Identification of Suppressors of top-2 Embryonic Lethality in Caenorhabditis elegans

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ABSTRACT Topoisomerase II is an enzyme with important roles in chromosome biology. This enzyme relieves supercoiling and DNA and RNA entanglements generated during mitosis. Recent studies have demonstrated that Topoisomerase II is also involved in the segregation of homologous chromosomes during the first meiotic division. However, the function and regulation of Topoisomerase II in meiosis has not been fully elucidated. Here, we conducted a genetic suppressor screen in Caenorhabditis elegans to identify putative genes that interact with topoisomerase II during meiosis. Using a temperature-sensitive allele of topoisomerase II, top-2(it7ts), we identified eleven suppressors of top-2-induced embryonic lethality. We used whole-genome sequencing and a combination of RNAi and CRISPR/Cas9 genome editing to identify and validate the responsible suppressor mutations. We found both recessive and dominant suppressing mutations that include one intragenic and 10 extragenic loci. The extragenic suppressors consist of a known Topoisomerase II-interacting protein and two novel interactors. We anticipate that further analysis of these suppressing mutations will provide new insights into the function of Topoisomerase II during meiosis.

KEYWORDS top-2 C. elegans meiosis topoisomerase II

Type II topoisomerases are highly conserved, ATP-dependent enzymes that alleviate DNA entanglements by passing one DNA strand through a transient break they create in a separate strand (Nitiss 2009). In this way, type II topoisomerases (Topo II) solve a variety of topological problems that arise during mitotic processes such as DNA replication, transcription, sister chromatid separation, and recombination (Nitiss 2009). In contrast to mitosis, meiosis has two nuclear divisions (Meiosis I and Meiosis II). In Meiosis I, homologous chromosomes segregate, which requires pairing, synapsis, and recombination. In Meiosis II, sister chromatids segregate from each other similar to mitosis. Studies in mammals, yeast, Drosophila, and our recent studies in C. elegans demonstrate that Topo II plays a role in homologous chromosome segregation at Meiosis I (Rose and Baillie 1980; Hartsuiker et al. 1998; Li et al. 2013; Hughes and Hawley 2014; Mengoli et al. 2014; Jaramillo-Lambert et al. 2016). We previously identified a temperature-sensitive (ts) allele of the single C. elegans Topo II homolog, top-2(it7ts). At the nonpermissive temperature (24°C), top-2(it7ts) disrupts the segregation of homologous chromosomes during the first meiotic division resulting in chromatin bridges during spermatogenesis. Chromatin bridging leads to a failure in the proper segregation of homologous chromosomes during Meiosis I resulting in anucleate sperm and embryonic lethality after fertilization (Jaramillo-Lambert et al. 2016). Interestingly, this allele does not display any chromosome segregation defects during oocyte meiosis. However, where Topo II functions within the genetic pathway of meiosis, as well as its meiosis interacting partners, have not been elucidated.

We sought to identify putative genes that interact with top-2 during meiosis through the application of a genetic suppressor screen. Genetic screens have the advantage of an unbiased approach in the identification of genes essential for a given pathway (Hodgkin 2005). In addition, these types of screens often lead to the identification of genes that were not previously believed to function in the pathway of interest.
Compromised or reduction-of-function alleles rather than null alleles are ideal for suppressor screens because they increase the likelihood that a second-site, extragenic mutation will ameliorate the phenotype of a given process (Jorgensen and Mango 2002). For these reasons we used the 	extit{C. elegans} temperature-sensitive mutant strain, top-2(it7ts), for our screen. With this approach, we report the identification and characterization of 11 suppressors of top-2(it7ts) embryonic lethality. The suppressors include both dominant and recessive alleles and are found both in intragenic and extragenic loci. We used one-step whole-genome sequencing (WGS) and single-nucleotide polymorphism (SNP) mapping to identify the responsible mutations for all 11 of the suppressor lines. This approach identified genes/proteins that have been previously shown to interact with Topo II in other systems as well as two novel interactors.

**MATERIALS AND METHODS**

**Nematode strains**

	extit{C. elegans} strains (Table S1) were maintained using standard conditions (Brenner 1973).

