# ANALYZING THE STRUCTURAL AND FUNCTIONAL CHANGES OF TOP-2 VARIANTS IN *CAENORHABDITIS ELEGANS*

by

Tommy Wilmoth

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Summer 2019

**ANALYZING THE STRUCTURAL AND FUNCTIONAL CHANGES OF TOP-2 VARIANTS IN *CAENORHABDITIS ELEGANS***

by

Tommy Wilmoth

Approved: _____

Aimee Jaramillo-Lambert, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Velia Fowler, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____

John A. Pelesko, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____

Douglas J. Doren, Ph.D.
Interim Vice Provost of Graduate and Professional Education and
Dean of the Graduate College

**ACKNOWLEDGEMENTS**

-Aimee Jaramillo-Lambert, for her unwavering guidance and support and for being so understanding throughout the past two years. She accepted me into her lab when other P.I.'s were hesitant due to my continuing sports career. Her many hours in teaching me lab techniques, how to place requests on Quartzy, and persuading me to say TOP-2 "crystal" structure, instead of "crystalline," have helped me to better myself as a graduate student and as a person.

-My committee members, Deni Galileo, Ph.D., and Jess Tanis, Ph.D., for their help towards the focus of my project.

-My professional friends and colleagues, especially my labmates, who have helped and supported me during my graduate career. I could always count on them as friends with which to get along, joke around, and sometimes debate:

Nirajan, Dina, Christine, "T," Dave, Gavin (soft G), Alekya, and others

This manuscript is dedicated to

-My dad and mom, Bruce and Donna Wilmoth, and my sisters, Mary Kate, Maureen, and Kathleen, for their unconditional love and support.

-Michelle Dellalana, for her love and support throughout graduate school.

-The late Donald Monagle, whose legacy and constant signs of his presence continue to inspire and encourage me to strive to achieve great accomplishments.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## ABSTRACT

During meiosis, cells undergo one round of DNA replication followed by two rounds of cell division to form haploid gametes. In meiosis I, homologous chromosomes segregate while in meiosis II, the sister chromatids segregate before cytokinesis to form haploid cells. Topoisomerase II is an enzyme that helps to facilitate DNA disentanglements. This enzyme has been studied extensively in mitosis but not in meiosis. Previously, we demonstrated that a temperature sensitive mutant, *top-2(it7),* causes chromosomes to fail to segregate in spermatogenesis when incubated at 24°C. The *top-2(it7)* mutation changes an arginine at amino acid 828 to cysteine. This mutation lies within an α/β fold of TOP-2 called the tower subdomain that is located within the catalytic domain, a region that has been proposed to interact with DNA. Structural modeling of the TOP-2 protein indicates that the wild-type arginine makes several interesting contacts with other amino acids, including a salt bridge with glutamate at amino acid site 960. We hypothesize that the arginine at position 828 is particularly important for the structure and function of the enzyme. I have characterized several amino acid substitutions at Arg828. I found that an Arg828Ala change has high embryonic viability at 15°C but significantly reduced viability when incubated at 25°C. This mutation has intermediate segregation defects at 25°C compared to *top-2(it7).* An Arg828Trp change results in sterility due to the absence of germline development. Characterization of an Arg828Lys change reveals reduced viability when incubated at 24°C that is exacerbated at 25°C. Examination of the germline shows chromosomal segregation defects that are not as penetrant as those seen in *top-2(it7).* Future directions

will include the examination of protein levels in the various *top-2* mutants, the characterization of additional amino acid substitutions, and assays to determine the effects of the mutations on TOP-2 enzymatic activity.

# Chapter 1

# INTRODUCTION

## 1.1 Meiosis: An Overview

Meiosis is a form of cell division that results in the formation of four haploid daughter cells that are each genetically unique compared to their parent cell. It consists of one round of DNA replication followed by two rounds of cell division, known as meiosis I and meiosis II. Unlike in mitosis, where identical sister chromatids separate during one division, meiosis displays two divisions: one in which homologous chromosomes segregate and one in which sister chromatids segregate.

Before meiotic divisions occur, cells undergo an "S" phase, where chromosomes are duplicated and generate identical sister chromatids. Following S phase and preceding the nuclear divisions is an extended prophase, unique to meiosis, which lays the foundation for the subsequent chromosomal and cytoplasmic divisions. The extended meiotic prophase is broken into five substages: leptotenema, zygonema, pachynema, diplonema, and diakinesis. In early prophase I, stages known as leptonema and zygonema, the DNA condenses, homologous chromosomes find each other, and undergo pairing within the nucleus (Pawlowski and Cande, 2005). ). As zygonema progresses into a substage called pachynema of prophase I, pairing is complete when a proteinaceous structure called the synaptonemal complex (SC) is formed between the homologous chromosomes. While accurate chromosomal pairing has been suggested to be largely dependent on recombination mechanisms in eukaryotes, including mice (Baudat et al.,

2000), *C. elegans* models have shown pairing to be initiated in an independent manner (Dernberg et al., 1998). Within the context of the SC, the chromosomes exchange DNA in a special process called homologous recombination leading to the physical linkage of the homologous chromosomes following SC removal. The physical linkage of homologous chromosomes manifests as a special structure called the chiasmata. The chiasmata are crucial for proper alignment of homologous chromosomes along the metaphase I plate. During anaphase I, this prevents daughter cells from having too few or too many chromosomes (Alberts et al., 2002). Finally, during diplonema and diakinesis, bivalents are formed as the SC degrades and the chromosomes further condense.

Metaphase I, Anaphase I, and Telophase proceed somewhat similarly compared to mitosis, though the significant difference remains that the parent cell's first division will yield two haploid cells due to segregation of homologous chromosomes. In metaphase I, the chromosomes align along the metaphase plate as the meiotic spindle is formed and begins contact with the chromosomes via kinetochores. In Anaphase I, homologous chromosomes are pulled toward their respective centrosome via microtubule shortening of the spindles (Freeman, 2011). Additionally, cohesin, a protein complex that binds chromosomes and chromatids together, begins to degrade. In contrast to mitosis, the cohesion between chromatids remains intact to prevent premature sister chromatid separation in meiosis I, facilitated by a protein called Shugoshin (Pierce, 2009). At the end of meiosis I, during telophase, the chromosomes have segregated to their respective poles and the cytoplasm divides.

Following the first division, the two cells will eventually undergo the second division without further genome duplication. Meiosis II proceeds quite similarly to mitosis, where the sister chromatids separate prior to cellular division. However, unlike having an end product of identical diploid daughter cells seen in mitosis, meiosis will yield haploid cells that have genetic variation due to recombination events. In prophase II, the chromatids condense and the spindle fibers begin to form again. In metaphase II, the chromosomes align along the metaphase plate and the meiotic spindles attach at the centromeres. In anaphase II, the spindles shorten and pull at the sister chromatids that, without Shugoshin's functional presence, segregate to their respective poles (Pierce, 2009). In telophase II and cytokinesis, the spindles begin to break down and the cytoplasm divides once more. Many of the factors and pathways facilitating this entire process have yet to be fully elucidated.

## 1.2 Type II Topoisomerases

Topoisomerases are a class of enzymes that mainly function to regulate the winding nature of DNA and its double helical build through topological mechanisms (Champoux, 2001). There are two main kinds of Topoisomerases: Type I and Type II topoisomerases. While both function to prevent major topological issues in DNA, working to relax DNA supercoils or decatenate DNA that is intertwined, Type II topoisomerases make double-stranded breaks in the DNA and require ATP hydrolysis (Hsieh, 1990). Gyrase, a prokaryotic form of topoisomerase II, consists of four subunits, two A and two B. The B subunits deal with ATP hydrolysis while the A subunits perform

DNA strand breakage and religation. In contrast, eukaryotic type II topoisomerases (topo II) have the combined capacity in its subunits for ATP hydrolysis as well as binding, cleavage, and resealing of DNA (Wang, 2002). Eukaryotic topoisomerase II functions as a homodimer ($A_2$), consisting of three distinct domains: an N-terminal ATPase domain, a DNA-binding catalytic domain, and a C-terminal domain. While the N-terminus and the DNA binding core and catalytic domain are fairly conserved among homologs, the C-terminus of topo II is not conserved and its function is not as well understood as the other two domains. However, studies have shown that the C-terminus may contribute to protein regulation and post-translational modifications (PTMs), using sites for SUMOylation (Lee and Bachant, 2009) and phosphorylation (Vassetzky et al., 1994).

Topo II function has been mainly studied in mitosis. It first starts by making contact with and binding to a strand of DNA, designated the "G" or gate strand, with its dimeric catalytic zone. Once ATP binds to the ATPase domain of both subunits, topo II awaits capture of a second strand, deemed the "T" or transport strand, through dimerization of the ATPase domain. Once the bound ATP is hydrolyzed, the DNA-binding core of the catalytic zone will cleave the "G" strand, which will allow subsequent passage of the "T" strand through the catalytic domain. The "T" strand then enters into the C-terminal domain, in which its dimerization acts as a gate. As the "G" strand is re-ligated, the C-terminal gate will open and the transport strand will exit (**Fig. 1.1**), yielding two segments of disentangled DNA or relaxing the supercoil, depending on the need for topo II. Though much has been characterized about Topo II in regards to its functions and interactions in mitosis, its meiotic implications are less well known. Continuing to

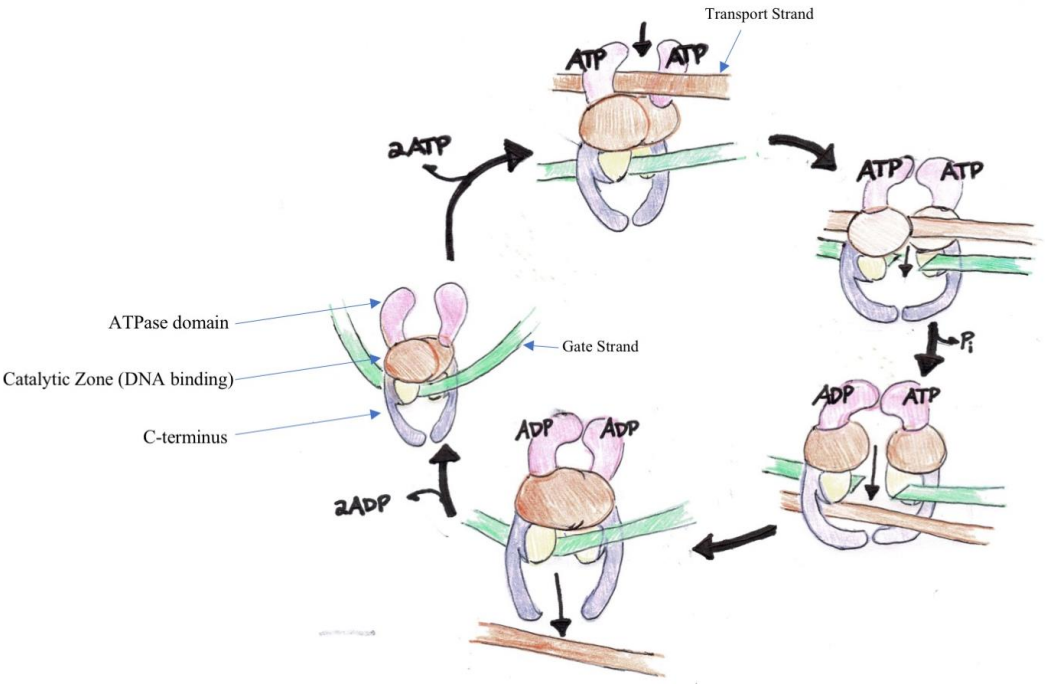


Figure 1.1: Passage strand mechanism of Topoisomerase II. Image based off of model from Vos et al., 2011. (https://www.nature.com/articles/nrm3228)

examine its enzymatic necessity and roles in germline models such as *C. elegans* can shed more light on its utility during meiotic processes.

## 1.3 *C. elegans* as a Model Organism

The roundworm *Caenorhabditis elegans* is a free-living nematode that is found naturally in soils and rotting vegetation throughout the world. Their main food source is bacteria, in particular *E. coli* that is found in the vegetable compost (Barrière and Félix, 2014). In nature, the vast majority of *C. elegans* are hermaphrodites and are capable of

self-reproduction, while naturally-occurring males come about at a frequency of less than 1 in 500 (Corsi et al., 2015). The body makeup is rather simplistic, as an adult hermaphrodite is 959 somatic cells, in addition to a proliferating germ line.

In 1963, Sydney Brenner developed the idea of using roundworms as a powerful and useful system of studying fields including neurobiology and general development (Brenner, 2002). *C. elegans* became a tremendous tool to utilize in these research fields, as thousands of laboratories have adopted this model (Corsi et al., 2015). The main advantages of *C. elegans* include a short life cycle, body transparency and germline visibility, progeny size, and genetic malleability.

*C. elegans* have a rather rapid life cycle, where wild-type (WT) worms (designated as N2) maintain a life span ranging from 12-18 days at the normal growth temperature of 20°C (Gems and Riddle, 2000). Under normal conditions, *C. elegans* go through seven stages of development: an embryonic stage that occurs both *in utero* and *ex utero*; four larval stages (denoted as L1, L2, L3, and L4); young adulthood; and reproductive adulthood (**Fig. 1.2**). Roughly, it takes 3-4 days to develop from a laid embryo, through the larval stages, and reach adulthood. A hermaphrodite can produce up to 300 fertilized embryos through self-fertilization. The large progeny number makes it advantageous when addressing germline and genetic questions.

