human *Top2 α* (Wei *et al.* 2005) and etoposide bound to *Top2 β* (Wu *et al.* 2011), we predict that *Top2³⁵⁻⁵* may alter the ATP-binding pocket; *Top2¹⁷⁻³* may alter DNA binding; and *Top2³⁵⁻¹*, *Top2³⁵⁻⁶*, and *Top2³⁵⁻¹²* might display altered binding to the *Top2* inhibitor etoposide.

We determined the levels of *Top2* protein produced in the 14 *Top2* strains that carry lethal alleles. 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 3B, Table 1, and data not shown). We found that *Top2* was at low levels or absent in 7 strains, including all 4 strains carrying nonsense mutations and 3 strains carrying missense mutations, while the remaining 7 strains carrying missense mutations displayed intermediate to high protein levels. In general, *Top2* proteins

Table 1 Properties of *Top2* alleles

Allele name	Amino acid change ^a	Affected domain ^b	Protein accumulation ^c	Interallelic complementation ^d
17-1	S791F	WHD	+/-	Yes
17-2	I198N	ATPase	+/-	No
17-3	L471Q	TOPRIM	+++	Yes
17-5	L318Q, Q1201 ^e	NA	+/-	No
17-6	Y592N	TOPRIM	+++	Yes
17-7	L774 ^e	NA	—	No
35-1	R197C, C347S, P465S	ATPase, TDD, TOPRIM	+++	Yes
35-2	K1300 ^e	NA	+/-	No
35-3	M1L	NA	+	No
35-4	T712I	WHD	ND	ND
35-5	D134G	ATPase	+++	Yes
35-6	S743F	WHD	++	No
35-12	G740E	WHD	++	No
35-13	R773H	WHD	++	Yes
35-14	Q186 ^e	NA	—	No

ND, not determined; NA, not applicable.

^a Boldface type: invariant between human *Top2* α and $-\beta$, mouse *Top2* α and $-\beta$, and yeast *Top2*.

^b TDD, **T**ransducer **d**omain; TOPRIM, **T**opoiso**merase/Primase** domain; WHD, **W**inged **h**elix **d**omain.

^c Protein accumulation as assayed by Western analysis (+++, protein levels equal to wild type; ++, intermediate levels; +, low levels; +/-, very low levels; —, no protein relative to wild-type).

^d Complementation with other *Top2* alleles.

^e Denotes nonsense mutation.

that carried changes in the TOPRIM domain accumulated high levels of protein, while those with amino acid substitutions in the ATPase domain or WHD produced varying levels of protein. *Top2*³⁵⁻³ produces low levels of protein even though the translation initiation ATG codon is mutated. This protein may arise from a low frequency of initiation at the nearby in-frame ATG codon located 12 codons downstream of the *bona fide* initiator, leading to a loss of the first few amino acids in *Top2*. A low level of full-length *Top2* was also found in protein extracts obtained from *Top2*³⁵⁻¹⁴/*Top2*^{Df⁹⁰⁴³} larvae, a surprising finding because *Top2*³⁵⁻¹⁴ carries a nonsense mutation at codon 186 and is predicted to generate a 20-kDa protein. The observed full-length protein might result from context-specific readthrough of the 186 nonsense codon, a possibility supported by the absence of this band in extracts obtained from other lines carrying nonsense mutations. Further, recent findings suggest that stop codon readthrough in *Drosophila* is more prevalent than previously realized (Jungreis *et al.* 2011). We were surprised by the low level of protein produced by the *Top2*³⁵⁻² allele that carries a nonsense mutation of codon 1300 because a similarly sized truncated *Drosophila* *Top2* protein was functional *in vitro* and complemented mutations of the yeast *Top2* gene (Crenshaw and Hsieh 1993). A possible explanation may lie in the observation that a nuclear localization signal is present in the last 60 amino acids (Crenshaw and Hsieh 1993), amino acids that are lost in the *Top2*³⁵⁻² protein. The absence of a nuclear localization signal in *Drosophila* may prevent *Top2*³⁵⁻² nuclear entry and lead to protein degradation. Taken together, our Western analyses show that some of the lethal *Top2* alleles accumulate high levels of protein. Previous studies in *Saccharomyces cerevisiae* have shown that lethality resulting from *Top2* depletion and lethality caused by catalytic

inactivation of *Top2* result from different mechanisms (Baxter and Diffley 2008). Here we demonstrate that our collection of *Top2* mutations includes those that produce no protein and those that produce near wild-type levels of a functionally compromised protein. Thus, our mutations have the capacity to elucidate 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 *y*^{1w67c23}/*Y*; *Top2*^m/*CyO*, *y*⁺ females to *y*^{1w67c23}; *Top2*^{Df⁹⁰⁴³}/*CyO*, *y*⁺ males, collected eggs for 2 days at room temperature, and then transferred progeny to 18° for the remainder of development. These studies identified one genotype, *Top2*¹⁷⁻²/*Top2*^{Df⁹⁰⁴³}, that produced adults at the lower temperature. Based on the number of *Top2*^m heterozygous siblings, we estimate that 29% of *Top2*¹⁷⁻²/*Top2*^{Df⁹⁰⁴³} flies survived to adulthood (Table S2 and Figure 4A). Although *Top2*¹⁷⁻²/*Top2*^{Df⁹⁰⁴³} females died shortly after emerging from their pupal cases, adult males survived for several days. To determine whether continued growth at 18° was essential for *Top2*¹⁷⁻²/*Top2*^{Df⁹⁰⁴³} survival, *y*^{1w67c23}/*Y*; *Top2*¹⁷⁻²/*CyO*, *y*⁺ females were crossed to *y*^{1w67c23}; *Top2*^{Df⁹⁰⁴³}/*CyO*, *y*⁺ males at 18° and progeny were shifted to 25° at different developmental times. We found that a shift to 25° at 1 day (embryonic shift) or 12 days (pupal shift) resulted in a loss of adults (Figure 4A), suggesting that *Top2* function is required both early and late in development, consistent with an essential function throughout development.