**EMS suppressor screen**

unc-4(e120) top-2(it7ts) L4 hermaphrodites were soaked in 48 mM EMS for 4 h at 15°, washed three times in M9, placed on a fresh 100 mm MYOB plate with OP50 and allowed to recover for 4 h at 15°. 25 L4s (P0s) were picked to each of 40 100 mm MYOB plates with OP50 and incubated at 15°. ~60 h later the P0s were removed from the plates. Gravid F1 adult progeny (84,720 haploid F1 genomes) were subjected to hypochlorite treatment to isolate F2 embryos. The F2 progeny were grown at 24° and their progeny were screened for viable animals. Eleven stable lines from independent P0s were identified.

**Whole-genome sequencing and SNP mapping**

The molecular identification of the top-2(it7ts) suppressors were determined by Hawaiian single-nucleotide polymorphism (SNP) mapping and whole-genome sequencing (WGS) (Doitsidou et al. 2010). All suppressors were crossed with Hawaiian (CB4856) males and screened for F2 suppressors as in (Jaramillo-Lambert et al. 2015). For suppressors ude2, ude3, ude4, ude5, ude6, ude7, and ude24 libraries were prepared and sequenced as in (Jaramillo-Lambert et al. 2015). Libraries and sequencing for suppressors ude13, ude14, ude15, and ude16 were prepared and sequenced by the University of Delaware DNA Sequencing and Genotyping Center. Suppressing mutations were identified by a pipeline of BMBP (Bushnell), SAMtools (Li et al. 2009), FreeBayes (Garrison and Marth), and ANNOVAR (Wang et al. 2010); see (Smith and Yun 2017) for detailed command-line instructions.

**Embryonic viability assays**

Individual L4 hermaphrodite larvae were placed on a single 35 mm MYOB plate spotted with OP50 or 	extit{E. coli} HT115[DE3] containing plasmids for RNAi knockdown. Each hermaphrodite was allowed to lay embryos at 24° for 24 h. Every 24 h period, adult worms were transferred to fresh plates until no additional embryos were produced. Percent hatching was calculated as the number of hatched larvae divided by the total number of embryos laid.

**Genetic analyses**

**Dominance/recessive tests:** To determine whether each top-2(it7ts) suppressor was either a dominant or recessive mutation, each suppressor line [unc-4(e120) top-2(it7ts); sup] was crossed with top-2(it7ts); him-8(e1489) males at 15°. Non-unc L4 cross progeny [unc-4(e120) top-2(it7ts);unc-4(+); top-2(it7ts); sup+] were picked to individual plates for embryonic viability assays at 24°. Nonviable progeny (<10% viable) indicated that the suppressing mutation was recessive and viable progeny indicated that the suppressing mutation was dominant (>50% viable) or semi-dominant (10–49% viable).

**Linkage analysis:** To distinguish between linked and unlinked suppressor mutations, F1 progeny [unc-4(e120) top-2(it7ts);unc-4(+) top-2(it7ts); sup/+ ] from the above cross were picked to new plates and allowed to produce self-progeny at 15°. 16-48 Unc F2 progeny [either unc-4(e120) top-2(it7ts)/ unc-4(e120) top-2(it7ts); +/+, unc-4(e120) top-2(it7ts)/ unc-4(e120) top-2(it7ts); sup/+ , or unc-4(e120) top-2(it7ts)/ unc-4(e120) top-2(it7ts); sup/sup] were picked to individual plates for embryonic viability assays. Dominant suppressors were determined to be unlinked from top-2 if 3/4 of F2 animals had viable progeny. Recessive suppressors were determined to be unlinked from top-2 if 1/4 of F2 animals had viable progeny. These results were confirmed by WGS/SNP analysis.

**RNAi**

RNA was introduced into worms using the feeding method of (Timmons et al. 2001). RNAi clones were obtained from the Ahringer library (smd-1, nrf-1) or the OpenBiosystems library (vib-1, ncp-1, tdpt-1). The nlp-38 clone was made by amplifying a 540 bp DNA fragment using primers CCGTGTTGGAGATGAAAAG and CTTCCTCCAAA-GACCTTGCC. The top-3 clone was made by amplifying a 481 bp DNA fragment using primers CAAGTGATCAGCTATGGC and GACCTTATTAGGAAATCTCCC. These fragments were inserted into pDEST-L4400 using Gateway technology (Invitrogen) and transformed into 	extit{E. coli} HT115[DE3].