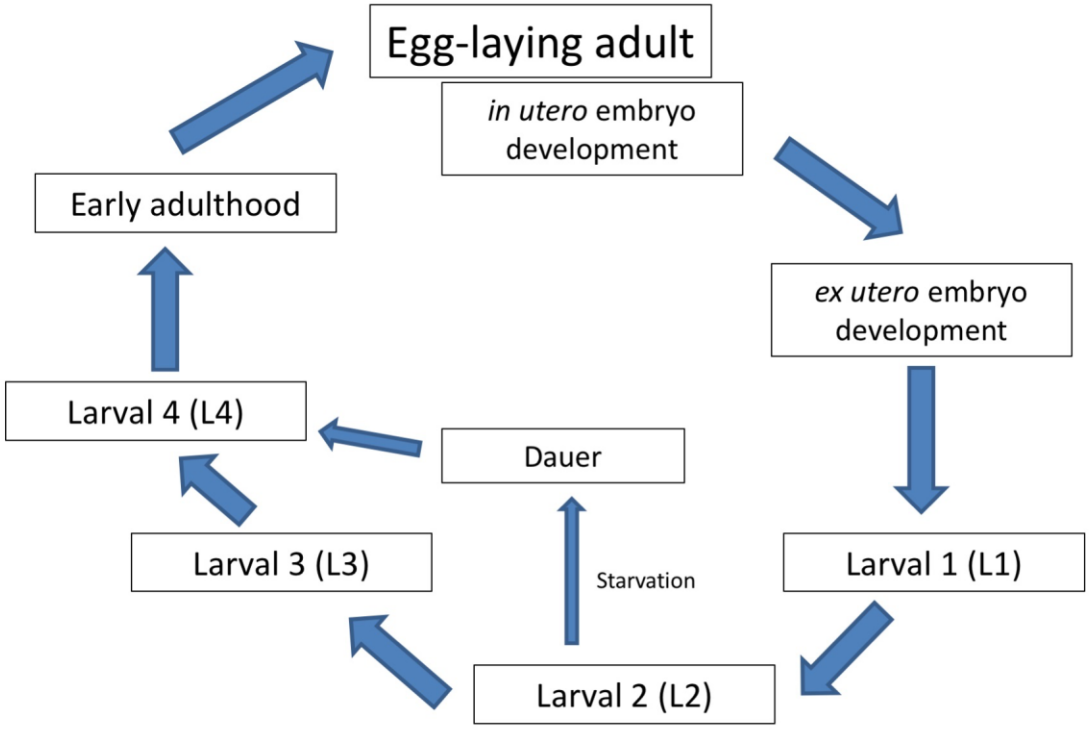


Figure 1.2: *C. elegans* life cycle at normal growth temperature. Image based off of model from WormAtlas: Introduction to *C. elegans* Anatomy (Altun and Hall, 2009). (https://www.wormatlas.org/hermaphrodite/introduction/mainframe.htm)

Under abnormal conditions, environmental factors can influence the rate at which these nematodes grow. For example, it was found that the mean life span changes from approximately 23 days at 16°C to about 9 days at 25°C (Klass, 1977). Another factor that can influence *C. elegans* life span is food source availability. When nematodes in the first and second larval stages (L1 & L2) encounter a dearth of food, they undergo an alternate life stage known as the dauer. In this state, the worms cease to grow and form a cuticle specific to this stage that prevents dehydration. Potentially due to these adaptations that conserve energy, their life span can increase by several months, as long as they are in the

dauer state. Once food is made available again to *C. elegans* dauers, they undergo morphological and physiological changes to return to their normal state, where they re-enter a normal life cycle (Klass and Hirsh, 1976).

### 1.3.1 The *C. elegans* Germ Line

Because of the transparent cuticle of *C. elegans*, there is high germline visibility in both hermaphrodites and males under a general dissection scope. Hermaphrodites have a gonad consisting of two U-shaped arms that converge on a central uterus. The distal tips contain proliferating mitotic cells, and along the path down the gonadal arms lie the various phases of germ cell differentiation (Hubbard, 2007). On both sides of the uterus, there is a special feature of the *C. elegans* gonad where generated sperm are stored and through which oocytes must pass for fertilization: a structure called the spermatheca (**Fig. 1.3**). Hermaphrodites produce their own amoeboid sperm during the L4 stage, accumulating around 150 sperm per gonad arm. However, as they transition to adulthood and undergo molting, a physiological switch promotes exclusive oocyte production for the rest of their lives (McGovern, 2007).

The male gonad is J-shaped with a layout similar to the hermaphrodite gonadal arm, but the germline connects with a somatic gonad extension, consisting of the vas deferens. The somatic gonad joins with a copulating organ on the tail end. The copulatory structure is located in a morphologically distinct posterior that resembles a tail fin (**Fig. 1.3**). Males begin sperm production in the L4 stage and continue to produce sperm throughout adulthood. While hermaphrodites may give rise to approximately 300

progeny through self-fertilization, mating between a male and hermaphrodite can yield a brood size of around 1400 (Hubbard, 2007).

Within the gonad, several stages of the meiotic pathway can be observed in order. This is due to unique feature of the *C. elegans* gonad where there is a spatiotemporal layout of the germ line, as germ cells are located at certain points along the gonad depending on what meiotic sub-stage through which they are progressing. At the distal tip of the gonad, there is a mitotic zone, in which cells proliferate and are not undergoing meiosis. The transition zone follows, characterized by cells that begin to switch from mitotic proliferation to meiosis (**Fig. 1.4**). The next stage that is visible is pachytene, a sub-stage of prophase I. The "condensation zone" is comprised of diplotene and diakinesis, where the chromosomes become more compact bivalents. One significant difference in the condensation zones between spermatogenesis and oogenesis is the clustering of the chromosomes into a central mass, known as the karyosome, in spermatogenesis (Shakes et al., 2009). The condensation zones are followed by the subsequent meiotic divisions (**Fig. 1.4**).

### 1.3.2 The Genome and Genetics of *C. elegans*

The entire *C.* elegans genome is contained within six pairs of chromosomes in hermaphrodites (five autosomal pairs and one sex pair), while males harbor five autosomal pairs and one sex chromosome (Riddle et al., 1997). In late 1998, its genome was the first to be sequenced among multicellular organisms (*C. elegans* Sequencing

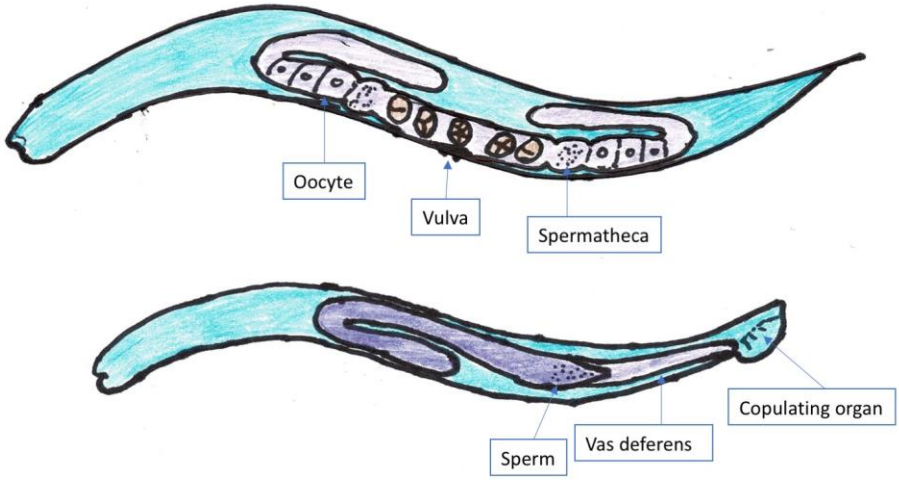


Figure 1.3: Morphological structure of the hermaphrodite (top) and male (bottom) gonad. Based off of image from Dr. Brian E. Staveley.
(http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_11/fig11_23.jpg)

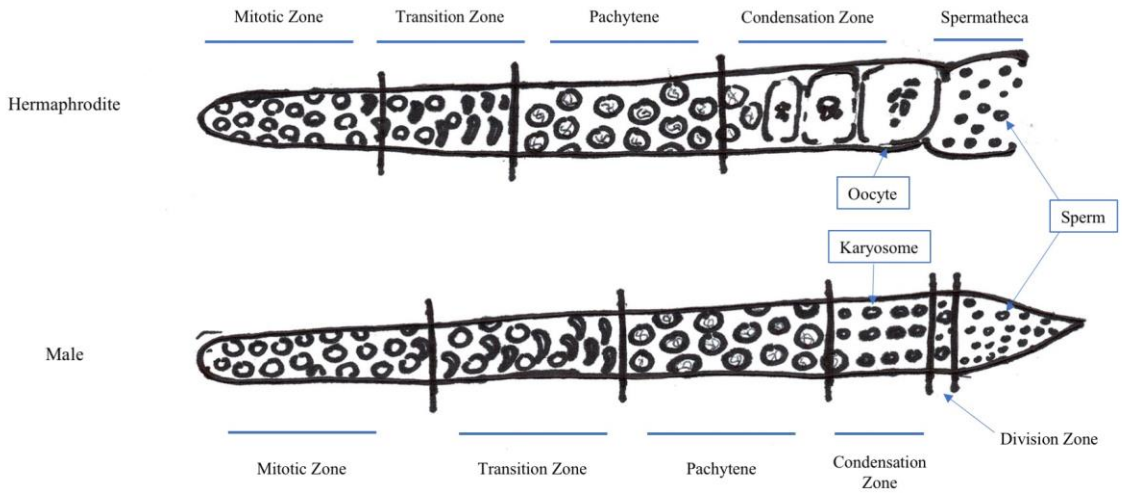


Figure 1.4: Spatiotemporal layout of the *C. elegans* germline. Based off of image from Shakes et al., 2009. (https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1000611)

Consortium, 1998). The genome is nearly 100 megabases whose coding regions account for around 20,000 genes, and at least 40% of the putative products are orthologous to those in other organisms (*C. elegans* Sequencing Consortium, 1998). In particular, nearly 38% of the protein products have a form of human homolog (Shaye and Greenwald, 2011). The likeness in genes and translated proteins becomes a powerful tool throughout genetic studies when examining effects of mutations on molecular and physiological processes.

The advantages of *C. elegans*, most notably their swift growth to mature adults as well as their ability to self-reproduce, make them a genetic model that can be utilized with relative ease. When *C. elegans* were first being studied, a process known as "forward genetics" was used to map out genes and their roles in nature. Forward genetics involves the generation of mutations and screening for specific phenotypes, especially those that deviate from WT behavior, morphology, or function (Corsi et al., 2015). Mutations can arise in various ways, including exposure to EMS, or ethane methylsulfonate, which can cause random single nucleotide polymorphisms (SNPs). Once a mutation is introduced, it is easy for the strain to become true breeding (or homozygous) through hermaphrodite self-fertilization, where a parent generation (P0) gives rise to individual mutant progeny in subsequent generations (Corsi et al., 2015). From there, the gene of interest can be sequenced without difficulty due today's tremendous improvements in genome sequencing.

In modern times, since a vast majority of the genes in *C. elegans* have been identified and holistically examined, a newer process known as "reverse genetics" has

become popular (Ahringer, 2006). Reverse genetics involves creating strains by mutating and/or silencing genes that have been successfully mapped out. RNA interference (RNAi) is a frequently used method of reverse genetics that allows for transient knockdown of genes. This can be accomplished quite simply when incorporated into the bacterial source that is fed to *C. elegans.* Another method that is potentially becoming the forefront of *C. elegans* reverse genetic studies is CRISPR/Cas9 genome editing. This significant advancement allows for highly-specific targeted mutations at virtually any part of the genome (Frøkjær-Jensen, 2013). The mutations can be screened by isolating genomic DNA from the F2 generation, amplifying the region of interest, and determining if the mutation is present based off of factors such as fragment size or presence of a designed alteration (e.g. restriction site). Then, selecting for the mutant in subsequent generations can yield a strain that is homozygous for the mutation (Corsi et al., 2015).