Considering that *Top2¹⁷⁻²* encodes a full-length protein with a substitution in the highly conserved motif in the ATPase domain (I198N; Table 1), it is possible that the temperature sensitivity of *Top2¹⁷⁻²* results from defects associated with an inability of ATP binding or hydrolysis, properties needed to impart conformational changes needed to alter DNA topology. Alternatively, improved survival at the lower temperature may result from changes in *Top2¹⁷⁻²* accumulation, resulting from altered translation or degradation. 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 outside of the ATPase domain and were clustered near the active site. These data suggest that missense mutations in multiple functional *Top2* domains can generate temperature sensitivity.

We were interested in defining whether our newly generated *Top2* alleles represent complete or partial loss of function. To this end, we assessed the viability of *Top2^m/Top2^{Df9043}* mutants, predicting that compared to complete loss of *Top2* function, partial loss of function would permit increased survival. We crossed *y^{1w67c23}/y^{1w67c23}; Top2^m/CyO, y⁺* females to *y^{1w67c23}/Y; Top2^{Df9043}/CyO, y⁺* males and followed development of *y^{1w67c23}; Top2^m/Top2^{Df9043}* larvae, identified by the absence of cuticle pigmentation. As a control, we tested two *trans*-heterozygous null genotypes (*Top2^{Df17}/Top2^{Df9043}* and *Top2^{Df35}/Top2^{Df9043}*), anticipating that these genotypes 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 <5% escaping into the third-instar larval stage (Ramos *et al.* 2011). Unexpectedly, we found that 49–66% of *Top2^{Df17}* or *Df35/Top2^{Df9043}* null larvae survived to pupation (Table 2), implying a lethal phase that is later than previously reported. Similar results were obtained for *Top2^m/Top2^{Df9043}* mutant larvae carrying nonsense alleles, wherein 38–66% of mutant larvae survived to pupal stages (Table 2). Finally, we studied *Top2^m/Top2^{Df9043}* larvae carrying *Top2* missense alleles. We found that seven *Top2^m/Top2^{Df9043}* genotypes demonstrated significantly enhanced survival relative to the known null genotypes, wherein 80–92% of mutant larvae became pupae ($P < 0.04$; Table 2). We note that one genotype, *Top2³⁵⁻³/Top2^{Df9043}* ($P < 0.0002$; Table 2) demonstrated significantly decreased survival relative to the null genotypes. Whether this difference reflects an unrelated influence of the genetic background or a special feature of *Top2³⁵⁻³* is unclear. For example, *Top2³⁵⁻³* may exert antimorphic effects, as it is predicted to carry a small amino-terminal deletion. Based on these data, we conclude that several newly identified missense mutations include hypomorphic *Top2* alleles.