**CRISPR/Cas9-mediated genome editing**

CRISPR-mediated genome editing via the clone-free method (Paix et al. 2015) with dpy-10 as a co-CRISPR marker (Arribere et al. 2014) was performed to recreate the ude5 and ude6 candidate mutations in KK381 [unc-4(e120) top-2(it7ts)]. All injections were done using an injection mix of Cas9 protein (25 μg), dpy-10 crRNA (3.2 μg, Dharmacon, GE Life Sciences, Pittsburgh, PA), dpy-10(Δnt64) repair oligonucleotide (0.2 μg), universal tracrRNA (20 μg, Dharmacon), an allele-specific crRNA (8 μg) and an allele-specific repair oligonucleotide (3.8 μg). To recreate the top-2(ude6) allele, which changes Asp809 to Asn, a crRNA targeting the sequence AATCTGGCTCAGATTACGTG and a repair template (GGAGAACAGTGCTTGATTAGGAAATTTGTG- AATCTCTAGCAGAATTTGTCGCTCCACAACACATCAGCTG- TCCTTTATCAGGAC) with sequence to edit codon 809 (bold), mutations to prevent additional cleavage by Cas9 (italics), and an AluI recognition site (underlined) for genotyping were used. tdpt-1(ude5), which changes Gly270 to Asp, was recreated using a crRNA targeting the sequence CCGGGCCGGCTGTGCTT and a repair template (GTTCGGTTGAAATCAGCAGCAGACACCGG- GCCCCTGGTCTTTTTTGGCAGATTTAAACTTTAGAGA- CGAGAGGTCCAGCGCTGGCTCGAGG) with sequence to edit codon 270 (bold), mutations to prevent additional cleavage by Cas9 (italics), and a DraI recognition site (underlined) for genotyping.

**DAPI staining**

Hermaphrodites were synchronized by picking L4s and incubating at either 15° or 24° for 16-24 h. Gonads were dissected on a coverslip in
30 μL 1X Egg buffer (118 mM NaCl, 48 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM Heps, pH 7.4) plus 0.1% Tween 20. Following dissection and extrusion of the gonads, 15 μL of the Egg buffer was removed and discarded. 15 μL of 2% paraformaldehyde solution in 1X Egg buffer plus 0.1% Tween 20 (16% paraformaldehyde, Electron Microscopy Sciences, Hatfield, PA) was then added to the slide. This was followed by removal of 15 μL of the paraformaldehyde/Egg/Tween solution and a Superfrost Plus microscope slide (Fisher Scientific) was placed on top. The samples were allowed to fix for 5 min and then frozen on dry ice. The slides were then placed in -20°C methanol for 1 min and washed three times in PBST (1X PBS, 0.1% Tween 20) for five min each. Slides were incubated in the dark with DAPI (2 μg/ml) for 5 min followed by a final wash in PBST for 5 min. Slides were mounted with Vectashield (Vector Laboratories) and the edges sealed with clear nail polish.

**Image collection and processing**

Z-stack images of post-meiotic sperm were obtained using a Zeiss LSM780 confocal microscope (Carl Zeiss, Inc., Gottingen, Germany). For each sample, the appropriate range of focal planes for z-stack projections was chosen with a constant slicing of 0.2 μm. Image processing and analysis was done via Fiji Is Just ImageJ [Fiji, (Schindelin et al. 2012)]. Although images were obtained using identical imaging parameters, brightness and contrast were adjusted to allow better visualization.

**Antibody production**

*C. elegans*-specific anti-TOP-2 antibodies were produced by injecting rabbits with the synthetic peptide (CQRDPMNTIKTINKEKNE) (YenZym Antibodies, LLC, San Francisco, CA). Polyclonal antibodies were purified by antigen-specific affinity purification (YenZym Antibodies, LLC, San Francisco, CA). Specificity of the antibody was validated by Western blot and reduction of protein in the top-2(ok1930) deletion line and worms depleted of TOP-2 through RNAi (Figure S2).

**Preparation of whole-worm protein lysates and Western blotting**

Seven to ten L4 worms from each strain were picked to six 60 mm plates and grown until a majority of their progeny had reached L4 stage. Worms were then shifted to 24°C and grown until viability above 10% with more variability in progeny survival among the suppressors, which each suppressor line rescued the embryonic lethality phenotype of the mutants (see Materials and Methods). Ten of the suppressors were unlinked to the deletion line and worms depleted of TOP-2 through RNAi (Figure S2).

