New Role for an Old Protein: An Educational Primer for Use with "The Identification of a Novel Mutant Allele of *topoisomerase II* in *Caenorhabditis elegans* Reveals a Unique Role in Chromosome Segregation During Spermatogenesis"

Ruby Boateng and Anna K. Allen[1]
Department of Biology, Howard University, Washington, DC 20059

ABSTRACT Modern experimental techniques, such as whole-genome sequencing and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 endogenous genome editing, are enabling researchers to identify and further characterize the roles of proteins that were previously thought of as well defined. In the December 2016 issue of *GENETICS*, an article by Jaramillo-Lambert *et al.* identified a new role for the enzyme topoisomerase II in *Caenorhabditis elegans* male meiosis. This Primer article is designed to provide essential background information on *C. elegans* spermatogenesis and the relevant scientific techniques that will assist students and instructors in their understanding and discussion of the related article.

Related article in *GENETICS*: Jaramillo-Lambert, A., A. S. Fabritius A. S., T. J. Hansen T. J., H. E. Smith H. E., and A. Golden A., 2016 The identification of a novel mutant allele of topoisomerase II in *Caenorhabditis elegans* reveals a unique role in chromosome segregation during spermatogenesis. *Genetics* **204:** 1407–1422.

KEYWORDS *Caenorhabditis elegans*; meiosis; spermatogenesis; oogenesis; topoisomerase

TABLE OF CONTENTS

doi:https://doi.org/10.1534/genetics.117.300482
[1]Corresponding author: Department of Biology, Howard University, 415 College St., NW, Just Hall, Rm. 244, Washington, DC 20059. E-mail: anna.allen@howard.edu

WHILE the function of DNA in encoding gene products is essential, it is also equally important that DNA maintains the ability to make copies of itself via replication. Upon division of an existing cell into new daughter cells, via either mitosis or meiosis, the DNA must be copied with a high degree of accuracy and one complete set of chromosomes (either in a diploid or haploid state depending on the division cycle) must end up in each of the newly generated cells. This ensures that the new cells contain the entire genome of the organism. Errors during DNA replication, such as a failure to appropriately copy a portion of the DNA or unrepaired breaks in the DNA strands, can lead to a genetic mutation and have serious consequences for the resultant daughter cells. If these altered DNA sequences arise during mitotic cell divisions, the defects are passed down from one cellular generation to the next within an individual organism. However, if the alteration occurs during a meiotic cell division, the defects can be passed to the subsequent generation and result in progeny within which every cell contains the genomic defect.

An additional genetic conundrum can arise during both replication and transcription as the double-stranded DNA molecule is unwound to provide access for the replication and transcription machinery. This unwinding at one location in a DNA molecule results in overwinding at another location in that same DNA molecule. Overwinding results in topological stress on the DNA molecule. This stress must be alleviated, and that occurs via a special set of enzymes called DNA topoisomerases that come in two flavors: type I and type II enzymes (Nitiss 2009). Type I enzymes introduce a transient single-strand break to alleviate the topological stress, while type II enzymes introduce a transient double-strand break (DSB) to accomplish the same purpose. The key word here is transient, as the strand break occurs solely to provide tension/stress relief and the break is typically quickly repaired. The action of type I and type II DNA topoisomerases has been extensively studied in mitosis, but less information is known about their roles during meiosis.

Jaramillo-Lambert *et al.* (2016) have taken advantage of a novel, previously uncharacterized, genetic allele of the topoisomerase II gene (*top-2*) in the worm *Caenorhabditis elegans*. Their research on this particular novel *top-2* mutation has identified a new role for the TOP-2 enzyme in male meiosis. It is genetic research such as this study, utilizing an established model organism system, that enables us to uncover unexpected and new roles for proteins previously thought to be well characterized and understood.

The Model System: The C. elegans Germ Line

The nematode *C. elegans* is a powerful genetic system due to its short generation time, completely sequenced genome, relative ease with which it can be worked with under laboratory conditions, optical transparency, and for the various tools available to conduct genetic experiments [reviewed in Corsi *et al.* (2015)]. *C. elegans* exist as two sexes: hermaphrodites (genetically XX) that are capable of producing oocytes and sperm, and males (genetically XO) that produce just sperm. Both sexes pass through an embryonic stage and four successive larval developmental stages (L1, L2, L3, and L4) before developing into a sexually mature adult. During larval stages, the animal undergoes periods of growth in size along with development of the various organ systems, such as the reproductive system.