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 pre-

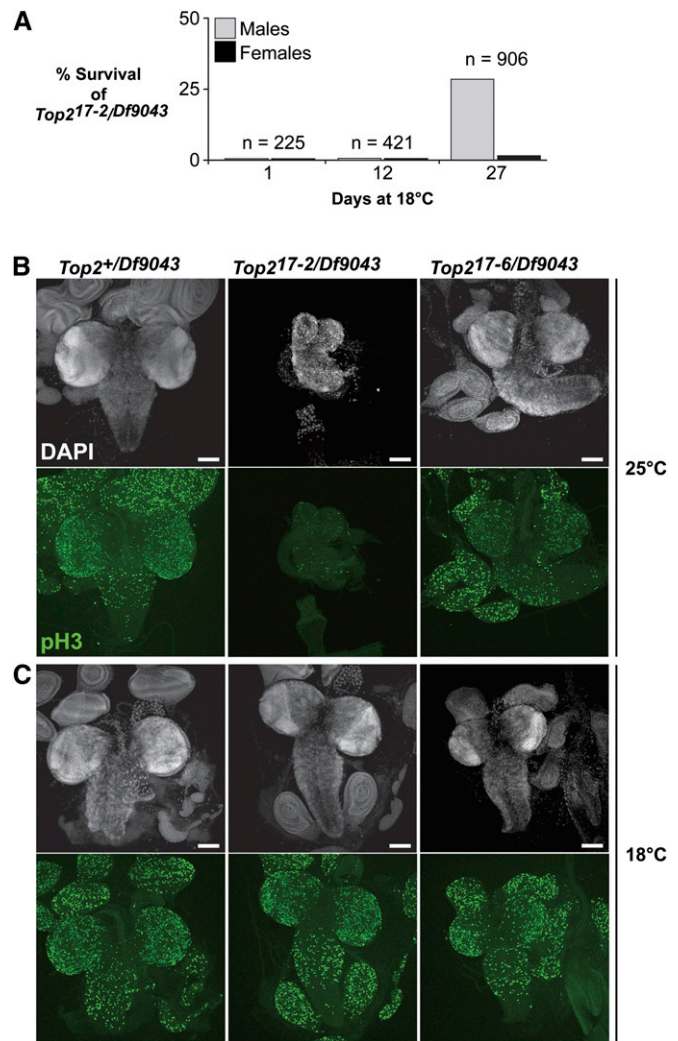


Figure 4 *Top2¹⁷⁻²* is a temperature-sensitive allele. (A) Temperature-shift experiments revealed that one *Top2* mutation, *Top2¹⁷⁻²*, was temperature sensitive. Once identified, the developmental requirement for *Top2* was determined by crossing *Top2¹⁷⁻²/CyO, y⁺* flies to *Top2^{Df9043}/CyO, y⁺* flies at 18° and shifting to 25° at 1, 12, or 27 days. The numbers of *Top2¹⁷⁻²/Top2^{Df9043}* adults were counted, with percentage of survival estimated from the number of *Top2¹⁷⁻²/CyO, y⁺* siblings. Only *Top2¹⁷⁻²/Top2^{Df9043}* individuals raised continuously at 18° reached adulthood, suggesting that *Top2* is required throughout development. The *Top2¹⁷⁻²/Top2^{Df9043}* females died shortly after eclosion. (B and C) *Top2* is required for development of third-instar larval brains and imaginal discs. Tissues were isolated from *Top2⁺/Top2^{Df9043}*, *Top2¹⁷⁻²/Top2^{Df9043}*, and *Top2¹⁷⁻⁶/Top2^{Df9043}* mutants, carrying hypomorphic missense alleles. Larvae were raised at a nonpermissive (25°) or permissive (18°) 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. Bar, 100 μm.

vious studies in *Drosophila* cell culture have demonstrated that chemical inhibitors of *Top2* decrease mitosis (Coelho *et al.* 2008). To test this postulate, we studied imaginal disc tissues isolated from multiple *Top2* mutant strains, which revealed that imaginal discs and brains were larger in strains carrying hypomorphic alleles than in strains carrying null

Table 2 Analysis of survival of *Top2^m/Top2^{Df9043}* larvae at 25°

Allele name ^a	Allele classification	% pupation ^b
<i>Top2⁺</i>	Wild-type	98 ^b (100) ^c
<i>Df 17</i>	Deletion	49 (106)
<i>Df 35</i>	Deletion	66 (103)
<i>17-1</i>	Missense	73 (100)
<i>17-2</i>	Missense	72 (100)
17-3	Missense	92 (120)
<i>17-5</i>	Missense, Nonsense	51 (142)
17-6	Missense	80 (120)
<i>17-7</i>	Nonsense	49 (135)
35-1	Missense	82 (100)
<i>35-2</i>	Nonsense	66 (110)
<i>35-3</i>	Missense	26 (141)
35-5	Missense	88 (100)
35-6	Missense	83 (100)
35-12	Missense	80 (100)
35-13	Missense	82 (136)
<i>35-14</i>	Nonsense	38 (104)

Boldface type indicates hypomorphic alleles, strains that showed significant viability ($P < 0.04$) relative to both *Top2^{Df17}* and *Top2^{Df35}*.

^a Maternally transmitted mutant *Top2^m*, paternally transmitted mutant *Top2^{Df9043}*.

^b Percentage of pupation was determined by dividing the total number of observed pupae by the total number of larvae placed in each vial, multiplied by 100.

^c Total number scored.

alleles. For example, *Top2¹⁷⁻⁶/Top2^{Df9043}* larvae that produce high levels of mutant Top2 protein contain near wild-type-sized brains and small discs (Figures 3B and 4B), although these tissues display an altered cellular organization (data not shown). In contrast, *Top2¹⁷⁻²/Top2^{Df9043}* larvae that produce very low levels of a mutant protein contain small brains and no imaginal discs when grown at the nonpermissive temperature, but larger brains and discs when grown at the permissive temperature (Figures 3B and 4B). To assess the level of mitosis in brains and imaginal discs obtained from *Top2¹⁷⁻⁶/Top2^{Df9043}* and *Top2¹⁷⁻²/Top2^{Df9043}* larvae, dissected tissues were stained with an antibody against phosphorylated serine 10 of histone H3, which is a highly selective marker for mitotic cells (Hendzel *et al.* 1997). These experiments revealed that the reduced size of brains and discs correlates with decreased staining of phosphorylated serine 10 of histone H3 (Figure 4, B and C), consistent with reduced levels of mitosis. Taken together, these data suggest that defects in imaginal disc growth may contribute to pupal lethality.