**RESULTS AND DISCUSSION**

Isolation and mapping of suppressors of top-2(it7ts) embryonic lethality

Previously, we identified top-2(it7ts), a temperature-sensitive, missense mutation in a conserved residue within the catalytic domain of TOP-2 (Jaramillo-Lambert et al. 2016). At 15°C, top-2(it7ts) animals produce viable progeny (94.8% hatching). In contrast, top-2(it7ts) hermaphrodites raised at 24°C produce inviable progeny (0.5% hatching) due to chromosome segregation defects during spermatogenesis [Figure 1B, (Jaramillo-Lambert et al. 2016)]. To identify suppressors of top-2(it7ts) embryonic lethality, we exposed L4 top-2(it7ts) hermaphrodites to EMS at the permissive temperature (15°C), allowed the worms to grow for two generations, shifted the F2 generation to 24°C, and collected rare survivors among the F3 progeny (Materials and Methods, Figure 1A). Of >80,000 haploid genomes screened, we recovered 11 independent suppressor lines.

We performed embryonic viability assays to determine the extent to which each suppressor line rescued the embryonic lethality phenotype of top-2(it7ts). Eight of the 11 lines showed strong suppression rescuing embryonic viability >50%. Six had nearly wild-type levels of embryonic viability with average percent viabilities of >80% (Figure 1B). One of the suppressors, ude16, rescued embryonic viability to 37.3%. The remaining two suppressors, ude14 and ude15, rescued embryonic viability above 10% with more variability in progeny survival among individual worms (Figure 1B).

We determined the dominance or recessiveness of the suppressor mutations by crossing each suppressor line with unmutagenized top-2(it7ts); him-8(e1489) males (see Materials and Methods). Of the 11 suppressor lines, ten of the suppressors were either dominant (>50% viability) or semi-dominant (10–49% viability) mutations and one suppressor mutation was recessive (<10% viability, Table 1). To differentiate between suppressors that are linked vs. unlinked to the top-2 gene, we performed embryonic viability assays on Unc F2 progeny from unc-4(e120) top-2(it7ts)/unc-4(+) top-2(it7ts); sup/+. F1 animals (see Materials and Methods). Ten of the suppressors were unlinked from top-2 and one suppressor was one linked (Table 1). Lastly, we sequenced a portion of the top-2 gene containing codon 828 in the 11 suppressor lines to determine if any of the suppressors had mutations that reverted the original it7ts mutation (Arg828Cys) back to a wild-type arginine amino acid. All 11 suppressors contained the it7ts mutation (Table 1).

We used a combination of Hawaiian SNP mapping and WGS (Doitsidou et al. 2010; Jaramillo-Lambert et al. 2015), to simultaneously map and identify the suppressing mutations. We identified a single nonsynonymous protein-coding candidate mutation for the one linked
suppressor, ude6, which is a novel missense mutation within top-2 (Table 2).

Of the 10 unlinked suppressors, seven of the suppressors contained a single candidate mutation on LGI (Table 2). Two of these seven suppressors carried the same mutation. Although they contain the same mutation, they were isolated in two independent screens and likely carry different background mutations. Thus, we assigned them different allele designations, ude5 and ude24 (Tables 1 and 2). These seven suppressors, ude2, ude3, ude4, ude5, ude7, ude13 and ude24, contain different mutant alleles within the same gene, tdpt-1. TDPT-1 is the C. elegans homolog of tyrosyl DNA phosphodiesterase 2 (Tdp2). Tdp2 is a multifunctional protein involved in DNA repair, gene transcription, and signal transduction during mitosis (Pommier et al. 2016) and has not previously been shown to play a role during meiosis.