The adult hermaphrodite reproductive system is a bi-loped U-shaped structure that consists of two gonad arms connected to a common shared uterus, which contains the vulva or exit point for embryos (Figure 1A). The adult male reproductive system is a single J-shaped structure that opens to the exterior via the posterior male copulatory apparatus (Figure 1B) (Lints and Hall 2009b). During embryogenesis and the L1–L2 stages, the somatic and germ line components of the reproductive systems are specified and growing (Hubbard and Greenstein 2005; Pazdernik and Schedl 2013). From late L3 through the L4 stage, mitotically dividing germ cells at the distal end of the hermaphrodite and the male gonads undergo spermatogenesis (male meiosis) to produce haploid spermatids. The spermatids are stored in the spermathecae in hermaphrodites and the seminal vesicle in males (Figure 1) (L'Hernault 2006). The male germ line produces spermatids throughout the animal's life span; however, after the L4 molt, the hermaphrodite germ line switches to undergoing oogenesis (female meiosis) resulting in haploid oocytes. The hermaphrodite will continue to produce oocytes throughout its adult life. *C. elegans* researchers take advantage of this switch in the hermaphrodite between L4 spermatogenesis and adulthood oogenesis to study genes involved in male and female meiosis within the same animal.

An advantage to studying meiosis in the *C. elegans* gonad is the distinct germ line architecture that enables one to easily identify and follow the various stages of meiosis in a linear progression [reviewed in Hillers *et al.* (2015)]. At a very basic level, both the hermaphrodite and male gonads are one-directional tubes, sending meiotically dividing cells from the blunt (distal) end of the tube to the tube exit. The DNA stain DAPI can be utilized to visualize changes in germ cell chromosome morphology associated with progression through the various stages of meiosis, including the substages of prophase I (leptotene, zygotene, pachytene, diplotene, and diakinesis). Germ cells initially proliferate or increase in number by undergoing mitosis in the most distal end of the gonad (Figure 1) (Hall *et al.* 1999; Hubbard and Greenstein 2000). There is then a transition zone where germ cells leave the mitotic cell cycle and enter the reductional division of meiosis I. The transition zone is comprised of nuclei in leptotene and zygotene of meiotic prophase I. In leptotene, the chromosomes are shortening, and during zyogtene the chromosomes pair with their homologous partner and begin to synapse (Shakes *et al.* 2009). Nuclei in leptotene/zyogtene are easily identifiable via half-moon-shape DAPI staining located toward one side of the nucleus (Figure 2). The nuclei then enter pachytene, where paired homologous chromosomes undergoing recombination, or crossover events, are located around the edges of the nucleus. This phenotype has been described as appearing like a "bowl of spaghetti" (Figure 2) (Hubbard and