### 1.4 The *top-2(it7)* Mutation

Though the role of topo II during mitosis has been well-characterized, its role in meiosis is not as apparent. Recently, a temperature sensitive mutation in *C. elegans*, *top-2(it7)*, has revealed TOP-2 to be crucial during spermatogenesis. When incubated at 15°C, *top-2(it7)* mutants display a WT phenotype and give rise to progeny with a normal brood size during embryonic viability assays. However, at 24°C, the mutants show a significant drop in embryonic viability, averaging below 5%. Further tests helped to characterize *top-2(it7)* as a paternal-effect lethal allele (Jaramillo-Lambert et al., 2016). The high embryonic lethality was explained when examining the different stages of

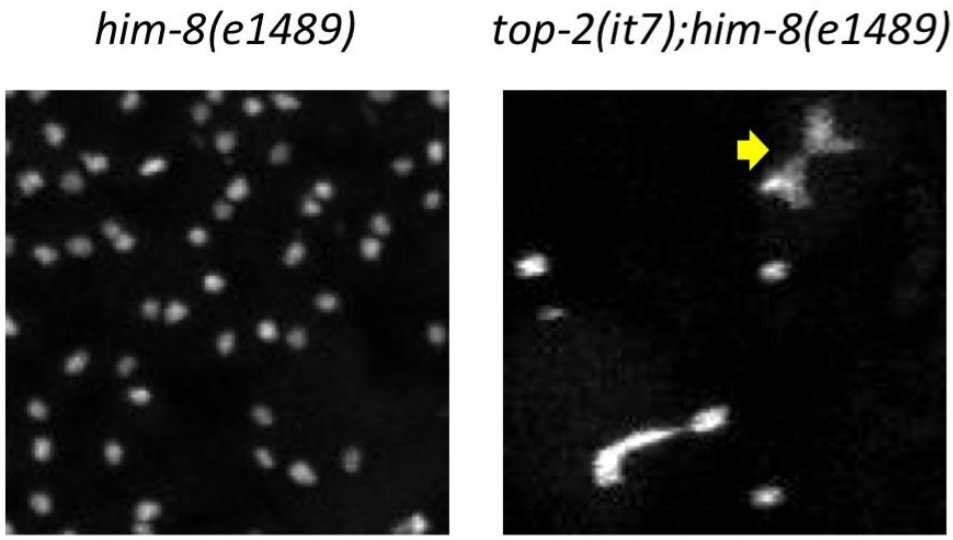


Figure 1.5: Representative images of chromosomal segregation in *him-8(e1489)* (WT) and *top-2(it7);him-8(e1489)* males following a 16 h incubation at 24°C. Arrows indicate examples of chromatin bridges.

meiosis in the mutant's germ line. Following a 24°C incubation, while normal alignment of the chromosomes along the metaphase plate was seen in both WT and the mutant, *top-2(it7)* cells in anaphase of meiosis I showed severe chromosomal segregation defects (**Fig. 1.5**). At the end stages of meiosis II, though the chromosomes have not properly segregated, the spermatocytes of the mutants proceed with spermatid budding. Consequently, the DNA becomes trapped in the central residual body, and the spermatids bud off to yield anucleate sperm (Jaramillo-Lambert et al., 2016).

*top-2(it7)* consists of a single-point, missense mutation that changes an arginine at amino acid 828 of TOP-2 to a cysteine (CGT→TGT) following translation. A crystal structure of TOP-2 that was modeled from yeast topoisomerase II reveals that the

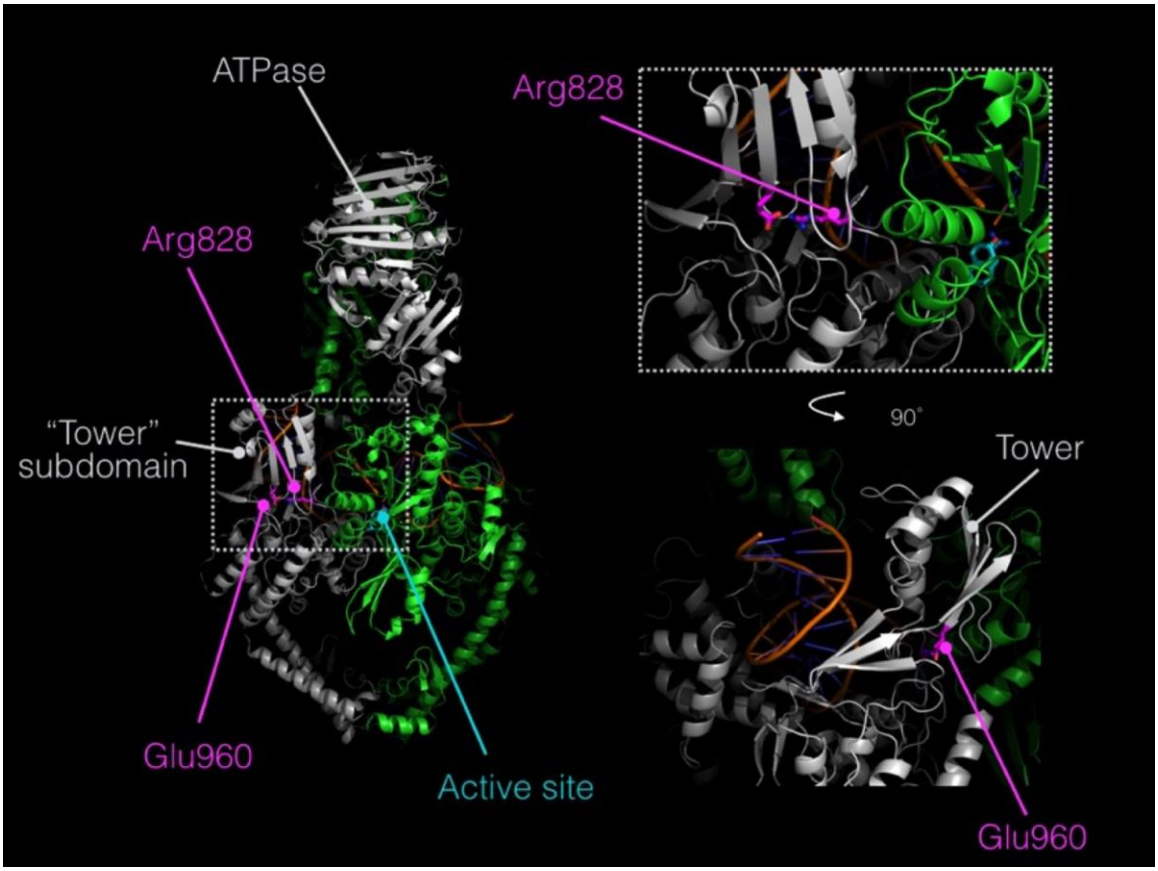


Figure 1.6: Crystal structure model of TOP-2 based off of yeast topo II. Image obtained from James Berger, personal communication.

mutation occurs in the DNA-binding catalytic domain, within an α/β fold of what is known as the "tower" subdomain (**Fig. 1.6**). Modeling of this protein also indicates that the Arg828 makes several interactions with other amino acids, including a salt bridge with a glutamate at amino acid site 960 (personal conversation with James Berger). Still, explanations behind the importance and necessity of Arg828 and the tower domain in which it resides have yet to be elucidated.

### 1.5 Proposed Hypothesis and Research Aims

As previously mentioned, the functional significance and role of topo II within the meiotic pathway has not been well defined. Previous research has at least shown TOP-2 in *C. elegans* to be necessary, specifically in spermatogenesis, where a single amino acid change generated a significant rift in the normal meiotic operations (Jaramillo-Lambert et al., 2016). Still, there is much to clarify in order to better understand this factor as a whole in the meiotic pathway.

The overall goal of this project was to illuminate the role of TOP-2 in meiosis of *C. elegans*. More specifically, the goal was to use structure/function analysis to define the importance of TOP-2 within the germ line as well as certain amino acid sites of TOP-2. My main proposed hypothesis is that an amino acid site of TOP-2, Arg828, is necessary for proper and ideal structural integrity as well as function of the enzyme. The first objective was to establish different mutants of Arg828 by exchanging the arginine for amino acids of different properties, followed by performing embryonic viability assays on the mutants to determine the mutant's viability potential. The second objective was to analyze the germline of the mutants and characterize any defects in chromosomal segregation compared to the previously generated Arg828Cys mutant *top-2(it7)*. The third objective was to compare relative protein levels of TOP-2 in the established mutants and potentially correlate these levels with their other phenotypes.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 *C. elegans* strains and maintenance

Each strain was maintained at their respective temperature: N2, *him-8(e1489)*, *top-2(ude9)/*mIn1, and *top-2(ok1930)* were incubated at 20°C; all others strains were incubated at 15°C.

**Table 2.1** consists of a list of the *C. elegans* strains that were used during development of this thesis. AJL indicates strains that were generated in the Jaramillo-Lambert Laboratory at the University of Delaware.

## 2.2 Preparation of whole-worm protein lysates and Western blotting

Seven to ten L4 worms from each strain were picked to six medium plates and grown until a majority of the progeny had reached L4 stage. Worms were then shifted to 24°C for 24 h. Following incubation, plates were washed with M9 buffer and the worms placed into a 1.5 mL tube. Worms were washed three times with M9, pelleted in a centrifuge at 2,000 revolutions per minute (RPM) for two min, and the M9 removed, leaving a worm pellet. An equivalent volume of 3X-SDS Sample buffer (3% SDS, 30% glycerol, 188mM Tris, 0.01% Bromophenol blue, 15% β-Mercaptoethanol) was added to the pellet. Next, samples were frozen at -80°C for 15 min, boiled at 95°C for 10 min, and vortexed for 10 min. Samples were then centrifuged at 14,000 RPM for 30 min at 4°C. The resulting

Table 2.1: List of strains of *C. elegans*

| Strain | Description | Comments |
|---|---|---|
| **N2 (Bristol)** | Wildtype | Control |
| **CB1489** | *him-8(e1489)* | Control; induces sex chromosome nondisjunction, resulting in higher incidence of male progeny |
| **AG261** | *top-2(ok1930)/mIn1;him-8(e1489)* | TOP-2 KO; genetically balanced with mIn1 for maintenance |
| **AG259** | *top-2(it7)*[Arg828Cys]*;him-8(e1489)* | temperature-sensitive |
| **AG334** | *top-2(av88)* [Arg828Ala] Line #1 | temperature-sensitive |
| **AG337** | *top-2(av88)* [Arg828Ala] Line #2 | temperature-sensitive |
| **AJL8** | *top-2(ude8)*[deltaC-T]*/mIn1* | 761 bp deletion removing codons 1295-1517 of the C-terminus; genetically balanced with mIn1 for maintenance |
| **AJL9** | *top-2(ude9)*[Arg828Trp]*/mIn1* | genetically balanced with mIn1 for maintenance |
| **AJL23** | *top-2(ude18)* [Arg828Lys] | temperature-sensitive |

supernatants were extracted and placed in fresh tubes. If not immediately used, lysates were stored at -20°C. 20 µL of each sample was loaded onto a 4-15% precast gel. The gel was run at 200 V and then was transferred onto a 0.45 µm nitrocellulose membrane. The membranes were blocked using a 5% milk solution in TBST (Tris-buffered saline with .1% Tween 20 (Sigma)) for 1 h. The membranes were blotted for TOP-2 using anti-TOP-2 primary antibody (1:750 dilution in a 5% milk solution in TBST) and for anti-Alpha tubulin (1:5000 dilution in a 5% milk solution in TBST, DM1α, Sigma), overnight at 4°C. Membranes were washed four times for five min each with TBST. The membranes were incubated with secondary antibodies (anti-rabbit-HRP antibody and anti-mouse-HRP, Life Technologies, 1:10,000 dilution in a 5% milk solution in TBST), at room temperature for 1 h. Membranes were washed five times for five min each in TBST, then placed in Tris-buffered saline (TBS) prior to imaging.

### 2.3 TOP-2 antibody production

*C. elegans*-specific anti-TOP-2 antibodies were produced by injecting rabbits with the synthetic peptide (CQRDPKMNTIKITINKEKNE) (YenZym Anitbodies LLC). Polyclonal antibodies were purified by antigen-specific affinity purification (YenZym Anitbodies LLC). Specificity of the antibodies was validated by Western blot and reduction of protein in worms depleted of TOP-2 through RNAi (Jaramillo-Lambert, unpublished antibody).

### 2.4 Methanol fixation for whole-mount animals

10 worms of each strain were picked into 5 µL of M9 buffer on regular glass slides (Fischer Scientific). M9 was removed, leaving only the worms on the slide. 15 µL of 100% room temperature methanol were applied to the worms for fixation. As soon as the methanol evaporated, 12 µL of 2 µg/mL DAPI (4′,6-diamidino-2-phenylindole) solution (in M9) were added to the worms. A coverslip was applied on top and the edges were sealed with nail polish. The slides were incubated in the dark for 30 min prior to observation.

### 2.5 DAPI staining of dissected gonads

Gonad dissections were performed on *him-8(e1489), top-2(it7);him-8(e1489), top-2(av88)* #1 and #2, and *top-2(ude18).* 20 L4 stage male worms of each strain were picked to new MYOB plates and incubated for 24 h at 24°C. 30 µL of M9 were placed onto a

Superfrost plus-charged glass slide (Fisher Scientific). Worms were picked to the M9 and their heads were severed beneath the pharynx using a surgical blade to release the gonad. 15 µL of M9 was removed, leaving the worm carcasses and gonads left untouched. 15 µL of 2% paraformaldehyde (16% (Electron Microscopy Sciences), diluted in sterile water) was added to the worms, pipetted several times to assist in gonad extrusion, 15 µL of paraformaldehyde (pFA) was removed, a coverslip was placed on top, and slides were allowed to fix for five min. Slides were then placed on dry ice, and the coverslips were removed. Slides were then immediately placed in -20°C methanol for one min. Slides were washed once in PBST (PBS with 0.1% Tween 20) for five min. Excess liquid was removed from the slides, and 40 µL of 2 µg/mL DAPI was added to the area of the slide containing the dissected gonads. Slides were incubated in the dark for five min then washed in PBST for five min. Slides were mounted by adding 8 µL of Vectashield (Vector Laboratories) followed by placing a coverslip on top. The edges of the coverslip were sealed with nail polish.