The remaining three unlinked suppressors yielded more than one candidate mutation for each. ude14 had one candidate mutation on LGII (nurf-1), and one candidate mutation on LGIV (mep-1, Table 2). nurf-1 is a nucleosome remodeling complex homolog (Andersen et al. 2006) and mep-1 encodes a zinc-finger protein that is part of a chromatin remodeling complex that functions in cell fate determination (Unhavaithaya et al. 2002). ude16 had two candidate mutations in viln-1 and nlp-38 in an interval on LGI. viln-1 is a homolog of human supervillin. The predicted gene function of viln-1 on Wormbase (www.wormbase.org) is to bind actin filaments. nlp-38 is predicted to encode a neuropeptide-like protein. ude15, contained an excess of unique mutations (20 out of 56) on LGIII, making identification of the suppressing mutation difficult. However, among the unique mutations is an allele of another topoisomerase, top-3, which has been shown to have roles in recombination during meiosis (Wicky et al. 2004). Because of this function, we focused on top-3 as the potential suppressor candidate for ude15 (Table 2).
### Table 1: Suppressor genetic analysis

| Suppressor Allele | Revertant* | Dominant/Recessive (% Embryonic Viable) | Linked or Unlinked from top-2(it7ts) |
|-------------------|------------|----------------------------------------|-------------------------------------|
| ude2              | No         | Dominant (68.7%)                       | Unlinked                            |
| ude3              | No         | Dominant (68.3%)                       | Unlinked                            |
| ude5              | No         | Dominant (85.8%)                       | Unlinked                            |
| ude4              | No         | Semi-dominant (41%)                    | Unlinked                            |
| ude6              | No         | Dominant (83.3%)                       | Linked                              |
| ude14             | No         | Semi-dominant (25.8%)                  | Unlinked                            |
| ude15             | No         | Recessive (5.9%)                       | Unlinked                            |
| ude13             | No         | Dominant (73.9%)                       | Unlinked                            |
| ude16             | No         | Semi-dominant (17.9%)                  | Unlinked                            |
| ude24             | No         | Dominant (84.1%)                       | Unlinked                            |
| ude7              | No         | Dominant (84.8%)                       | Unlinked                            |

*This column indicates whether the suppressor line is due to a reversion of the top-2(it7ts) R82BC mutation back to wild type.

**P percent embryonic viability of heterozygous animals (Suppressor/+). Suppressors with >50% viability are dominant, 10–49% viability are semi-dominant, <10% viability are recessive.

ude = the allele designation assigned to the Jaramillo-Lambert laboratory.

### Identification of the suppressor loci

To validate the molecular lesion responsible for suppression in each suppressor line, we first tested candidates for phenocopy of suppression through RNAi knockdown. We knocked-down tdpt-1, top-3, nlp-38, viln-1, nurf-1, and mep-1 in unc-4(e120) top-2(it7ts) hermaphrodites raised at 24°C and scored for the production of viable progeny. RNAi of tdpt-1 verified the candidate gene for ude2, ude3, ude5, ude7, ude13, and ude24. Depletion of tdpt-1 resulted in suppression of embryonic lethality in unc-4(e120) top-2(it7ts) with a penetrance of 91.9% (Table 2). RNAi knockdown of tdpt-1 in wild-type (N2) animals does not result in a decrease in embryonic viability (Figure S1A). Given that the tdpt-1 suppressing mutations are dominant alleles, it is surprising that loss of tdpt-1 expression through RNAi can recapitulate suppression. This indicates that the tdpt-1 suppressing alleles are haploinsufficient and sensitive to the dose of TDPT-1 protein, thus requiring two copies of the wild-type allele to function properly. One function of Tdp2 is to remove trapped Top2-DNA covalent complexes (Top-2cc) that inhibit DNA replication and transcription (Pommier et al. 2014). The identification of seven mutant alleles of tdpt-1 that suppress the top-2(it7ts) phenotype suggests that mutant TOP-2 proteins may bind and cleave DNA during meiosis, however, they may be unable to complete their topoisomerase activity forming Top-2cc. Perhaps, when TOP-2 cannot function properly, as in the case of the top-2(it7ts) mutant, wild-type TDPT-1 removes TOP-2 from DNA disrupting chromosome structure/chromatin function, resulting in chromosome missegregation. In this context, if TDPT-1 is rendered inactive (suppression mutations), residual TOP-2 activity will eventually lead to chromosome segregation. RNAi knockdown of tdpt-1 in wild-type (N2) animals is not haploinsufficient because no Top-2cc are present. This is one possible hypothesis. Future studies will elucidate the mechanism of tdpt-1-mediated suppression.

For ude14 knockdown of the individual gene candidates, mep-1 and nurf-1, did not suppress unc-4(e120) top-2(it7ts) embryonic lethality (1.0% and 1.7% viable progeny, Table 2). However, previous studies found that mep-1 and nurf-1 interact genetically (Andersen et al. 2006). We used RNAi to knockdown both mep-1 and nurf-1 in unc-4(e120)....