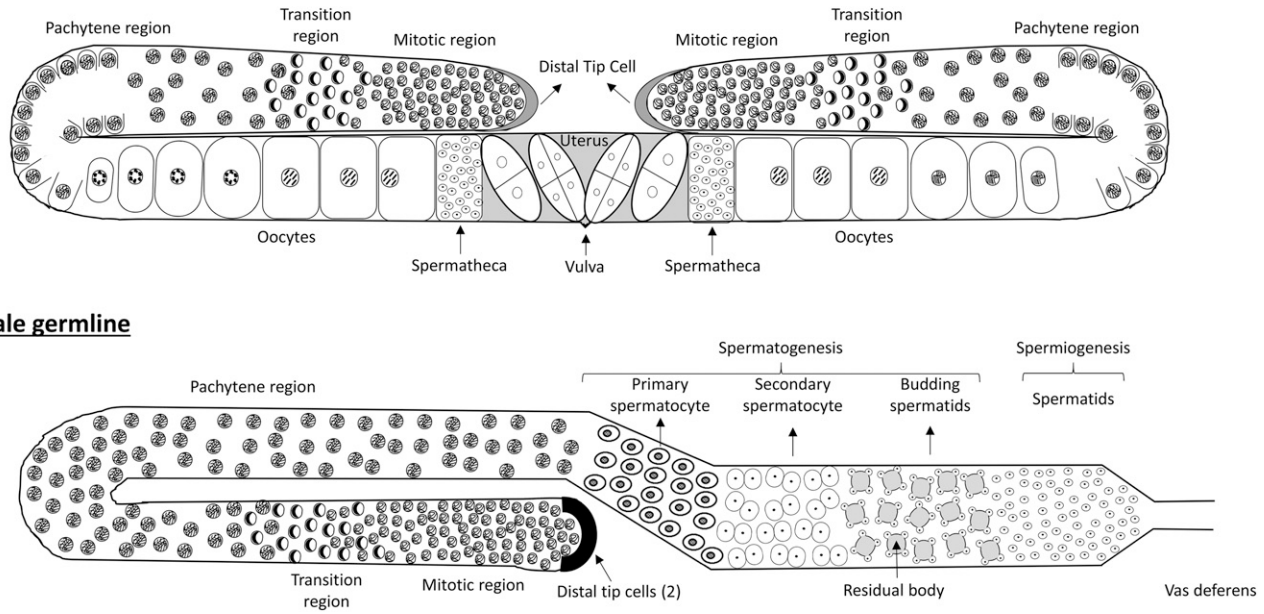

Figure 1 The hermaphrodite and male *C. elegans* reproductive systems. (A) A schematic of the hermaphrodite bilobed, U-shaped gonad. The distal end contains the mitotically dividing germ cells, and germ cells progress toward the spermatheca (sperm storage organ) undergoing the various stages of prophase I of meiosis. The uterus contains embryos undergoing early development, which will ultimately be laid through the vulva to the external environment. (B) A schematic of the male gonad with germ cells undergoing mitosis at the distal end and progressing through the various stages of meiosis as they move toward the vas deferens. For both (A) and (B), the chromosome configurations can be utilized to identify specific stages of meiosis.

Greenstein 2000; Lints and Hall 2009a). By late pachytene, the synaptonemal complex holding the paired chromosomes together starts to break down and, upon entering diplotene, the chromosomes begin to condense such that six bivalents, or pairs of homologous chromosomes, are observed (Figure 2). There is a spermatogenesis-specific feature of meiosis that occurs at this point, and the male chromosomes go through extensive aggregation such that individual chromosomes are not resolvable by microscopy and form a structure known as the karyosome (Figure 2, top panel) (Shakes *et al.* 2009). While the karyosome structure has been described in multiple species, its function is unclear. The final substage of prophase I, diakinesis, is characterized in oogenesis by further condensation of the bivalents to their greatest extent, and in spermatogenesis by observing aggregated chromosomes in the karyosome separating slightly (Figure 2).

In most female animal species, the end of prophase I is marked by an extended period of meiotic arrest in oocytes that does not occur in germ cells undergoing spermatogenesis (Pazdernik and Schedl 2013). In *C. elegans*, oocytes arrest in diakinesis and await a signal from the sperm stored in the spermatheca to complete meiosis (Kim *et al.* 2013). Once the signal has been received, the oocyte will resume meiosis I, but requires fertilization to complete the meiotic division, ultimately generating one haploid oocyte and two polar bodies (Figure 2, bottom panel) (Kim *et al.* 2013; Hillers *et al.* 2015). In contrast, cells undergoing spermatogenesis proceed through meiosis without arrest or interruption (Chu and Shakes 2013). A 4N primary spermatocyte undergoes prophase I, metaphase I, and anaphase I to generate two 2N secondary spermatocytes (Figure 2, top panel). The secondary spermatocytes can either undergo complete cytokinesis, where the 2N cells totally separate from each other, or incomplete cytokinesis, resulting in partially separated 2N cells. Subsequently, each secondary spermatocyte then undergoes metaphase II and anaphase II, followed by incomplete cytokinesis to generate a total of four haploid spermatids. Incomplete cytokinesis in meiosis I results in four haploid spermatids emanating from a central anucleate body termed the residual body, to which cytosolic components are transferred (Figure 2, top panel) (L'Hernault 2006; Chu and Shakes 2013). Complete cytokinesis results in two separate secondary spermatocytes undergoing meiosis II, and the formation of two residual bodies each with two haploid sperm budding off.