**2.6 Embryonic viability assays**

Single L3 or L4 stage hermaphrodites were picked to individual plates seeded with OP50 *E. coli*. The plates were then immediately incubated at the desired temperature for a 24 h period. After 24 h, the adult worms were transferred to new individual 3 cm plates, and again incubated at the desired temperature. This process was repeated until no additional embryos were laid. Plates were observed 24 h after the adult worms were removed, and the number of dead embryos and live larvae were counted, making up the total progeny

for each plate. Viability was calculated by dividing the sum of the live larvae by the number of total progeny. For each strain, the viabilities were averaged. Each experiment was repeated at least three times.

### 2.7 Image collection and processing

Slides, for the purpose of image acquisition, were observed using the ZEISS LSM780 Multiphoton Confocal Microscope. Images were acquired with a 40X objective with z-stacks of 0.2 μm. Slides with DAPI-stained extruded gonads were observed and chromosome segregation defects were scored using the ZEISS Axio Observer-D1 Inverted Fluorescence Microscope. Gonads were imaged using a 63X objective.

### 2.8 Quantification of chromosome segregation defects

Worm gonads were observed using the ZEISS Axio Observer-D1 Inverted Fluorescence Microscope, the proximal gonad end was located, and the focus was adjusted until the approximate center of the gonad was in view. All defects in chromosome segregation within frame were counted. Gonads were grouped according to how many defects were observed in each frame: 0-1; 2-5; 6-10; and greater than 10. For each strain, the groups were shown as a percentage of the total gonads observed.

### 2.9 CRISPR/Cas9-mediated gene editing

CRISPR-mediated gene editing was performed to create amino acid changes at the codon site 828 of TOP-2 in the N2 background via the clone-free method (Paix *et al.*,

2015), using *dpy-10* as a co-CRISPR marker (Arribere *et al.* 2014). All of the following injections were done using an injection mix of Cas9 protein (10 mg), *dpy-10* CRISPR RNA (crRNA) (3.2 mg), *dpy-10(cn64)* repair oligonucleotide (0.2 mg), universal trans-activating crRNA (tracrRNA) (20 mg, Dharmacon, GE Life Sciences), *top-2* sequence targeting crRNA (CUUCUUCCAAUCGGACAGUUGUUUUAGAGCUAUGCUGUU UG, 8 mg), and a gene-/allele-specific repair oligonucleotide (0.8 mg). To convert the arginine 828 to lysine, N2 young adult hermaphrodites were injected with the above mix containing a repair oligonucleotide containing the sequence to edit the wild-type codon CGT to AAA (bold), additional nucleotide changes to prevent Cas9 cleavage after repair (italics), and a *Pst*I restriction enzyme recognition site (underline) to distinguish between edited and non-edited lines (CTCGCTCAAGATTACGTTGGCTCCAACAACATCAA CCTGCTTCTTCCAAT*A*GG*G*CA*A*TT*C*GGTACT**AAA**CTGCAGGGTGGAAAGGAC AGTGCTTCAGCTCGTTACATCTTCACTCAACTGTCGCC).

To convert arginine 828 to alanine, N2 young adult hermaphrodites were injected with the above mix containing a repair oligonucleotide containing the sequence to edit the wild-type codon CGT to GCT (Bold), additional nucleotide changes to prevent Cas9 cleavage after repair (italics), and a *Pst*I restriction enzyme recognition site (underline) to distinguish between edited and non-edited lines (CTCGCTCAAGATTACGTTGGCTCC AACAACATCAACCTGCTTCTTCCAAT*A*GG*G*CA*A*TT*C*GGTACT**GCT**CTGCAGGG TGGAAAGGACAGTGCTTCAGCTCGTTACATCTTCACTCAACTGTCGCC).

To convert arginine 828 to tryptophan, N2 young adult hermaphrodites were injected with the above mix containing a repair oligonucleotide containing the sequence

to edit the wild-type codon CGT to TGG (Bold), additional nucleotide changes to prevent Cas9 cleavage after repair (italics), and a *Pst*I restriction enzyme recognition site (underline) to distinguish between edited and non-edited lines (CTCGCTCAAGATTAC GTTGGCTCCAACAACATCAACCTGCTTCTTCCAAT*A*GG*G*CA*A*TT*C*GGTACT**TG G**CTGCAGGGTGGAAAGGACAGTGCTTCAGCTCGTTACATCTTCACTCAACTG TCGCC).

# Chapter 3

# RESULTS

## 3.1 TOP-2 variants at Arg828 site reduce embryonic viability

To determine whether arginine at amino acid site 828 is necessary to maintain the structural and functional integrity of TOP-2, we used CRISPR/Cas-9 genome editing to substitute the arginine for amino acids with varying sizes and properties (outlined in **Table 3.1**). After homozygous lines for edits to the Arg828 site were established, characterization of that change began through embryonic viability assays. It has been shown that an Arg828Cys change, or *top-2(it7)*, results in paternal-effect embryonic lethality (Jaramillo-Lambert et al., 2016). In contrast to cysteine, an amino acid such as alanine is smaller, nonpolar, and nonreactive. Due to these properties we asked if an Arg828Ala substitution would be less disruptive of an amino acid substitution than Arg828Cys. We used CRISPR/Cas9 genome editing to change CGT at codon 828 to GCT to code for alanine. After establishing two identical Ala828 homozygous lines [allele designation: *top-2(av88)* #1 and #2], I performed viability assays at 15°C and 24°C. At 15°C, *top-2(av88)* embryonic viability was comparable to N2 (99.3%) and *him-8(e1489)* (93.3%), with an average of 95.7% for line #1 and 96.5% for line #2 (**Fig. 3.1**). At 24°C, the restrictive temperature for *top-2(it7)*, *top-2(av88)* had a reduction in viability (line #1 at 86.6% and line #2 at 66.9%), although this was not statistically different from either N2 (99.2%) or *him-8(e1489)* (80.1%) (**Fig. 3.1**). Because the *top-2(av88)* lines showed a consistent, although insignificant, reduction in viability, we asked whether increasing the incubation temperature would result in increased embryonic

Table 3.1: Arg828 substitutions and amino acid properties

| Genotype | Amino Acid Change | Properties | Predicted Phenotype |
|---|---|---|---|
| *top-2(it7)* | Arg828Cys | reactive, sulfur group | ts embryonic lethality |
| *top-2(av88)* | Arg828Ala | small, nonpolar | viability similar to WT |
| *top-2(ude18)* | Arg828Lys | positively charged | mimic Arg828, viability similar to WT |
| *top-2(ude9)* | Arg828Trp | large, aromatic side chain | disrupt structural stability |
| N/A | Arg828Asp | negatively charged | repel Glu960, disrupt structural stability |
| N/A | Arg828Met | fairly nonreactive, hydrophobic | viability similar to WT |

lethality. At 25°C, viability was significantly reduced in both lines, with line #1 at 52.1%, line #2 at 46.4%, and WT *him-8(e1489)* at 99.5%. However, the decrease in embryonic viability is not as severe as observed in *top-2(it7)* (2.7%) (**Fig. 3.1**). These results demonstrate that an Ala substitution at Arg828 is not ideal for TOP-2 function, but it is not as deleterious of a change as Arg828Cys.

A second amino acid substitution we made was to change Arg828 to Trp. Tryptophan is a larger amino acid with an aromatic side chain that prefers to be buried in hydrophilic cores and can be involved in amino acid stacking. Because of its unique chemistry and size, we predicted that an Arg828Trp substitution would be more disruptive to TOP-2 structural integrity than the original Arg828Cys. The substitution was created and the mutant was given the allele designation of *top-2(ude9). top-2(ude9)* was not temperature sensitive and was maintained by picking balanced heterozygous *top-2(ude9)/mIn1* animals. Embryonic viability assays were performed on WT *him-8(e1489),*

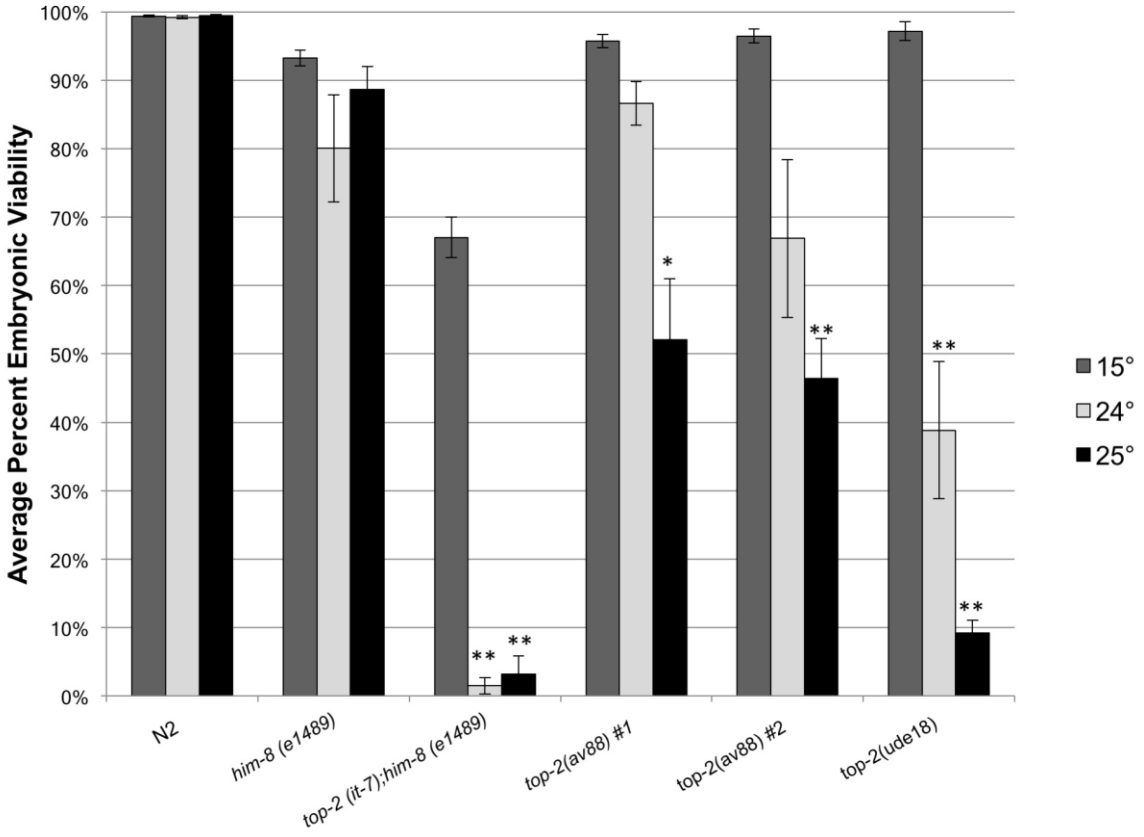


Figure 3.1: TOP-2 variants at Arg828 site reduce embryonic viability. Average percent embryonic viability of N2, *him-8(e1489), top-2(it7);him-8(e1489), top-2 (av88) #1, top-2(av88) #2,* and *top-2(ude18)* at 15°C, 24°C, and 25°C. The progeny of at least 25 hermaphrodites were scored for each strain at each temperature. Error bars represent the SEM of the averages of at least 3 separate experiments. (*) indicates a p-value<0.01 and (**) indicates a p value<0.001.

*top-2(ude9)/mIn1,* and *top-2(ude9)/top-2(ude9)* hermaphrodites. Assays revealed that both WT [*him-8(e1489)*] and *top-2(ude9)/mIn1* heterozygotes displayed high viability at 20°C, and *top-2(ude9)top-2(ude9)* homozygotes laid no progeny and were sterile (**Fig. 3.2**). These results show that the Trp substitution at Arg828 has a more severe effect than Arg828Cys.

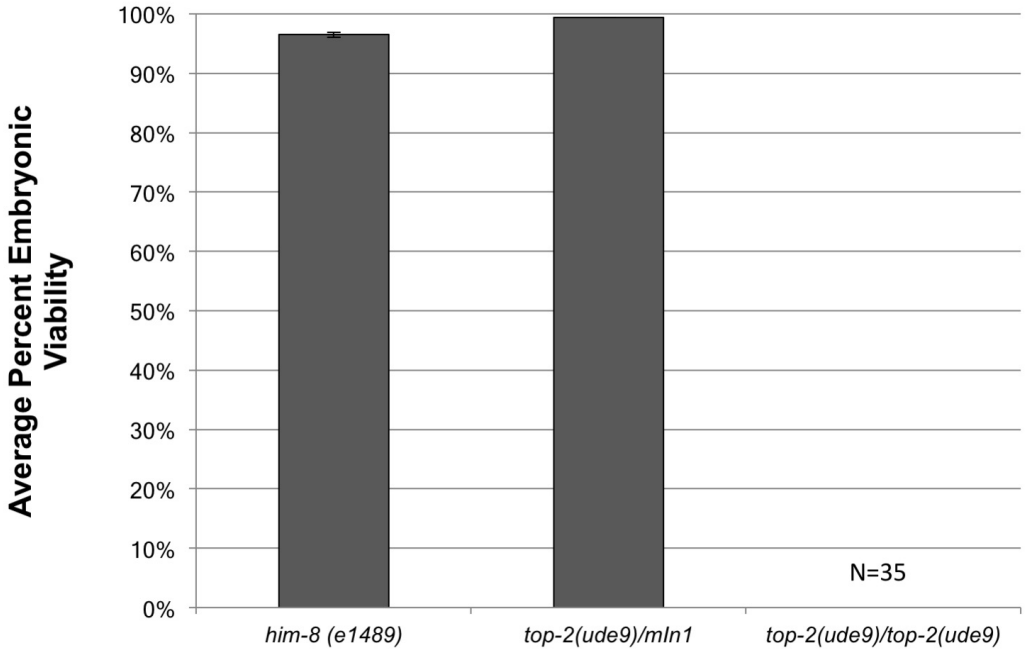


Figure 3.2: Average percent embryonic viability of *him-8(e1489), top-2(ude9)/mIn1,* and *top-2(ude9)/top-2(ude9)* at 20°C. The progeny of at least 28 hermaphrodites were scored for each strain at 20°C. Error bars represent the SEM of the averages of three individual replicate experiments.