### Table 2: Candidate genes for the suppressors

| Intragenic/Extragenic | Suppressor allele | Candidate gene | Chromosome | Mutation | RNAi of top-2(it7ts)* |
|-----------------------|-------------------|----------------|------------|----------|----------------------|
| Intragenic            | ude6              | top-2          | II         | D809N    | 0.8% (n = 8167) NP    |
| Extragenic            | ude2              | tdpt-1         | I          | G328E    |                      |
|                       | ude3              | tdpt-1         | I          | G219E    |                      |
|                       | ude4              | tdpt-1         | I          | G117R    |                      |
|                       | ude5              | tdpt-1         | I          | G270D    | 91.9% (n = 2253)^**  |
|                       | ude24             | tdpt-1         | I          | G270D    |                      |
|                       | ude7              | tdpt-1         | I          | G270S    |                      |
|                       | ude13             | tdpt-1         | I          | A355ST   |                      |
|                       | ude14             | mep-1          | IV         | G57D     | 1.0% (n = 4624)^**   |
|                       | ude23             | nurf-1         | II         | E1954K   | 1.7% (n = 4253)^**   |
|                       |                   |               |            |          | 9.2% (n = 3460)*     |
|                       |                   |               |            |          |                      |
|                       |                   |               |            |          |                      |
|                       |                   |               |            |          |                      |

*Percent of viable progeny from unc-4(e120) top-2(it7ts) hermaphrodites fed RNAi bacteria against the gene listed in column three at 24°C.

NP = not performed.

n = the number of embryos scored.

*P < 0.05, one-tailed student’s T-test.

**P < 0.005, one-tailed student’s T-test.

*Indicates the data are not significant by the one-tailed student’s T-test.

ude = the allele designation assigned to the Jaramillo-Lambert laboratory.
top-2(it7ts). The combined knockdown of mep-1 and nurf-1 resulted in suppression of embryonic lethality in unc-4(e120) top-2(it7ts) at 24°C (9.2%, Table 2). Individual RNAi knockdown of either mep-1 or nurf-1 as well as the combined knockdown of mep-1 and nurf-1 in wild-type (N2) animals does not result in a decrease in embryonic viability (Figure S1A). From these data we conclude that the combination of nurf-1 and mep-1 mutations can suppress top-2(it7ts)-induced embryonic lethality. In the light of this conclusion, we kept the original allele designation of ude14 assigned to the mep-1 G57D mutation and assigned the mep-1 E1954K mutation the allele designation of ude23. Hence, the genotype of the original suppressed line should more accurately be top-2(it7ts) nurf-(ude23); mep-(ude14). Both nurf-1 and mep-1 are involved in chromatin remodeling suggesting that a particular chromatin state is required for proper chromosome segregation. TOP-2 may be involved in providing this particular chromatin conformation.

ude15 contains the only recessive suppressing mutation. For ude15, RNAi knockdown of top-3 in the unc-4(e120) top-2(it7ts) background failed to validate top-3 [P699S] as a suppressor of top-2(it7ts) embryonic lethality (0.9% viable, Table 2). Previous studies reported that RNAi knockdown of top-3 shows various phenotypes from normal, healthy worms to animal sterility (Kim et al. 2000). We found that wild-type animals treated with top-3 RNAi were both fertile and produced viable progeny (Figure S1A). This could be due to insufficient knockdown of top-3. We delivered the double-stranded RNA (dsRNA) to the animals using the RNAi feeding method (Timmons et al. 2001), while the previous study of top-3 performed RNAi via injection of dsRNA; a more direct delivery method. RNAi by feeding is not always as potent as injection of dsRNA (Ahringer 2006). As ude15 contained 20 unique mutations, it is highly possible that the P699S mutation identified in top-3 is not the suppressing mutation. Additional studies will need to be performed to determine the identity of the suppressing mutation for ude15.