RNA interference (RNAi): A Method for Reducing Gene Expression

RNAi is an innate response present within animals and plants, wherein the presence of double-stranded RNA (dsRNA) is recognized as "foreign" and the host activates specific machinery to degrade the potentially damaging dsRNA (Wilson and Doudna 2013). It is proposed that this inherent response arose as a defense mechanism against infection by viruses, some of which utilize dsRNA as their genomic material. RNAi can also be used to reduce the level of gene expression for a given gene of interest by directly targeting the amount of messenger RNA

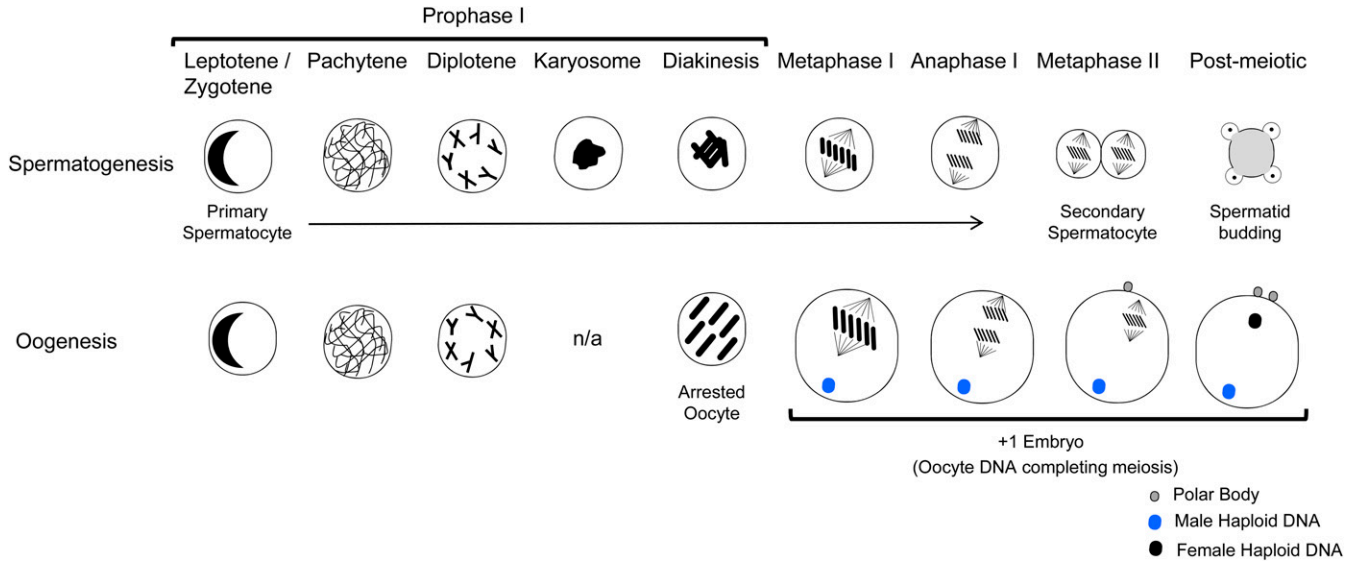

Figure 2 Chromosome dynamics during *C. elegans* spermatogenesis and oogenesis. Following chromosome appearance can help determine which stage of meiosis a germ cell is in. In this figure, the chromosomes appear as dark lines. Karyosome formation is only observed during spermatogenesis. Note that spermatids complete meiosis before fertilization, while oocyte meiosis completion is triggered upon fertilization in the +1 embryo. n/a, not applicable.

(mRNA) available for translation (Hannon 2003). The mechanism of RNAi was elucidated first in *C. elegans* by Andrew Fire and Craig Mello, for which they were awarded the Nobel Prize in Physiology or Medicine in 2006 (Nobelprize.org 2006).