Arginine is a positively-charged and polar amino acid. Interestingly, Arg828 is predicted to interact with Glu960 (potential salt bridge, James Berger personal communication). To test whether Arg828 is necessary to maintain TOP-2 integrity, I substituted Arg828 with an amino acid of the same charge, lysine, via CRISPR/Cas9 genome editing. A single homozygous line was generated and given the allele designation *top-2(ude18)*. Embryonic viability assays were performed at 15°C, 24°C, and 25°C. At 15°C, *top-2(ude18)* had an average viability of 97.2%, which was comparable

to WT (N2= 99.4%) (**Fig. 3.1**). At 24°C, *top-2(ude18)* showed a significant reduction in embryonic viability compared to WT, with *top-2(ude18)* at 38.8% and WT N2 at 99.2%, but it is not as severely reduced as *top-2(it7)*. Embryonic viability assays were then performed at 25°C to determine if *top-2(ude18)* would display a further decrease in viability similar to *top-2(av88)*. Indeed, at 25°C, embryonic viability of *top-2(ude18)* decreased to 9.2% compared to WT N2 at 99.5%. These results demonstrate that although Lys, like Arg, is a positively charged, polar amino acid, it cannot substitute for the Arg at amino acid site 828.

### 3.2 TOP-2 variants at Arg828 site show germ line defects

Previously, we showed that at 24°C, *top-2(it7)* chromosomes fail to segregate during Anaphase I of meiosis of spermatogenesis, resulting in anucleate sperm (Jaramillo-Lambert et al, 2016). To determine if the low embryonic viability observed in *top-2(av88)* and *top-2(ude18)* are due to defects in chromosome segregation similar to Arg828Cys, I examined both *top-2(av88)* and *top-2(ude18)* male germ lines by DAPI staining after 16 h incubation at 24°C. Both *top-2(av88)* and *top-2(ude18)* lines displayed chromosome segregation defects (**Fig. 3.3**). In contrast to the defects observed in *top-2(it7)* where proper chromosome segregation was almost never observed, both *top-2(av88)* and *top-2(ude18)* contained cells with chromosome segregation defects and cells with proper chromosome segregation (**Fig. 3.3**). Because *top-2(av88)* and *top-2(ude18)* have an increase in embryonic lethality at 25°C compared to 24°C (**Fig. 3.1**), we asked if

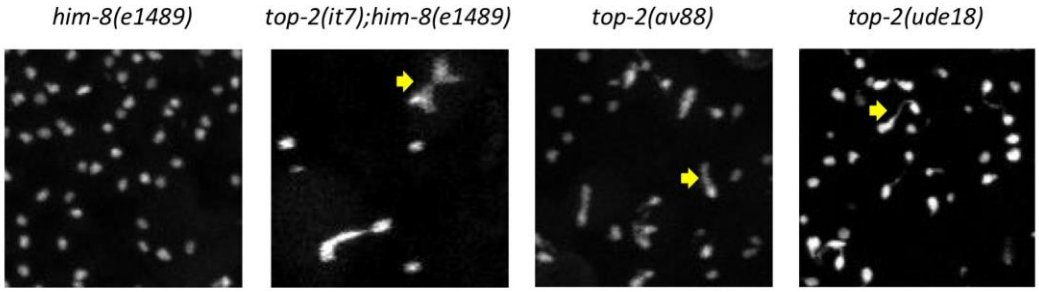


Figure 3.3: TOP-2 variants at Arg828 site show germline defects. Representative images of chromosomal segregation in *him-8(e1489), top-2(it7);him-8(e2489), top-2(av88),* and *top-2(ude18).* Images were taken of chromosomes at the proximal gonad center of males of each strain following a 16 h incubation at 24°C. Arrows indicate examples of observed defects.

the reduced viability seen at 25°C is due to an increase in defects within the germ line compared to 24°C. Male germ lines were DAPI stained and inspected after a 16 h incubation at 25°C and imaged for defects. Indeed, quantification revealed that *top-2(av88)* lines had an average increase in the number of defects observed when incubated at 25°C, with ten or more defects seen in 72.7% of the line #1 gonads and 100% of the line #2 gonads (**Fig. 3.4**). *top-2(ude18)* gonads also showed an increase in observed defect number, with ten or more defects seen in 92.3% of the gonads (**Fig. 3.4**). These results show that the lower viabilities seen at the higher temperatures in *top-2(av88)* and *top-2(ude18)* are due to chromosomal segregation defects, and that the prevalence of the defects within the gonad are temperature-dependent.

Earlier, embryonic viability assays showed that Arg828Trp homozygotes were sterile (**Fig. 3.2**). Sterility in *C. elegans* commonly manifests for two reasons: the germ line does not properly develop; or there is an issue disrupting fertilization of the

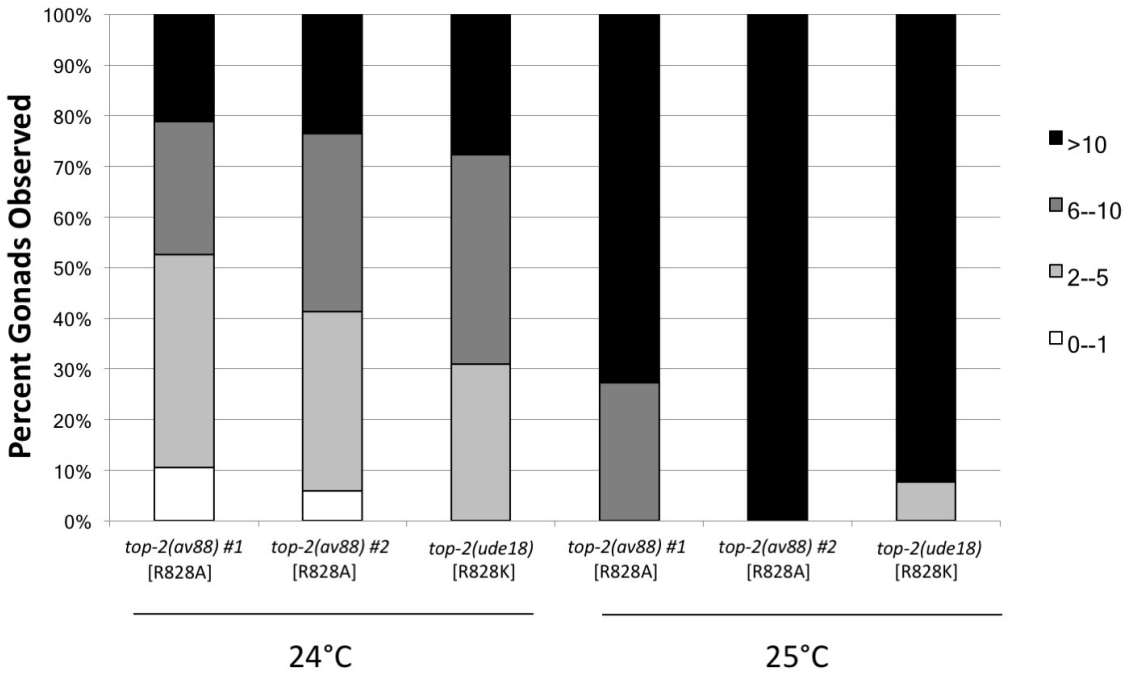


Figure 3.4: Comparative quantification of observed segregation defects in *top-2(av88)* males following a 16 h incubation at either 24°C or 25°C. Gonads were quantified by focusing on the center of the proximal end and scoring the total number of defects seen within the frame. Gonads were then placed in one of four categories: 0-1 defect seen; 2-5 defects; 6-10 defects; and greater than 10 defects.

eggs by sperm. To differentiate between these two possibilities, I examined *top-2(ude9)* homozygotes compared to *top-2(ude9)/mIn1* through whole mount DAPI staining. Imaging of the whole worm revealed that *top-2(ude9)* adult males and hermaphrodites failed to develop germ lines while *top-2(ude9)/mIn1* males and hermaphrodites showed normal germ line formation (**Fig. 3.5**). These results reveal that an Arg828Trp change results in failure to proliferate a germ line that causes *C. elegans* to be sterile.

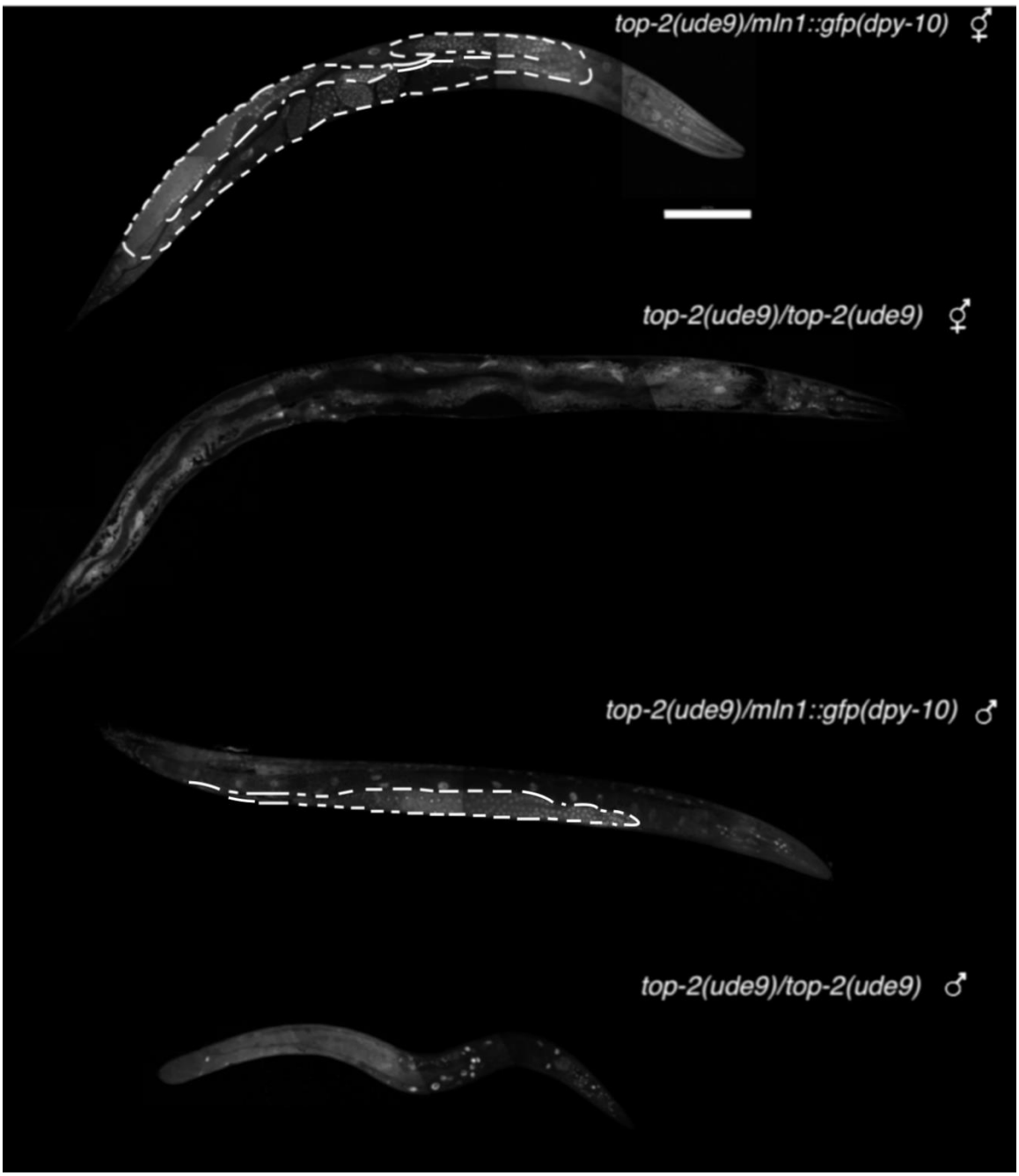


Figure 3.5: Representative images *top-2(ude9)* homozygotes and *top-2(ude9)/mIn1* heterozygotes. Images were taken following whole mount DAPI staining of both males and hermaphrodite adults for each strain. The dotted lines in the heterozygous worms indicate the germ line.

### 3.3 TOP-2 protein levels for 828 site mutants

To determine if protein levels are affected in the different *top-2* arginine 828 mutant lines, Western blotting was performed following 15°C and 25°C incubations. Data shows that at 15°C incubations, *top-2(av88)* lines display TOP-2 protein levels similar to *top-2(it7)* while *top-2(ude18)* displays protein levels similar to WT (N2) (**Fig. 3.6**). *top-2(ude9)* displayed virtually no TOP-2 protein levels (**Fig. 3.6**). In the blot following a 25°C incubation, it appears that *top-2(av88)* and *top-2(ude18)* lines have higher TOP-2 protein levels compared to N2 (**Fig. 3.6**). However, the α-tubulin loading control suggests that inconsistent amounts of lysate used for blotting in N2, *top-2(it7)*, and *top-2(ude18)*, making the results inconclusive. At 25°C, *top-2(ude9)* again displays virtually no TOP-2 protein levels compared to the other strains (**Fig. 3.6**).