For ude16, the individual RNAi knockdown of viln-1 and nlp-38 in the unc-4(e120) top-2(it7ts) background did not suppress embryonic lethality (1.0% and 2.7% viable respectively, Table 2). We also used RNAi to knockdown both viln-1 and nlp-38 in unc-4(e120) top-2(it7ts). The combined knockdown of viln-1 and nlp-38 in unc-4(e120) top-2(it7ts) at 24°C resulted in 6.9% embryonic viability, however, this was not statistically significant (P > 0.05, Table 2). RNAi knockdown of viln-1 and nlp-38, as well as the combined knockdown of viln-1 and nlp-38 in wild-type (N2) animals did not decrease embryonic viability (Figure S1A). From these data, RNAi in the unc-4(e120) top-2(it7ts) background did not positively identify either viln-1 [R552C] or nlp-38 [G77R] as the suppressing mutations. However, as the ude16 suppressing mutation is dominant, we reasoned that it may also be a gain-of-function mutation. To that end we performed RNAi in the suppressed line to determine if gene knockdown would reverse suppression. Using this approach, we found that RNAi of viln-1, nlp-38, and the viln-1/nlp-38 combination did not reverse suppression (Table S2). This suggests that WGS has not identified the correct suppressing mutations or that RNAi knockdown of these genes is not efficient in eliciting the suppressing phenotype. Additional studies will need to be performed to determine the identity of the suppressing mutations of the ude16 suppressor.

As further validation that mutations in tdpt-1 result in top-2(it7ts) suppression of embryonic lethality, we used CRISPR/Cas9 genome editing to recreate one of the identified tdpt-1 mutations. We identified three suppressors that resulted in missense alleles at amino acid 270 of TDPT-1, suggesting that this site is very important for TDPT-1 function. For this reason, we recreated the tdpt-1(ude5) and tdpt-1(ude24) G270D missense mutation in the unc-4(e120) top-2(it7ts) strain originally used for the suppressor screen. We generated one line and assigned it the allele designation ude22. While unc-4(e120) rol-1(e91) WT control viability was 94.2%, embryonic viability of unc-4(e120) top-2(it7ts) was 0.6% at 24°C (Figure 2). Embryonic viability of the tdpt-1 G270D mutation [unc-4(e120) top-2(it7ts); tdpt-1(ude24)] was 72.2% (Figure 2). We conclude, from this data along with the RNAi data, that mutations in tdpt-1 can suppress top-2(it7ts)-induced embryonic lethality. We also used CRISPR/Cas9 genome editing to validate the one intragenic suppressor through recreation of the top-2(ude6) D809N allele in the unc-4(e120) top-2(it7ts) line. We recovered two independent, edited lines, one linked and one unlinked from dpy-10, and assigned them the allele designation ude29. Embryonic viability of unc-4(e120) top-2(it7ts) was 0.6% (at 24°C) while embryonic viability of the recreated lines were 56.5% and 88.7% respectively [unc-4(e120) rol-1(e91)] control viability was 94.2%, Figure 2). These data confirm that the ude6 allele (D809N) of top-2 suppresses the embryonic lethality caused by the R828C mutation of top-2(it7ts). Interestingly, this suppressing mutation also falls within the topoisoerase domain perhaps compensating for structural changes caused by the R828C mutation in top-2(it7ts). Animals bearing a CRISPR recreated version of top-2(ude6) in a wild-type (N2) background were healthy and produced viable progeny at 24°C (Figure S1B). Future studies will determine the structural and functional roles of the R828 and D809 amino acids in TOP-2 function during meiosis.

Characterization of the suppressors

All 11 of the suppressors rescue the embryonic lethality of top-2(it7ts) with varying degrees of penetrance (Figure 1B). To determine whether the suppressors can rescue other top-2(it7ts) phenotypes, we examined post-meiotic sperm in the spermathecae of suppressor hermaphrodites for rescue of chromosome segregation defects by DAPI staining after 16 h incubation at 24°C (shift at L3/L4 larval stage). Post-meiotic sperm of wild-type hermaphrodites [unc-4(e120) rol-1(e91)] have a round and compact morphology (Shakes et al. 2009) while unc-4(e120) top-2(it7ts)
post-meiotic sperm have abnormal chromosome morphology with chromatin bridges [Figure 3A and 3B, (Jaramillo-Lambert et al. 2016)]. We found that all 11 suppressors can rescue chromosome segregation defects with varying penetrance (Figure 3A and 3B). tdpt-1(ude3) and tdpt-1(ude13) suppressor lines were the most similar to wild type with 0% of germ lines displaying abnormal chromosome morphology (Figure 3B). ude15, ude16, and the double mutant mep-1(ude14); mep-1(ude23) were the least efficient suppressors with 66.7%, 83.3%, and 68.8% of gonads with abnormal chromosome structures. Interestingly, the intragenic suppressor, ude6, had one of the highest percent embryonic viabilities of the suppressors (77% viable, Figure 1B), but had one of the highest percent of gonads with at least one chromatin bridge (60% of gonads with chromosome structure defects, Figure 3A and 3B).