For over two decades, scientists have been utilizing RNAi as an experimental tool in the laboratory to manipulate or knock down the levels of specific gene targets by introducing exogenous dsRNA into an organism. The introduced dsRNA actives the RNAi pathway and results in the degradation of mRNA complementary to the initial dsRNA. In *C. elegans*, performing an RNAi experiment is accomplished by feeding, soaking, or injecting the animals with dsRNA that is complementary to the target gene of interest (Ahringer 2006). Through RNAi, *C. elegans* researchers have been able to investigate the function of many genes throughout development and during adulthood. Importantly, RNAi has provided an avenue to study the function of essential genes later on in the organism. One can allow the animal to develop to adulthood and then treat with dsRNA, thereby activating the RNAi pathway and knocking down the levels of that particular gene only in the adult and their progeny. Specifically, with regard to investigating the role of particular genes during meiosis (oogenesis or spermatogenesis), researchers can feed dsRNA to L3 hermaphrodites to deplete the gene product of interest during both spermatogenesis and oogenesis, or to L4 hermaphrodites to deplete the gene product during oogenesis only. This is one great advantage of asking reproductive questions in *C. elegans*.

Whole-Genome Sequencing (WGS): A Method to Uncover Genetic Mutations

Technological advances have eased our ability to identify the causative genetic change(s) that result in a specific mutant phenotype. Previously, one performed the time-consuming process of classical three-factor genetic mapping to narrow down a mutation location to a chromosomal region, and even with that effort there could potentially be numerous genes within the region! With WGS, a researcher uses next-generation sequencing technology to determine the complete DNA sequence of the mutant organism's genome (Shendure and Ji 2008). The resulting mutant genomic sequence is computationally compared against a reference wild-type genomic sequence and variations between the two sequences are determined. However, since mutagenesis can generate hundreds of base pair mutations, and there already exist naturally occurring nucleotide variations between different strains, this still leaves the researcher investigating multiple mutations to identify which one causes the phenotype of interest.

Thus, researchers have developed strategies to assist with mutational identification after WGS that include minimizing the possible number of identified mutations. One way combines single-nucleotide polymorphism (SNP) mapping with WGS (Doitsidou *et al.* 2010). A SNP is a DNA sequence variation that is common (found at $> 1\%$) in the population. In contrast, a mutation is a DNA variation that occurs in $< 1\%$ of the population. *C. elegans* strains isolated from different geographic locations have distinct SNPs already identified that allow researchers to discriminate between the isolates. Most *C. elegans* researchers utilize the N2 strain originally isolated in England; however, the Hawaiian strain is another isolate that has ~100,000 SNPs differentiating it from N2.

SNP mapping involves taking the mutant of interest, typically in an N2 strain background, and outcrossing or mating it to a different isolate, such as Hawaiian (Figure 3). Outcrossing N2 to Hawaiian introduces unrelated genetic material and generates recombinant chromosomes in the progeny that are a mix of N2 and Hawaiian DNA. If you outcross and select among the progeny for individuals exhibiting the

mutant phenotype of interest, the genomic region surrounding that mutation will still be made up of N2 sequence that originally harbored the mutation. Subjecting the outcrossed progeny to WGS results in regions away from the mutation site exhibiting an equal percentage of N2 *vs.* Hawaiian SNPs, while the genomic region close to the mutation will have a decreased number of Hawaiian SNPs and more N2 SNPs (Jaramillo-Lambert *et al.* 2015). Software tools are used to analyze the WGS data, and identify both the SNPs (compared to the N2 reference) and the ratio of Hawaiian *vs.* N2 sequence at each particular position along a chromosome (Figure 3). Combining SNP mapping and WGS makes the process of identifying the molecular identity of a mutation significantly faster.

CRISPR/Cas9: Endogenous Genome-Editing Technology

CRISPR stands for Clustered Regularly Interspersed Short Palindromic Repeats, while Cas9 is a CRISPR-associated protein 9. Experimental researchers have enthusiastically embraced the endogenous genome-editing technique CRISPR/Cas9 (Doudna and Charpentier 2014; Lander 2016). CRISPR/Cas9 genome-editing technology is based on the Type II *Streptococcus pyogenes* bacterial adaptive immune-like system that is utilized to respond to invading pathogens such as viruses (Jinek *et al.* 2012). The CRISPR/Cas9 system is a fast, inexpensive, and most importantly, specific and accurate genome-editing technique.