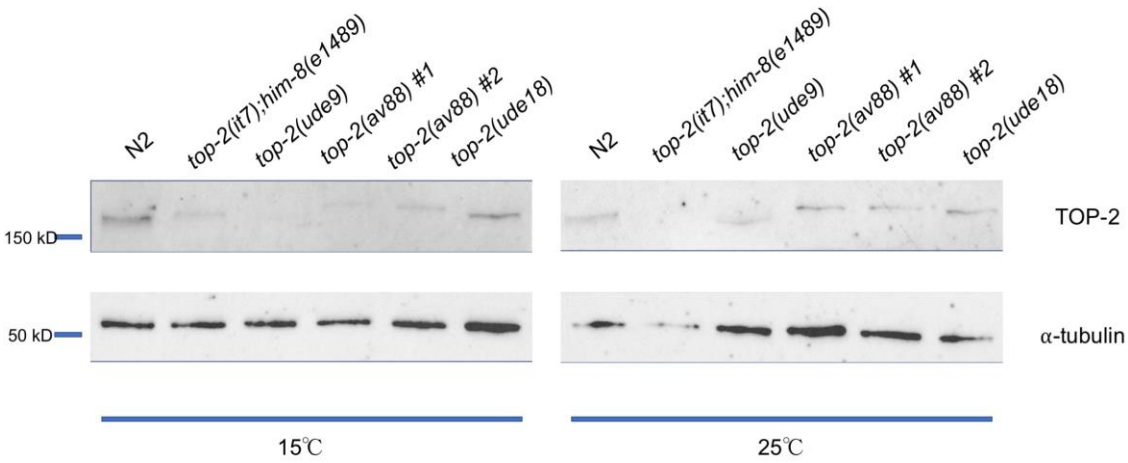


Figure 3.6: TOP-2 protein levels of R828 substitution mutants. Preliminary data of Western blots performed on N2, *top-2(it7);him-8(e1489)*, *top-2(ude9)*, *top-2(av88)* #1 and #2, and *top-2(ude18)*. 100 adult animals were picked for each strain's lysate following a 16 h incubation at either 15°C or 25°C. α-tubulin was used as a loading control.

# Chapter 4

# DISCUSSION

In this project, I have demonstrated that multiple mutants of Arg828 display reduced viability compared to WT. I have also shown that reduced viability is due to chromosomal segregation defects within the male germline that are not as severe as the *top-2(it7)* mutation but are still detrimental. Additionally, any sterility seen in mutants was a result of a failure to proliferate a germline. Western blots have suggested that Arg828 mutants may have reduced protein levels compared to WT.

## 4. 1 Meiotic Regulation is Crucial for Proper Gamete Production

Meiosis is the unique process of cellular division observed in a vast number of species, yet so much information is not known about its different intricacies and pathways. When not properly regulated, lacking an important component or having an unnecessary component, the outcomes can be very detrimental. When there are improper numbers of chromosomes, or aneuploidy, most cases lead to embryonic lethality. However, there are rare cases that lead to survival. Down syndrome or trisomy 21, a condition where a human's genome contains an extra or partial copy of chromosome 21, has become an issue growing in prevalence. Statistics reveal that the rate of babies born with Down syndrome has increased by approximately 30% between 1979 and 2003 (Center for Disease Control and Prevention, 2019). Many of the babies born with Down syndrome do not live past the first year, and the condition is accompanied by many other

conditions, especially congenital heart defects (Center for Disease Control and Prevention, 2019). There are other disorders that are characterized by sex-chromosome segregation issues. For example, human males born with Klinefelter's have a genome consisting of an extra X-chromosome. In these conditions, males are usually sterile and can present with underdeveloped testes, stunted puberty, and the development of breasts, or gynecomastia (Klinefelter's syndrome, 2019). Additionally, females can be born with Turner syndrome, a condition where an X chromosome is missing or detrimentally altered in structure (Turner syndrome, 2019). Females with Turner syndrome are mostly sterile, where properly developed ovaries is rare, and they may suffer from other complications, including heart defects (Turner syndrome, 2019). More severe cases of aneuploidy include trisomy 16, where non-mosaic (all cells have an extra copy) cases cause the individual to die early in development, usually prior to birth (Chromosome 16, 2019).

Understanding meiosis at a more complex level can also give insight into other diseases that are not directly involved in meiosis. A recent report has gathered research that suggests that cancers utilize meiotic-related regulator genes in mitotically-dividing cells during oncogenesis and its progression. More specifically in spermatogenesis, there are genes that are normally activated only in meiotic conditions that are deemed cancer/teste genes (CT genes) when their products are abnormally/excessively expressed (McFarlane and Wakeman, 2017). Furthermore, several of these CT genes have been implicated in various roles involved in cancer progression, including tumor maintenance, malignant angiogenesis, and metastasis (McFarlane and Wakeman, 2017). The study

highlights the strong evidence of a somatic-to-germline-like switch in cancer onset, though further elucidation will help guide researchers to a broader and more effective solution. When examining the previously discussed diseases, it is important to note that characterizing all the meiotic factors leading to these types of conditions and determining the risk factors involved can lead to a more refined approach in creating treatments and proactive measures preventing them altogether.

## 4.2 Meiotic effects of TOP-2 mutations on Embryonic Viability in *C. elegans*

One of the factors that is less well known regarding its roles within the meiotic pathway is topoisomerase II. Since mitotic studies and some meiotic studies have identified topo II as a conserved and crucial enzyme in cell division, it has become one of the main targets in cancer therapy. Drugs have been developed to inhibit human tumor development either through creating Top2:DNA covalent cleavage complexes (e.g. etoposide) or preventing Top2 catalytic activity altogether (e.g. bisdioxopiperazines) (Nitiss, 2009). However, these treatments tend not to distinguish cancer cells from normal cells, causing unnecessary cell death. Clarification of the mechanisms of topo II can lead to a refined perspective that can yield desired results while filtering out unwanted outcomes.

To better understand topo II, in this project, I use *C. elegans* as a model to study TOP-2, specifically in meiosis, utilizing a structure/function analysis approach to elucidate the significance of certain sites. I characterized embryonic viability in different mutants of the Arg828 site and compared the findings to WT and the previously

characterized *top-2(it7)* line. It was discovered that all of the mutants displayed reduced embryonic viability compared to WT. Both *top-2(av88)* lines showed significantly reduced viability at 25°C. *top-2(ude18)* mutants have significantly reduced embryonic viability at 24°C and 25°C. Why is an arginine residue so important at this site? Arginine is a highly interactive amino acid whose contacts include salt bridging and hydrogen bonding (Barnes, 2007). Arginine is also subject to PTMs, including methylation (Griffiths, 2016). Arg828 is proposed to interact with Glu960 via a salt bridge in TOP-2 (James Berger, personal communication). The results of the Arg828Ala substitution suggest that the salt bridge is necessary for structural integrity of TOP-2. However, the salt bridge connection with Glu960 is not the only critical function for Arg828. The results of the Arg828Lys substitution suggest that there are other potential interactions that make arginine necessary at amino acid site 828. The sterility seen in Arg828Trp indicates that tryptophan completely disrupts structural integrity of TOP-2, disallowing TOP-2 to function properly. We plan on further investigating the site by generating an Arg828Asp change, which is a polar, negatively-charged amino acid. We propose Asp828 would repel Glu960 and prevent any potential interaction between the two amino acids. This change could disrupt structural stability in a way similar to Arg828Trp, resulting in sterility. We also propose that an Arg929Met change may be able to restore embryonic viability. Methionine is relatively unreactive and hydrophobic, which allows it to be less affected by hydrophobic residues surrounding amino acid site 828. Additionally, we plan on creating an Arg/Glu swap of amino acid sites 828 and 960 in order to confirm that the salt bridge is not the only interactions that Arg828 is making.

## 4.3 Further Characterization of Generated Mutants

Previous research in our lab has shown that *top-2(it7)* displays embryonic lethality due to chromosomal segregation defects, specifically the formation of chromatin bridges during anaphase I of meiosis (Jaramillo-Lambert et al., 2016). This phenotype was seen exclusively in spermatogenesis, where unsegregated chromosomes became trapped in the residual body during spermatid budding, yielding anucleate sperm. Furthermore, when the anucleate sperm fertilized oocytes, this yielded haploid embryos, leading to embryonic lethality. Thus, our generated Arg828 mutants were characterized in the same manner. Following 24°C incubations, Arg828Ala [*top-2(av88)*] mutants show chromosomal segregation defects in the male gonad that are not as severe as *top-2(it7)*. This means that *top-2(av88)* gonads displayed normal-looking sperm as well as the chromatin bridge phenotype, while *top-2(it7)* gonads showed only chromatin bridging. The same observation was made in Arg828Lys [*top-2(ude18)*] mutants, which could explain the less severe embryonic lethality phenotype compared to *top-2(it7)*. After 25°C incubations, the prevalence of the defects, rather than the severity, increases in *top-2(av88)* and *top-2(ude18)*, potentially accounting for the further decrease in embryonic viability in both mutants. Unlike the other Arg828 mutants, Arg828Trp [*top-2(ude9)*] displayed total sterility, regardless of temperature, due to a failure in germline development. While it appears that maternal rescue provides enough TOP-2 for survival of the progeny, maternally-derived TOP-2 is insufficient for proliferating a germ line.

Preliminary data reveals that *top-2(it7)* has reduced TOP-2 protein levels compared to WT after a 24°C incubation. We sought to determine if our Arg828 substitution mutants would reveal a similar reduction in TOP-2 protein levels. Preliminary Western blotting of the mutants indicates that there may be reduced TOP-2 levels after incubation at the restrictive temperature (in this case, 25°C), though the experiment needs repetition. This again reinforces our thought that an insufficient amount of TOP-2 is potentially contributing to the spermatogenesis-specific phenotypes observed. Future experiments include performing decatenation assays, ATPase assays and DNA-binding assays on our Arg828 substitution mutants to shed light on to any enzymatic changes that are occurring with TOP-2.

Research into *C. elegans* spermatogenesis has shown a stage within the germline designated as the "karyosome" phase, where the DNA material aggregates into a central mass prior to meiotic divisions (Shakes et al., 2009). It is possible that exiting the karyosome stage requires higher levels and activity of TOP-2 in order to undo the DNA aggregates. Future studies will test this hypothesis.

## 4.4 Conclusions

Meiosis is an intricate and highly complex process, and its mechanisms and enzymatic factors need further clarification. Though the role of TOP-2 in meiosis has been preliminarily examined, there is much that remains unclear about its interactions with the meiotic machinery. I have isolated a certain site within the DNA-binding domain of TOP-2, Arg828, that I have shown is critical for the function of TOP-2 during

spermatogenesis. Future studies will further illuminate how the Arg828 site is necessary and what specific interactions with Arg828 are contributing to TOP-2 structural fidelity and proper function.

## REFERENCES


Ahringer, J. (2006). Reverse genetics. WormBook, ed. The *C. elegans* Research Community, WormBook, https://doi.org/10.1895/wormbook.1.47.1, http://www.wormbook.org.

Alberts, B., Johnson, A., Lewis, J., et al. (2002). Molecular Biology of the Cell. 4th edition. New York: Garland Science. Meiosis. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26840/

Altun, Z.F., & Hall, D.H.(2009). Worm Atlas. https://doi.org/10.3908/wormatlas.1.14. Edited for the web by Laura A. Herndon. Last revision: April 30, 2012

Barnes, M.R. (2007). Bioinformatics for Geneticists: A Bioinformatics Primer for the Analysis of Genetic Data. John Wiley & Sons. p. 326. ISBN 9780470026199.

Barrière, A., Félix, M. A. (2014). Isolation of *C. elegans* and related nematodes. WormBook ed. The C. elegans Research Community, WormBook, 1-19. https://doi.org/10.1895/wormbook.1.115.2

Baudat, F., Manova, K., Yuen, J. P., Jasin, M., & Keeney, S. (2000). Chromosome Synapsis Defects and Sexually Dimorphic Meiotic Progression in Mice Lacking Spo11. Molecular Cell, 6(5), 989-998. https://doi.org/10.1016/s1097-2765(00)00098-8

*C. elegans* Sequencing Consortium (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science, 282(5396):2012-8.

Centers for Disease Control and Prevention (2019). Data and Statistics on Down Syndrome. Retrieved from https://www.cdc.gov/ncbddd/birthdefects/downsyndrome/data.html

Chromosome 16 - Genetics Home Reference - NIH. (2019). Retrieved from https://ghr.nlm.nih.gov/chromosome/16#conditions

Clarke, D., & Azuma, Y. (2017). Non-Catalytic Roles of the Topoisomerase IIα C Terminal Domain. International Journal of Molecular Sciences, 18(11), 2438. https://doi.org/10.3390/ijms18112438

Corsi, A. K., Wightman B., & Chalfie M. (2015). A Transparent Window into Biology: A Primer on *Caenorhabditis elegans*. Genetics, 200(2), 387-407. https://doi.org/10.1534/genetics.115.176099

Dernburg, A. F., Mcdonald, K., Moulder, G., Barstead, R., Dresser, M., & Villeneuve, A. M. (1998). Meiotic Recombination in *C. elegans* Initiates by a Conserved Mechanism and Is Dispensable for Homologous Chromosome Synapsis. Cell, 94(3), 387-398. https://doi.org/10.1016/s0092-8674(00)81481-6

Ferrandiz, N., Barroso, C., Telecan, O., Shao, N., Kim, H.M., Testori, S., Faull, P., Cutillas, P., Snijders, A., Colaiácovo, M., Martinez-Perez, E (2018). Spatiotemporal regulation of Aurora B recruitment ensures release of cohesion during *C. elegans* oocyte meiosis. *Nature Communications* 9, 834.