Loss of *Top2* alters polytene chromosome structure

We studied the polytene chromosomes isolated from larval salivary glands to examine how loss of Top2 affects chromosome structure. These chromosomes contain ~1000 aligned DNA strands, providing a powerful system for directly visualizing interphase chromosomes. Here we studied two null or nearly null *Top2* genotypes, *Top2¹⁷⁻⁵/Top2^{Df9043}* and *Top2³⁵⁻¹⁴/Top2^{Df9043}*. Polytene chromosomes isolated from these female and male *Top2* mutant larvae were fragile and displayed a diffuse DNA-banding pattern (Figure 5). This phenotype was particularly apparent for the male X chromosome, which displayed a “puffy,” decondensed structure,

reminiscent of the structure associated with mutations in genes encoding chromatin-modifying proteins (Figure 5, A–C and A'–C') (Deuring *et al.* 2000; Wang *et al.* 2001; Badenhorst *et al.* 2002; Spierer *et al.* 2005; Furuhashi *et al.* 2006; Bai *et al.* 2007; Grau *et al.* 2008). These observations suggest that loss of Top2 affects global chromosome structure, with the male X showing the greatest sensitivity within the genome.

The response of the male X chromosome to Top2 loss suggested a connection to the dosage compensation (DC) pathway. A possible connection to the DC pathway was further supported by the shared phenotype associated with hypomorphic mutations of *JIL-1*, which encodes a kinase component of the MSL complex that is responsible for interphase phosphorylation of histone H3 Ser10 in interphase cells (Deng *et al.* 2005). The dosage compensation pathway is required for increasing transcription from the single male X through recruitment of the Male Specific Lethal (MSL) complex, which leads to increased acetylation of lysine 16 of histone H4 (H4K16ac) (Gelbart and Kuroda 2009; Conrad and Akhtar 2011). To investigate whether the puffy X phenotype in *Top2* mutant males resulted from altered dosage compensation, we examined X chromosome localization of components of the MSL complex in *Top2¹⁷⁻⁵/Top2^{Df9043}* and *Top2³⁵⁻¹⁴/Top2^{Df9043}* male larvae. We found that loss of Top2 neither affects recruitment of MSL1, MLE, or JIL-1 nor alters enrichment of H4K16ac on the male X chromosome (Figure 5, D–F and G–I; data not shown).

Previous studies 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 are linked to defects in nucleosome assembly due to reduced 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 the previously recognized 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, salivary gland proteins were isolated from *Top2⁺/Top2^{Df9043}*, *Top2¹⁷⁻⁵/Top2^{Df9043}*, and *Top2³⁵⁻¹⁴/Top2^{Df9043}* larvae and assessed for H1 levels, using Western analyses. These studies demonstrated that H1 accumulates at near wild-type levels (data not shown), suggesting that loss of Top2 does not affect H1 production. It remains possible that loss of Top2 alters H1 deposition, a possibility that we were unable to test due to the inability of our H1 antibodies to work in immunohistochemical analyses.

Top2 mutations display interallelic complementation

Previous *in vitro* studies have demonstrated that Top2 subunits can undergo cooperative interactions to overcome defective ATP binding by one subunit (Lindsley and Wang 1993). Based on these findings, we predicted that missense

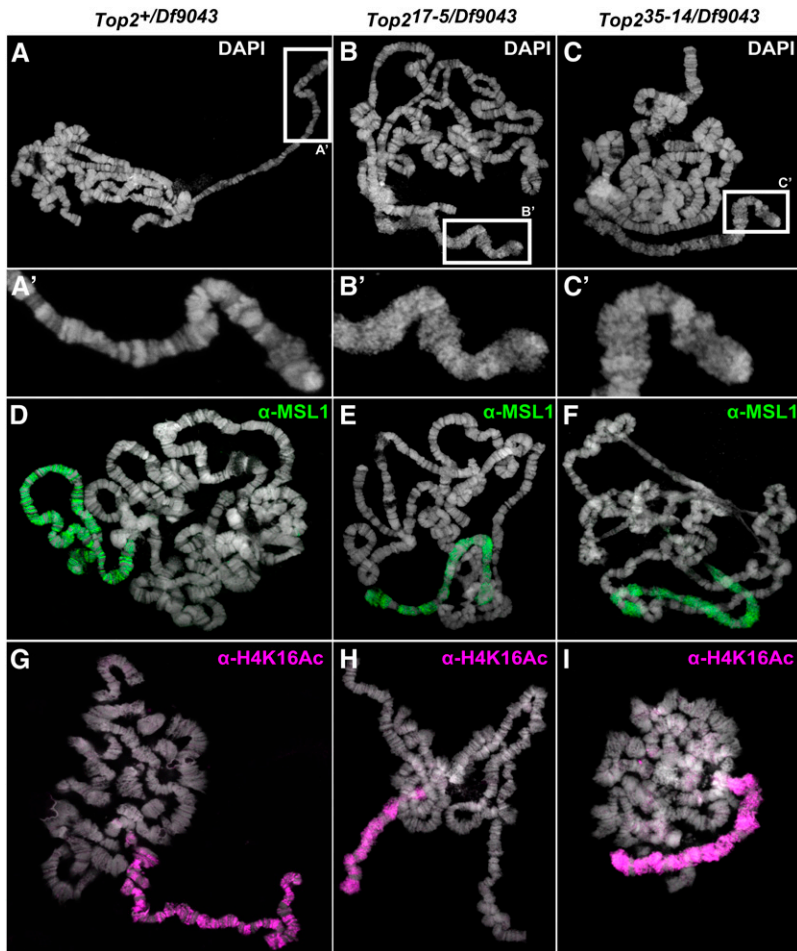


Figure 5 Loss of Top2 affects chromosome structure. Salivary gland polytene chromosomes were prepared from male third-instar larvae representing wild-type [*Top2*^{+/+}/*Top2*^{Df9043}] (A, D, and G) or mutant [*Top2*¹⁷⁻⁵/*Top2*^{Df9043}] (B, E, and H) and [*Top2*³⁵⁻¹⁴/*Top2*^{Df9043}] (*Top2* genotypes. Chromosomes were stained with DAPI (A–C), with enlargement of the male X chromosome shown to demonstrate the diffuse DNA-binding pattern (A'–C'). Recruitment of the MSL complex was assessed using antibodies specific to MSL-1 (D–F) and H4K16ac (G–I).