CRISPR/Cas9 is a multiple-step process by which an RNA-guided endonuclease Cas9 protein generates a DSB within a specific region in the genome, and then repairs the break either by error-prone Nonhomologous End Joining (NHEJ) or Homology-Directed Repair (HDR) (Figure 4) (Jinek *et al.* 2012; Doudna and Charpentier 2014). Two key molecules are required to generate the DSB: (1) the endonuclease enzyme Cas9 and (2) a piece of RNA, the single-guide RNA (sgRNA), which directs Cas9 to the desired point in the genome. The sgRNA is a fusion RNA molecule made up of a 20-bp RNA sequence complementary to the target genomic sequence (the CRISPR RNA or crRNA) and a *trans*-activating CRISPR RNA (a 74-bp RNA sequence that binds the Cas9 protein), thereby bringing the endonuclease to the desired locale for introduction of the DSB (Figure 4, upper) (Jinek *et al.* 2012). To initiate a DSB, the Cas9 enzyme must interact with a *protospacer-adjacent motif* (PAM) in the genome, which, for *S. pyogenes* Cas9, is denoted by the sequence "NGG," where N stands for any of the four DNA nucleotides and G is guanine (Dickinson *et al.* 2013). Upon interaction with the PAM site, Cas9 initiates a DSB three nucleotides upstream of the PAM site (Jinek *et al.* 2012). By manipulating the sequence of the crRNA, the ribonucleoprotein Cas9 complex can be programmed to precisely target any PAM site within the genome (Paix *et al.* 2015).

Once the DSB is generated by Cas9, the break is repaired by error-prone NHEJ, which can lead to the addition or subtraction of base pair sequences, or HDR, which involves the introduction of an exogenous donor repair template (Figure 4, bottom) (Addgene 2016). In the *C. elegans* field, HDR is commonly used since most researchers want to make specific changes (*i.e.*, deletions, insertions, or substitutions) within their targeted genomic region (Dickinson and Goldstein 2016). To precisely repair the DSB via HDR, either a single-stranded oligonucleotide (ssODN) or a dsDNA PCR product is utilized as a repair template. The ssODN is an oligonucleotide consisting of 30–80 bp of sequence homology on either side of the cut site, and includes the new genomic modifications (substitutions, additions, or deletions) that the researcher desires to introduce into the genome. For insertions of small tags (such as 3xFLAG) or Green Fluorescence Protein (GFP), a dsDNA PCR repair product can be used as the template. The PCR repair product consists of DNA sequence coding for the desired insert (for example, GFP) and 30–60 bp homology to the genomic sequence flanking the cut (Figure 4, bottom). The dsDNA repair is made by PCR amplification from the appropriate vector, and ensuring that the forward and reverse PCR primers have 30–35 bp sequence homology to the genomic insertion site and sequence homology to the tag (Paix *et al.* 2015).

Unpacking the Experiments

Jaramillo-Lambert *et al.* (2016) were interested in identifying sperm-provided components that are essential for embryogenesis. In many animal systems, the contribution of the female gamete to successful early development has been investigated in detail, while less effort has been devoted to thoroughly investigating the male contribution. Historically, the male gamete has been thought to provide only two items necessary for early embryogenesis: the haploid paternal DNA and centrosomes. Delegating the paternal contribution to these two specific roles is potentially a very faulty and dangerous generalization that could limit our understanding of the true contribution of the male gamete to fertilization and embryogenesis. This is the premise that Jaramillo-Lambert and colleagues based their research question around.