Freeman, Scott (2011). Biological Science (6th ed.). Hoboken, NY: Pearson. pp. 249-250.

Frøkjær-Jensen, C. (2013). Exciting prospects for precise engineering of *Caenorhabditis elegans* genomes with CRISPR/Cas9. Genetics 195: 635-642. Abstract

Gems D., Riddle DL. (2000). Defining Wild-Type Life Span in *Caenorhabditis elegans*, The Journals of Gerontology, A55(5), B215–B219. https://doi.org/10.1093/gerona/55.5.B215

Griffiths, JR, Unwin RD (2016-10-12). Analysis of Protein Post-Translational Modifications by Mass Spectrometry. John Wiley & Sons. ISBN 9781119250883.

Hubbard, E. J. (2007). Caenorhabditis elegans germ line: a model for stem cell biology. Developmental dynamics: an official publication of the American Association of Anatomists, 236(12), 3343–3357. https://doi.org/10.1002/dvdy.21335

Hsieh, T. S. (1990). 7 Mechanistic Aspects of Type-II DNA Topoisomerases. Cold Spring Harb. Monogr. Arch. 20, 243–263. 10.1101/087969348.20.243

Klass, M. R. (1977). Aging in the nematode *Caenorhabditis elegans*: Major biological and environmental factors influencing life span. Mech. Ageing Dev. 6:413–429.

Klass, M R, Hirsh, D. (1976). Nonaging developmental variant of *Caenorhabditis elegans*. Nature. 260:523–525.

Klinefelter syndrome - Genetics Home Reference - NIH. (2019). Retrieved from https://ghr.nlm.nih.gov/condition/klinefelter-syndrome#

Lee, M. T., & Bachant, J. (2009). SUMO modification of DNA topoisomerase II: trying to get a CENse of it all. DNA repair, 8(4), 557–568. https://doi.org/10.1016/j.dnarep.2009.01.004

Mcfarlane, R. J., & Wakeman, J. A. (2017). Meiosis-like Functions in Oncogenesis: A New View of Cancer. Cancer Research, 77(21), 5712-5716. https://doi.org/10.1158/00085472.can-17-1535

McGovern, M., Yu, L., Kosinski, M., Greenstein, D., & Savage-Dunn, C. (2007). A role for sperm in regulation of egg-laying in the nematode C. elegans. BMC developmental biology, 7, 41. https://doi.org/10.1186/1471-213X-7-41

Nakazawa, N., Arakawa, O., Ebe, M., & Yanagida, M. (2019). Casein kinase II dependent phosphorylation of DNA topoisomerase II suppresses the effect of a catalytic topo II inhibitor, ICRF-193, in fission yeast. Journal of Biological Chemistry, 294(10), 3772-3782. https://doi.org/10.1074/jbc.ra118.004955

Nitiss, J. L. (2009). Targeting DNA topoisomerase II in cancer chemotherapy. Nature reviews. Cancer, 9(5), 338–350. https://doi.org/10.1038/nrc2607

Pawlowski, W. P., & Cande, W. Z. (2005). Coordinating the events of the meiotic prophase. Trends in Cell Biology, 15(12), 674-681. https://doi.org/10.1016/j.tcb.2005.10.005

Pierce, Benjamin (2009). «Chromossomes and Cell Reproduction». Genetics: A Conceptual Approach, Third Edition. W.H. FREEMAN AND CO. p. 32

Riddle, D. L., Blumenthal, T., & Meyer, B. J. (1997). C. elegans II. Secton II: Fertilization. 2nd edition (2nd ed.).NY: Cold Spring Harbor Laboratory Press. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK19994/

Shakes, D. C., Wu, J. C., Sadler, P. L., Laprade, K., Moore, L. L., Noritake, A., & Chu, D. S. (2009). Spermatogenesis-specific features of the meiotic program in *Caenorhabditis elegans*. PLoS genetics, 5(8), e1000611. https://doi.org/10.1371/journal.pgen.1000611

Turner syndrome - Genetics Home Reference - NIH. (2019). Retrieved from https://ghr.nlm.nih.gov/condition/turner-syndrome#genes

Vassetzky, Y. S., Dang, Q., Benedetti, P., & Gasser, S. M. (1994). Topoisomerase II forms multimers in vitro: effects of metals, beta-glycerophosphate, and phosphorylation of its C-terminal domain. Molecular and cellular biology, 14(10), 6962–6974. https://doi.org/10.1128/mcb.14.10.6962

Vos, S. M., Tretter, E. M., Schmidt, B. H., & Berger, J. M. (2011). All tangled up: How cells direct, manage and exploit topoisomerase function. *Nature Reviews Molecular Cell Biology, 12*(12), 827-841. https://doi.org/10.1038/nrm3228

Wang, J.C. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 3: 430–440. https://doi.org/10.1038/nrm831

Zielinska, D.F., Gnad, F., Jedrusik-Bode, M., Wiśniewski JR, Mann, M. (2009). *Caenorhabditis elegans* has a phosphoproteome atypical for metazoans that is enriched in developmental and sex determination proteins. J Proteome Res. 8:4039-4049. https://doi.org/10.1021/pr900384k

# Appendix A

## DELETION OF THE TOP-2 C-TERMINUS RESULTS IN STERILITY DUE TO A FAILURE IN GERMLINE DEVELOPMENT

TOP-2 has three main domains in its homodimeric structure: The ATPase domain, the catalytic zone, and the C-terminus. The C-terminal domain is the least conserved portion among TOP-2 homologs (Sengupta et al. 2003). In other organisms, there are phosphorylation and SUMOylation sites within the C-terminus of Type II topoisomerases that are recognized as being important for both protein regulation and post-translational modifications in mitosis (Ryu et al. 2015). To initially determine if the C-terminus is necessary for TOP-2 function during meiosis in *C. elegans,* a c-terminal deletion mutant was created using CRISPR/Cas9 genome editing and was given the allele designation of *top-2(ude8). top-2(ude8)* is not temperature sensitive and is maintained by picking balanced heterozygous *top-2(ude8)/mIn1* animals. Embryonic viability assays were performed on *top-2(ude8)/mIn1* and *top-2(ude8)/top-2(ude8)* hermaphrodites. The assays showed that *top-2(ude8)/mIn1* heterozygotes displayed high viability at 20°C, and *top-2(ude8)top-2(ude8)* homozygotes laid no progeny and were sterile (**Fig. A.1**). To determine the cause of sterility, *top-2(ude8)* homozygotes were examined through whole mount DAPI staining and compared to *top-2(ude8)/mIn1*. Imaging of the whole worm revealed that *top-2(ude8)* adult males and hermaphrodites displayed an absence of a fully developed germ line while *top-2(ude8)/mIn1* males and hermaphrodites showed normal germ line formation (**Fig. A.2**).

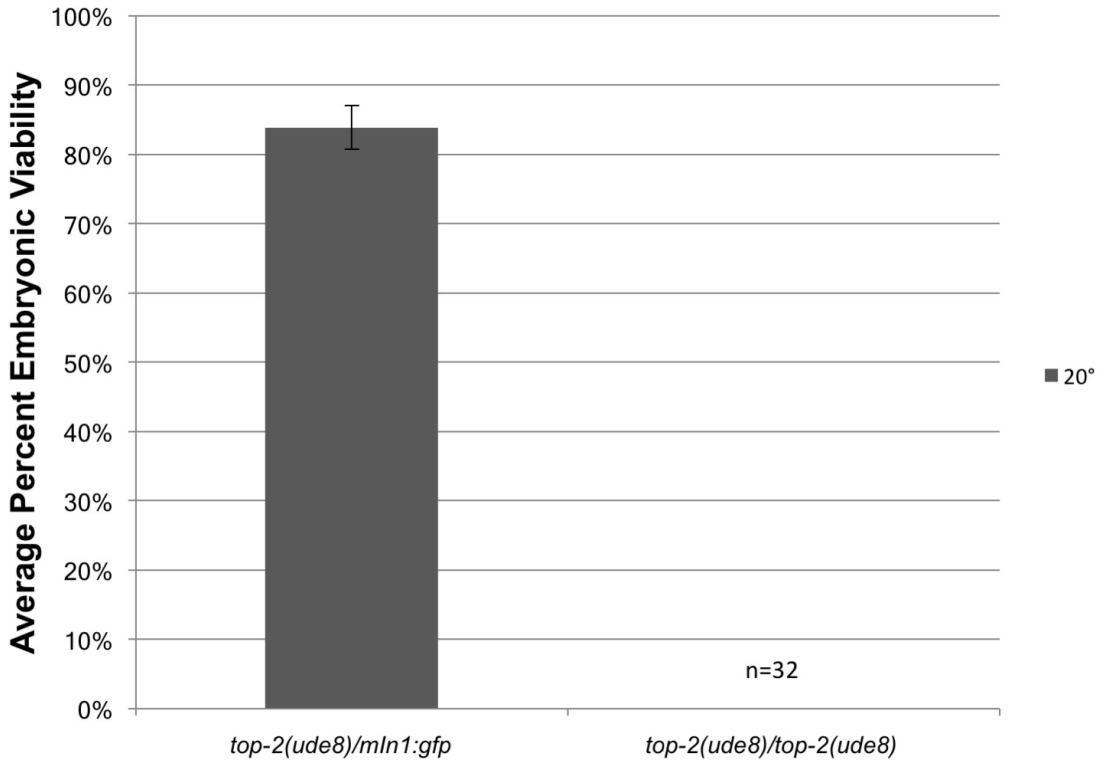


Figure A.1: Average percent embryonic viability of *top-2(ude8)/mIn1* and *top-2(ude8)/top-2(ude8)* at 20°C. n=32 hermaphrodites that were scored for *top-2(ude)* homozygotes at 20°C. Error bars represent the SEM of the averages of three individual replicate experiments.

## A.1 The C-terminus Has Several Potential Roles Needed for Both Structural Stability and Functionality of TOP-2

The C-terminus is the least conserved domain of topoisomerase II among homologs. Its purpose in eukaryotic topo II needs to be researched more, though there are some suggestions that it plays roles in regulation through PTMs. Recent reports found that phosphorylation of certain sites on the C-terminus play a role in decreasing topo II inhibition by anti-topo II drugs (Nakazawa et al., 2019). Another report studied SUMO in

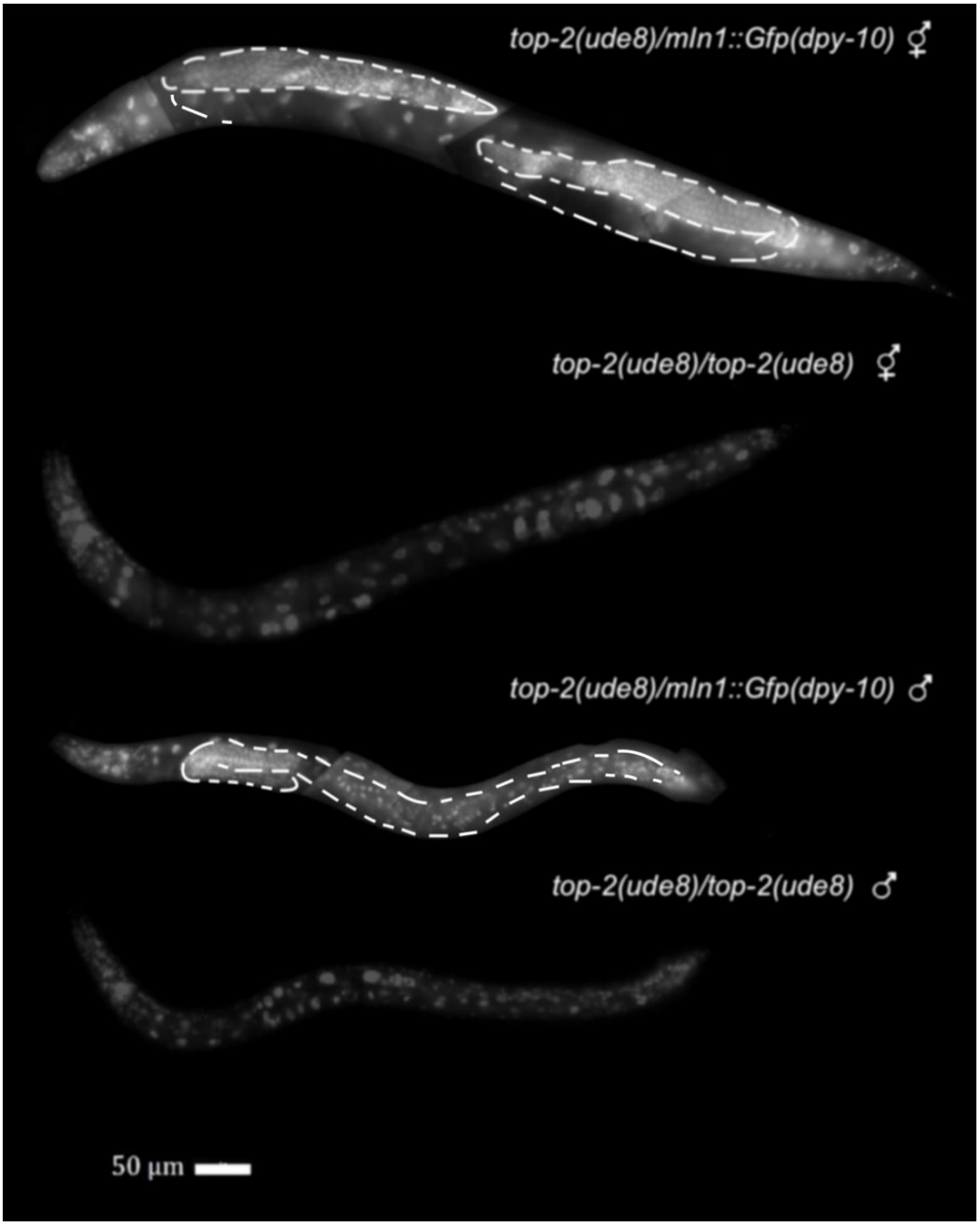


Figure A.2 C-terminal deletion mutants do not proliferate a germline. Representative images *top-2(ude8)* homozygotes and *top-2(ude8)/mIn1* heterozygotes. Images were taken following whole mount DAPI staining of both males and hermaphrodite adults for each strain. The dotted lines indicate where the gonad is.