alleles encoding Top2 proteins with mutations in distinct functional domains might display interallelic complementation. To this end, we conducted pairwise complementation tests between all *Top2* alleles, assaying each cross for the production of viable adults. Initial crosses determined that 6 of the 14 lethal alleles supported interallelic complementation, generating *Top2*^{m1}/*Top2*^{m2} adults (data not shown). We refer to these progeny as *Top2*-complementing adults. To determine the extent of complementation, we retested the 6 alleles, conducting crosses in both directions and scoring progeny daily (Table 3). Twenty-six crosses produced adults, revealing 13 heteroallelic genotypes capable of interallelic complementation. Based on the number of *Top2*^m heterozygous siblings obtained in each complementation cross, we estimate that the viability of *Top2*^{m1}/*Top2*^{m2} adults ranged from ~13% to 98% (Table 3). Over half of all mutant genotypes displayed >50% viability of *Top2*^{m1}/*Top2*^{m2} adults, indicating that interallelic complementation is robust.

Several conclusions were made from our *inter se* crosses. First, none of the nonsense alleles demonstrated interallelic complementation, suggesting that complementation requires full-length proteins. Second, alleles capable of interallelic complementation produced intermediate to high levels of Top2 protein (Table 1 and Figure 3). Even so, an absolute correlation between protein levels and complementation

was not observed. For example, *Top2*¹⁷⁻¹ complemented several alleles, even though *Top2*¹⁷⁻¹ produces very low levels of protein, while *Top2*³⁵⁻⁶ failed to complement any allele, but produced intermediate levels of protein (Table 1 and Figure 3). Third, 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 3). Fourth, complementation was observed between alleles encoding proteins with defects in the same 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 *in vivo* and suggest that significant cooperation can occur between defective Top2 subunits to restore *in vivo* function.

***Top2*-complementing adults show limited developmental defects**

We studied progeny from *inter se* crosses between *Top2* nonsense alleles to understand the extent of restored Top2 function. In general, we found that *Top2*-complementing adults emerged at least 1 day later and died earlier than did their

Table 3 Interallelic complementation between *Top2* mutants y^1w^{67c23}/Y ; *Top2^m/CyO*, $y^+ \times y^1w^{67c23}/y^1w^{67c23}$; *Top2^m/CyO*, y^+

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

Boldface type, 11–100% viability. Vials were scored daily.

^a *Top2³⁵⁻¹* has three amino acid changes; however, only one change is in an invariant amino acid (P465S in TOPRIM domain).

^b Percentage of viability is the number of Cy⁺ flies divided by half the number of Cy⁻ flies multiplied by 100.

^c Total number of Cy⁻ flies scored.

wild-type siblings. Nonetheless, these adults were morphologically normal, including having a body size that was similar to that of *Top2⁺* flies. Unexpectedly, the F₁ male-to-female ratio in 5 of 26 crosses showed significant deviation from the expected 1:1 ratio, wherein a single sex represented $\geq 70\%$ of the offspring (Table S4). Higher numbers of male offspring were observed in all but one case (*Top2¹⁷⁻³/Top2¹⁷⁻⁶*). These findings are reminiscent of our observations that *Top2¹⁷⁻²/Top2^{Df9043}* males produced by growth at 18° are healthier than *Top2¹⁷⁻²/Top2^{Df9043}* females (Figure 4A). The reason for the different viability of *Top2*-complementing males and females is unclear.

We assessed the fertility associated with *Top2*-complementing adults. In initial studies, fertility was judged by an ability of *Top2*-complementing males or females to generate adult progeny when mated to y^1w^{67c23} ; *Sco/CyO* flies. This assay established that male fertility was largely not affected, with the one exception representing the heteroallelic genotype *Top2¹⁷⁻³/*

Top2³⁵⁻¹ (Table 4). In contrast, all *Top2*-complementing females were sterile. To investigate the cause of female sterility, nonvirgin *Top2*-complementing females were placed in vials, which were examined for egg production. We found that all *Top2^{m1}/Top2^{m2}* females produced low numbers of eggs. None of these eggs hatched, although their appearance was normal. As activation of a meiotic checkpoint is linked to defects in eggshell patterning (Morris and Lehmann 1999), these findings suggest that oogenesis in *Top2*-complementing females occurs without checkpoint activation. Eggs from *Top2³⁵⁻⁵/Top2³⁵⁻¹³*- and *Top2¹⁷⁻⁶/Top2³⁵⁻¹*-complementing females were collected and stained with DAPI. Imaging using confocal microscopy showed that the vast majority of eggs did not undergo nuclear divisions (90 of 95 and 30 of 31, respectively). While the lack of development may have resulted from a lack of fertilization, the observation that a few eggs displayed evidence of nuclear division suggests that fertilization could occur. We consider that compromised Top2 activity may lead