Next, we assessed TOP-2 protein levels in the suppressor lines compared to wild type (N2) and unc-4(e120) top-2(it7ts) mutant lines. Although, we previously demonstrated that the primary defect of top-2(it7ts) is a failure to localize to chromosomes, we also showed that TOP-2 protein levels via quantification of pixel intensities indicated that TOP-2 protein is reduced in late meiotic prophase of top-2 mutant germ lines [top-2(av77)::3xFlag, a CRISPR recreation of top-2(it7ts)] (Jaramillo-Lambert et al. 2016). To more directly assess TOP-2 protein levels, we generated a C. elegans-specific anti-TOP-2 polyclonal antibody and examined protein levels by Western blotting. TOP-2 protein levels were reduced after RNAi-mediated knockdown of top-2 and in top-2(ok1930D) confirming antibody specificity (Figure S2). As expected, at 24°C, TOP-2 protein levels were reduced in unc-4(e120) top-2(it7ts) whole worm lysates compared to wild-type control (N2, Figure 4A and 4B). Although several of the suppressors did not restore TOP-2 protein levels [ude13, ude14; ude23, and ude15], seven of the suppressors, ude2, ude3, ude4, ude5, ude6, ude7, and ude16 had TOP-2 protein levels that were intermediate between unc-4(e120) top-2(it7ts) and wild type or restored to wild-type TOP-2 protein levels (Figure 4A and 4B). Quantiﬁcation by densitometry, indicates that TOP-2 protein levels are increased the most in ude6. As ude6 is the top-2 intragenic suppressor it is possible that the D809N suppressing mutation is creating some sort of structural compensation for the original top-2(it7ts) [R828C] mutation. Even though the primary defect of top-2(it7ts) is a failure to localize to meiotic chromosomes, this study demonstrates that top-2(it7ts) also has a defect in TOP-2 protein stability and that some of the suppressors can restore TOP-2 protein stability. Future studies will examine the mechanisms of suppression for the validated suppressors.

Figure 3: Suppressors ameliorate the chromosome segregation defects of top-2(it7ts). (A) Chromosomes in post-meiotic sperm of hermaphrodite young adults (24 h post L4 at 15°C and 24°C) of control lines [unc-4(e120) rol-1(e91) and unc-4(e120) top-2(it7ts)] and the suppressor lines. Chromosome segregation defects were assessed via confocal imaging of DAPI stained dissected gonads and spermathecae. Arrowheads in yellow indicate chromatin bridges. At least 30 gonads were examined for each genotype. Scale bar = 5 μm. (B) Quantification of chromosome segregation defects at 24°C from (A). Dark gray bar= percent of gonads with at least one chromosome segregation defect. Light gray bar= percent of gonads with no chromosome segregation defects (wild-type chromosome segregation). At least 30 gonads were examined for each genotype.
CONCLUSION
In summary, we identified 11 genetic suppressors of top-2(it7ts) embryonic lethality. We identified the molecular lesion responsible for suppression of nine of the suppressor candidates via whole-genome sequencing and single-nucleotide polymorphism mapping and verified either through RNAi phenocopy or through CRISPR/Cas9 recreation of the suppressing mutation. The genes identified in this screen comprise different functional classes including chromatin remodeling, DNA repair, and protein structure/stability. The intragenic mutation, ude6, most likely helps restore protein structure and stability as TOP-2 protein levels are decreased in the top-2(it7ts) mutant line and restored in the top-2(it7ude6) suppressor line (Figure 4). MEP-1 and NURF-1 are both involved in chromatin remodeling and interact genetically. TDPT-1 is involved in the removal of TOP-2 protein complexes and DNA repair. How these proteins interact with TOP-2 in the context of homologous chromosome segregation during meiosis is unknown. Future studies will help determine the mechanism of suppression of these different mutations and will elucidate the genetic pathways in which these genes interact with top-2 during meiosis.

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