yeast topo II and gathered that there is at least one SUMOylation site within the C-terminal domain (CTD) of several topo II homologs, and that it may help to release the enzyme in cases where it cannot completely facilitate its catalytic activity (Lee and Bachant, 2009). In *C. elegans* TOP-2, there are at least 11 identified phosphorylation sites and two potential SUMOylation sites within the C-terminal domain (Zielinski et al., 2009). Reports have mentioned that the presence of the CTD is dispensable in the strand-passage mechanism of TOP-2 (Clarke and Azuma, 2017). We questioned whether the C-terminus was necessary for proper TOP-2 functionality in *C. elegans*. The resulting sterility and failure in germline proliferation from our C-terminal deletion mutant *top-2(ude9)* highly support the thought that the C-terminus has functional significance. Future experiments include targeting the individual phosphorylation and putative SUMOylation sites as well as combinations of the mutations. We are in the process of using CRISPR/Cas9 to change the amino acids at those potential sites and characterizing the mutants to determine the importance or dispensability of those sites.

## Appendix B

## *top-2(it7)* SUPPRESSORS

The meiotic pathway consists of a series of protein-protein interactions that help mediate the complex pathways involved in karyokinesis and cytokinesis. The role and placement of TOP-2 in the meiotic pathway has not been fully illuminated, and discovering known upstream or downstream factors that genetically interact with *top-2* can help to shed light on its roles in meiosis. One way of finding potential genetic interactors of a gene is through screening for suppressors of mutant phenotypes of that particular gene. In this case, *top-2(it7)* mutants were exposed to EMS mutagenesis and the F2 progeny were screened through embryonic viability assays at 24°C. Progeny that restored embryonic viability to some degree were further screened and their genome analyzed to determine the molecular lesions resulting in suppression (Jaramillo-Lambert, unpublished data). This screen identified 11 suppressors of *top-2(it7)* embryonic lethality (**Fig. B1**). As a side project, our goal was to better characterize the different suppressor mutations (List of strains in **Table B.1**). Within this objective was the examination of overall TOP-2 protein levels of each suppressor following an incubation at the restrictive temperature.

### B.1 TOP-2 protein levels in suppressors of *top-2(it7)*

Eleven independent suppressor lines were recovered from 80,000 haploid genomes. Ten of these suppressors are due to dominant mutations and one is recessive.

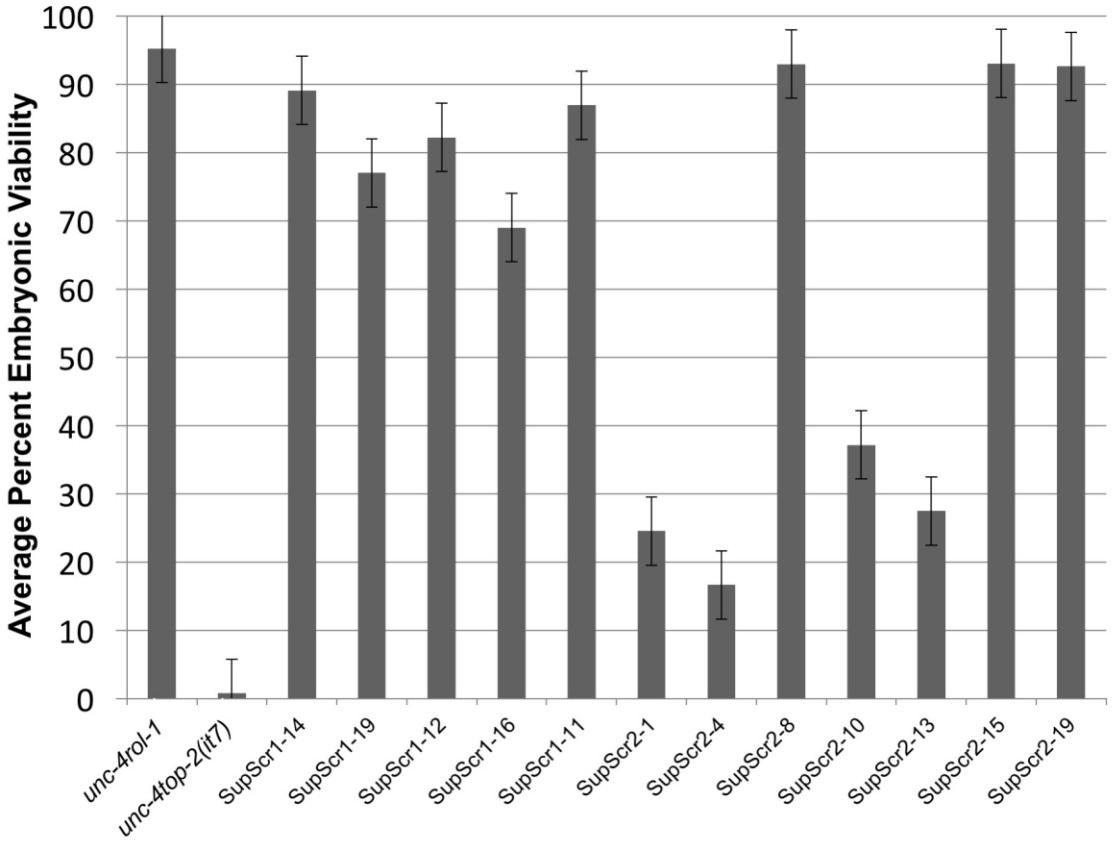


Figure B.1: Average percent embryonic viability performed on suppressor mutants during 24°C incubation. See Table 2.1 for corresponding strains/genotypes. Courtesy of Aimee Jaramillo-Lambert.

Further analysis found that ten of the suppressors are unlinked from *top-2(it7)* and one is linked and intragenic. Embryonic viability assays reveal that each mutant suppresses the lethality of *top-2(it7)* at 24°C to an extent, though the viabilities vary among them (**Fig. B.1**, Jaramillo-Lambert, unpublished data).

Western blotting performed on *top-2(it7)* mutants following a 24°C incubation indicate that TOP-2 protein levels are reduced (**Fig. B.2**). To determine whether the suppressors rescue the reduction of TOP-2 protein levels in *top-2(it7)*, Western blotting

Table B.1: Strains of *C. elegans* in suppressor project, in order by strain number.

| Strain | Description | Comments |
|---|---|---|
| **KK381** | *unc-4(e120)top-2(it7)* | uncoordinated phenotype; temperature-sensitive |
| **AJL2** | *unc-4(e120)top-2(it7);tdpt-1(ude2)* [SupScr1-11] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL4** | *unc-4(e120)top-2(it7);tdpt-1(ude4)* [SupScr1-16] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL5** | *unc-4(e120)top-2(it7);tdpt-1(ude5)* [SupScr1-14] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL6** | *unc-4(e120)top-2(it7ude6)*[SupScr1-19] | intragenic suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL7** | *unc-4(e120)top-2(it7);tdpt-1(ude7)* [SupScr2-19] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL16** | *unc-4(e120)top-2(it7);tdpt-1(ude11)* [SupScr12b] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL18** | *unc-4(e120)top-2(it7);tdpt-1(ude13)* [SupScr2-8] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL19** | *unc-4(e120)top-2(it7);ude14* [SupScr2-1] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL20** | *unc-4(e120)top-2(it7);ude15* [SupScr2-4] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |
| **AJL21** | *unc-4(e120)top-2(it7);ude16* [SupScr2-10] | suppressor of it7ts embryonic lethality; EMS mutagenesis of KK381 |

was performed following 24°C incubations on each suppressor. Preliminary data suggests that several of the suppressors show at least partial restoration of TOP-2 levels, with the exception of SupScr2-19, which remains unclear (**Fig. B.2**). It appears that several of the suppressors display a restoration of TOP-2 levels while others do not, but these experiments need repetition. Currently, we are validating the candidate mutations that have been identified. We are also currently performing TOP-2 localization experiments in

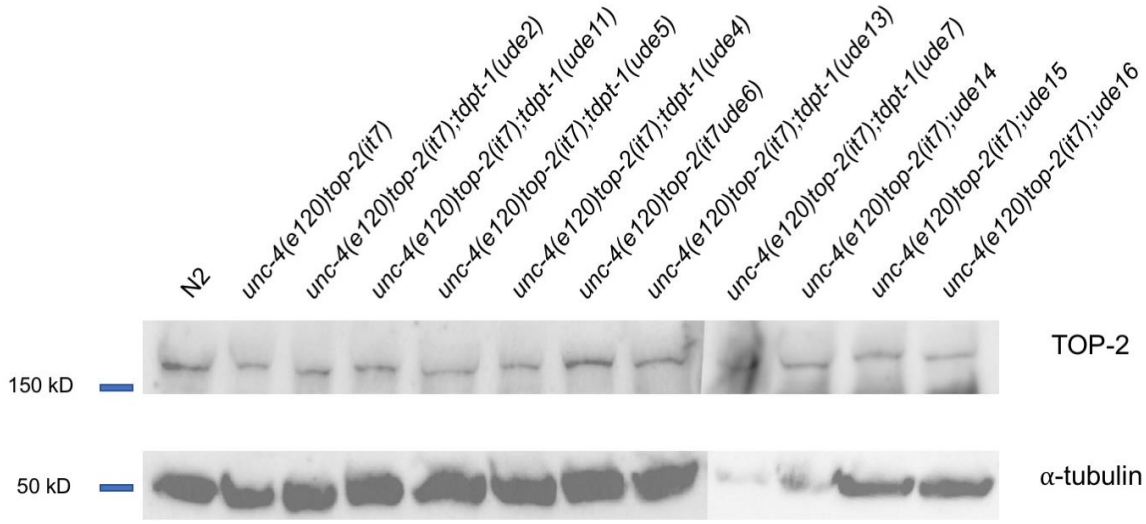


Figure B.2: TOP-2 protein levels of suppressors of *top-2(it7)*. Preliminary Western blotting of N2, *unc-4(e120)top-2(it7), unc-4(e120)top-2(it7);tdpt-1(ude5), unc-4(e120)top-2(it7ude6, unc-4(e120)top-2(it7);tdpt-1(ude11), unc-4(e120)top-2(it7);tdpt-1(ude4), unc-4(e120)top-2(it7);tdpt-1(ude2), unc-4(e120)top-2(it7);ude14, unc-4(e120)top-2(it7);ude15, unc-4(e120)top-2(it7);tdpt-1(ude13), unc-4(e120)top-2(it7);ude16,* and *unc-4(e120)top-2(it7);tdpt-1(ude7)*. Whole plate washes were performed for lysate preparation for each strain following a 16 h incubation at 24 °C. α-tubulin was used as a loading control.

the suppressors. In the future, we hope to elucidate the putative interactions between the candidate suppressor proteins and TOP-2 and determine the mechanism of suppression for each mutation.

Table B.2: Candidates genes identified by whole genome sequencing. Courtesy of Aimee Jaramillo-Lambert.

| Intragenic/ Extragenic | Suppressors | Candidate gene | Suppressor Allele | Mutation |
|---|---|---|---|---|
| **Intragenic** | SupScr1-19 | *top-2* | *ude6* | D809N |
| **Extragenic** | SupScr1-14 | *tdpt-1* | *ude5* | G270D |
| | SupScr1-12 | *tdpt-1* | *ude3* | G219E |
| | SupScr1-16 | *tdpt-1* | *ude4* | G117R |
| | SupScr1-11 | *tdpt-1* | *ude2* | G328E |
| | SupScr2-1 | *mep-1*<br>*nurf-1* | *ude14* | G57D<br>E1954K |
| | SupScr2-4 | *top-3* | *ude15* | P699S |
| | SupScr2-8 | *tdpt-1* | *ude13* | A355T |
| | SupScr2-10 | *viln-1*<br>*nlp-38* | *ude16* | R552C<br>G77R |
| | SupScr2-15 | *tdpt-1* | *ude5* | G270D |
| | SupScr2-19 | *tdpt-1* | *ude7* | G270S |