Table 4 Fertility of complementing *Top2* adults

Allele transmitted by male (M)	Allele transmitted by female (F)					
	17-1 WHD	17-3 TOPRIM	17-6 TOPRIM	35-1 TOPRIM	35-5 ATPase	35-13 WHD
17-1 WHD	NA	F NA	F NA	F sterile	F NA	NA
17-3 TOPRIM	F NA	NA	F sterile	F sterile	F sterile	F sterile
17-6 TOPRIM	M fertile	M fertile	NA	M fertile	M fertile	M fertile
35-1 TOPRIM	F sterile	F sterile	F sterile	NA	F sterile	F sterile
35-5 ATPase	M fertile	M sterile	M fertile	NA	M fertile	M fertile
35-13 WHD	F NA	F sterile	NA	F sterile	NA	F sterile
	M fertile	F fertile	F sterile	M fertile	M fertile	M fertile
	NA	F sterile	F sterile	F sterile	F sterile	NA
		M fertile	M fertile	M fertile	M fertile	

NA, not assessed. Males were considered fertile if any progeny eclosed; females were considered fertile if they produced eggs that hatched.

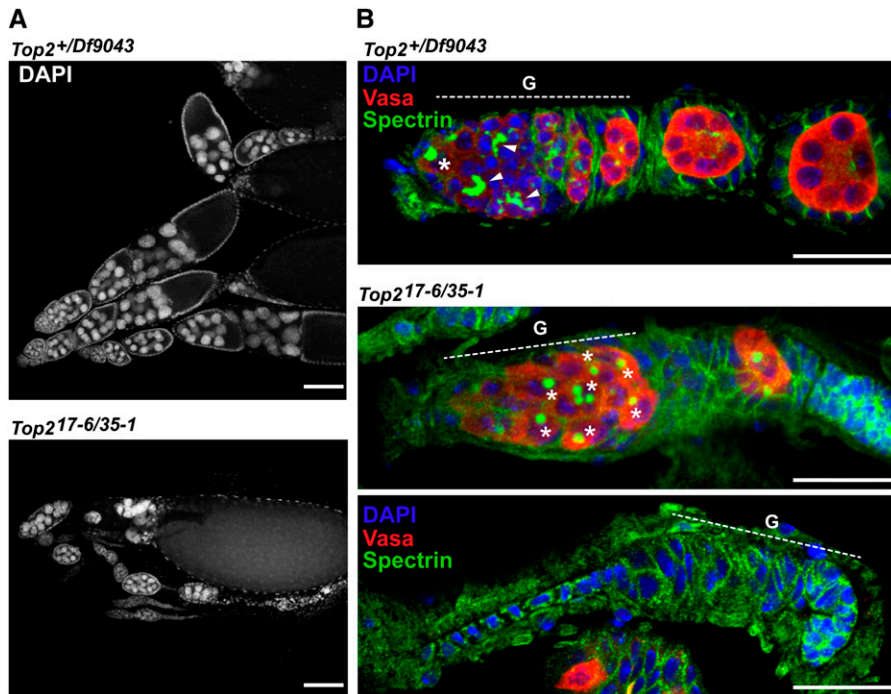


Figure 6 *Top2*-complementing females show complex ovary phenotypes. (A) Wild-type and heteroallelic *Top2*¹⁷⁻⁶/*Top2*³⁵⁻¹ mutant ovaries were dissected from 3-day-old females and stained with DAPI. The ovaries of *Top2*-complementing females were smaller than wild-type ovaries, but still retained strings of developing egg chambers. Bars, 25 μm. (B) Wild-type and heteroallelic *Top2*¹⁷⁻⁶/*Top2*³⁵⁻¹ 3-day-old ovaries were stained with DAPI and with antibodies against Vasa (red) to mark germ cells or with Spectrin (green) to mark spectrosomes (asterisks) present in germline stem cells and fusomes (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. Bars, 100 μm.

to defects in transcription during oogenesis, causing depletion of maternal products needed in embryogenesis. Alternatively, these eggs may carry defects in meiosis that inhibit embryogenesis.

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 and disorganized and contained evidence of egg chamber apoptosis (Figure 6A). 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 loss and gain of GSCs (Figure 6B). 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.

Concluding perspectives

Type II topoisomerases are molecular targets for chemotherapy against several types of cancers (Nitiss 2009b; Chikamori *et al.* 2010). However, treatment of cells with *Top2* chemotherapeutic agents can produce drug-resistant forms of the enzyme. For example, treatment of bacteria, yeast, and human cells with chemotherapeutic agents has caused *Top2*

mutations that alter drug binding, DNA binding, or 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 and TOPRIM domains and the WHD. 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*¹⁷⁻³, *Top2*³⁵⁻¹, *Top2*³⁵⁻⁶, and *Top2*³⁵⁻¹², mutations that have alterations in the TOPRIM domain and the WHD (Table 1) and correspond to residues L491, P485, S763, and G760 in human TopIIα, respectively. Among these mutations, *Top2*¹⁷⁻³ and *Top2*³⁵⁻¹ are hypomorphic alleles that support interallelic complementation, suggesting that certain heterodimers of drug-resistant proteins may similarly reconstitute enough *Top2* function for viability. Based on these data, it is possible that individuals carry *Top2* variants composed of defective monomers, which might confer different sensitivities to inhibitors and affect treatment outcomes.

Acknowledgments

We thank Donna Arndt-Jovin for the generous gift of *Top2* antibodies. We thank Chantal Allamargot, Kathy Walters, and other staff at the Central Microscopy Research Facility at the University of Iowa for their assistance. We thank members of the Geyer and Wu laboratories for helpful discussions and comments on the manuscript and Anna Moran for technical assistance. This work was supported by National Institutes of Health grants to P. Geyer (GM42539) and C.-ting Wu (GM085169) and by a National Science Foundation Graduate Research Fellowship to A. Hohl.

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GENETICS

Supporting Information

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Restoration of Topoisomerase 2 Function by Complementation of Defective Monomers in *Drosophila*

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Tao-Shih Hsieh, C.-ting Wu, and Pamela K. Geyer

Table S1 Primer sequences used for PCR amplification and genomic sequencing

Primer Name	Sequence (5' to 3')
Top2F + 1031 ^a	TAACTGGGTGGGTACTGTAGTCTTCTG
Top2R + 1703	AACGGTGAAGCTGGTGGAGAATATG
Top2F + 1545	TGACCATGCACAAGGAGCAGAAG
Top2R + 2356	ACCTGGAATGGCTTGATGTTGATGC
Top2F + 2145	TCCAACAGGTCTCGTTTGTCAACTC
Top2R + 2924	GCCATCCTGATCCTGATCTGTC
Top2F + 2773	TAATTTCAAGCAGCTTTCGGAGAATG
Top2R + 3483	AAGTGAACATCACCTTCCGCTGAC
Top2F + 3362	ATCACCTATGCAGACTTTATCAATCTGG
Top2R + 4061	TTCACTGATTTCTAAACGGTTTCCG
Top2F + 3959	TACAAGAACTTTTTAGGACGCATGG
Top2R + 4657	TTCTTAGCCTTTGAGCTGACACTGG
Top2F + 4534	ATATCGTCCCGATCCCGTCAAG
Top2R + 5222	ATCAAATTCGTCCACCTCATCG
Top2F + 5035	ACCCGTCGAGTTTAAGATCACCGAAG
Top2R + 5379	CTGAAACACTTACCTTGGCCTTGCTG
Top2F + 5157	AACCGAAGGGCAAGCAGATTAAGC
Top2R + 6249	CCTTTATTTCCCACTTCCGATTGTG

^a Primers are relative to the transcription start site (+1).

Table S2 Complementation tests to define *Top2* alleles y^1w^{67c23} ; *Top2^m/CyO*, $y^+ \times y^1w^{67c23}$; *Top2^{Df9043}/CyO*, y^+

Allele Name	18°C	25°C	25°C
	Maternally transmitted allele	Maternally transmitted allele	Paternally transmitted allele
	% viability ^a		
<i>Df17</i>	ND	0 ^a (604) ^b	0 (196)
<i>Df35</i>	ND	0 (424)	0 (695)
17-1	1 (710) ^c	0 (359)	0 (412)
17-2	29 (906) ^{c, d}	0 (193)	0 (170)
17-3	0 (737)	0 (263)	0 (189)
<i>17-4</i>	ND	137 (249)	110 (122)
17-5	0 (895)	0 (430)	0 (287)
17-6	0 (939)	0 (207)	0 (295)
17-7	0 (467)	0 (211)	0 (389)
35-1	0 (535)	0 (300)	0 (99)
35-2	0 (874)	0 (289)	0 (104)
35-3	0 (898)	0 (283)	0 (102)
<i>35-4</i>	ND	25 (438)	87 (453)
35-5	0 (803)	0 (423)	0 (815)
35-6	0 (631)	0 (120)	0 (160)
<i>35-7</i>	ND	127 (229)	159 (208)
<i>35-8</i>	ND	118 (320)	113 (238)
<i>35-9</i>	ND	205 (112)	124 (302)
35-12	0 (703)	0 (69)	0 (207)
35-13	0 (611)	0 (115)	0 (218)
35-14	0 (1065)	0 (226)	0 (162)

^a Percent viability is the # of Cy^+ flies divided by half the # of Cy^- flies multiplied by 100.

^b Total # of Cy^- flies scored.

^c *Top2^m/Top2^{Df9043}* flies become stuck in food.

^d *Top2¹⁷⁻²/Top2^{Df9043}* males are sterile; females immediately become stuck in food.

ND: Not determined.

Bold indicates the fourteen lethal alleles at 25°C.

Table S3 Numbers of progeny obtained from rescue crosses using the $P[Top2-w^+]$ transgene y^1w^{67c23} ; $Top2^{Df35}/CyO$; $P[Top2-w^+]/+ \times y^1w^{67c23}/Y$; $Top2^m/CyO, y^+$

Allele transmitted by female				
Allele transmitted by male	$Top2^m/Top2^{Df35}; +/+$	$Top2^m/Top2^{Df35}; P[Top2-w^+]/+$	$Top2^m \text{ or } Df35/CyO \text{ or } CyO, y^+; P[Top2-w^+]/+$	% Viability ^a
<i>Df17</i>	0	32	74	86 ^a (146) ^b
<i>Df35</i>	0	26	82	63 (161)
<i>17-1</i>	0	45	90	100 (185)
<i>17-2</i>	0	67	101	133 (213)
<i>17-3</i>	0	30	56	107 (127)
<i>17-5</i>	0	72	130	111 (256)
<i>17-6</i>	0	44	105	84 (205)
<i>17-7</i>	0	79	130	122 (269)
<i>35-1</i>	0	40	76	105 (157)
<i>35-2</i>	0	53	76	139 (129)
<i>35-3</i>	0	48	68	141 (154)
<i>35-5</i>	0	37	64	116 (131)
<i>35-6</i>	0	35	64	109 (155)
<i>35-12</i>	0	38	69	110 (124)
<i>35-13</i>	0	58	110	105 (210)
<i>35-14</i>	0	78	127	123 (280)

^a% viability is the # of Cy^+ , $P[Top2-w^+]$ flies divided by half the # of Cy^- , $P[Top2, w^+]/+$ flies multiplied by 100.

^bTotal # of Cy^- flies scored.

Table S4 Percentage of male and female offspring of viable heteroallelic *Top2* genotypes

Allele transmitted by male	Allele transmitted by female					
	17-1 WHD	17-3 TOPRIM	17-6 TOPRIM	35-1 TOPRIM	35-5 ATPase	35-13 WHD
17-1 WHD	NA	F: 9 M: 91 (23)^a	F: 42 M: 58 (24)	F: 47 M: 53 (139)	F: 50 M: 50 (105)	NA
17-3 TOPRIM	F: 12 M: 88 (101)	NA	F: 53 M: 47 (97)	F: 57 M: 43 (70)	F: 41 M: 59 (143)	F: 30 M: 70 (69)
17-6 TOPRIM	F: 60 M: 40 (30)	F: 76 M: 24 (63)	NA	F: 48 M: 52 (190)	NA	F: 27 M: 73 (79)
35-1 TOPRIM	F: 44 M: 56 (108)	F: 59 M: 41 (74)	F: 57 M: 43 (205)	NA	F: 51 M: 49 (92)	F: 43 M: 57 (143)
35-5 ATPase	F: 51 M: 49 (94)	F: 45 M: 55 (145)	NA	F: 45 M: 55 (154)	NA	F: 38 M: 62 (125)
35-13 WHD	NA	F: 38 M: 62 (93)	F: 47 M: 53 (73)	F: 46 M: 54 (228)	F: 57 M: 43 (115)	NA

^aTotal number of homozygotes scored.

NA: Not applicable.

Bold: statistically significant ($p < 0.05$).

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<i>17-5</i>	0	72	130	111 (256)
<i>17-6</i>	0	44	105	84 (205)
<i>17-7</i>	0	79	130	122 (269)
<i>35-1</i>	0	40	76	105 (157)
<i>35-2</i>	0	53	76	139 (129)
<i>35-3</i>	0	48	68	141 (154)
<i>35-5</i>	0	37	64	116 (131)
<i>35-6</i>	0	35	64	109 (155)
<i>35-12</i>	0	38	69	110 (124)
<i>35-13</i>	0	58	110	105 (210)
<i>35-14</i>	0	78	127	123 (280)

^a% viability is the # of Cy^+ , $P[Top2-w^+]$ flies divided by half the # of Cy^- , $P[Top2, w^+]/+$ flies multiplied by 100.

^bTotal # of Cy^- flies scored.

Table S4 Percentage of male and female offspring of viable heteroallelic *Top2* genotypes

Allele transmitted by male	Allele transmitted by female					
	17-1 WHD	17-3 TOPRIM	17-6 TOPRIM	35-1 TOPRIM	35-5 ATPase	35-13 WHD
17-1 WHD	NA	F: 9 M: 91 (23)^a	F: 42 M: 58 (24)	F: 47 M: 53 (139)	F: 50 M: 50 (105)	NA
17-3 TOPRIM	F: 12 M: 88 (101)	NA	F: 53 M: 47 (97)	F: 57 M: 43 (70)	F: 41 M: 59 (143)	F: 30 M: 70 (69)
17-6 TOPRIM	F: 60 M: 40 (30)	F: 76 M: 24 (63)	NA	F: 48 M: 52 (190)	NA	F: 27 M: 73 (79)
35-1 TOPRIM	F: 44 M: 56 (108)	F: 59 M: 41 (74)	F: 57 M: 43 (205)	NA	F: 51 M: 49 (92)	F: 43 M: 57 (143)
35-5 ATPase	F: 51 M: 49 (94)	F: 45 M: 55 (145)	NA	F: 45 M: 55 (154)	NA	F: 38 M: 62 (125)
35-13 WHD	NA	F: 38 M: 62 (93)	F: 47 M: 53 (73)	F: 46 M: 54 (228)	F: 57 M: 43 (115)	NA

^aTotal number of homozygotes scored.

NA: Not applicable.

Bold: statistically significant ($p < 0.05$).