Characterization and genomic identification of a new temperature-sensitive, paternal-effect lethal mutant allele

Jaramillo-Lambert *et al.* (2016) started their search by reanalyzing embryonic-lethal (Emb) mutants that had been previously generated via genetic screens and identifying mutants that could be rescued by male mating. A mutation is considered Emb if it results in the death of the animal during the embryonic period prior to organogenesis. Embryonic lethality can be a result of either zygotic lethality (abbreviated Zyg) or maternal-effect lethality (abbreviated Mel). An Emb mutation that is zygotic lethal (Zyg) must be maintained in its heterozygous state (one mutant copy, one wild-type copy) in order for the strain to survive. Individuals that receive two mutant copies would be Emb and not develop past the embryonic stage. Mel mutations are those that exhibit embryonic lethality only if the mother is homozygous for the mutation, and the genotype of the embryo is not significant. These two categories of mutations, Zyg and Mel, help

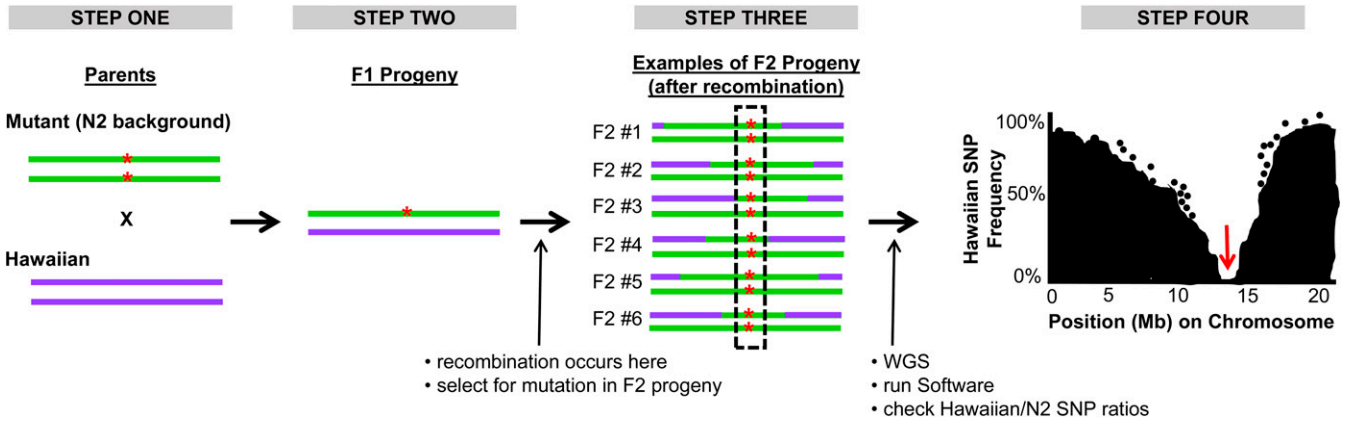

Figure 3 Strategy for one-step Hawaiian single-nucleotide polymorphisms (SNP) mapping and whole-genome sequencing (WGS). The red asterisks indicate the mutation of interest in an N2 background. Step 1 involves mating the strain carrying the mutation of interest to Hawaiian males. Step 2 involves allowing the F1 progeny to self-fertilize, thereby generating F2 progeny that have undergone recombination events between the original N2 and Hawaiian chromosomes. Step 3 involves isolating F2 animals exhibiting the mutation phenotype of interest and that represent a unique recombination event between the N2 (Bristol) and Hawaiian chromosomes. The progeny of the F2 hermaphrodites are then pooled, the DNA isolated, and WGS performed. Step 4 involves computationally analyzing the results of the WGS and the Hawaiian/N2 SNP ratios to determine the genomic loci, which has little to no Hawaiian SNPs, and thus is comprised of N2 genomic sequence. The researcher would then have a narrowed down genomic region to look at for the causative mutation (the boxed region in Step 3 and red arrow in Step 4).

discriminate whether the expression of a gene is needed maternally (provided by the mother into the oocyte) or zygotically (made by the newly fertilized zygote).

When an Emb mutant *C. elegans* hermaphrodite begins to lay viable progeny after mating with a wild-type male, it suggests that there are problems with the hermaphrodite mutant sperm, and that the hermaphrodite mutant oocytes are capable of being fertilized and undergoing successful early embryogenesis. This mutation should then be classified as a Pel mutation, standing for paternal-effect embryonic lethal. Jaramillo-Lambert and colleagues identified one intriguing temperature-sensitive allele that fit the above criteria, originally called *mel-15*(*it7*) because, when first isolated, it was thought to be a Mel allele. Temperature-sensitive mutants are genetic mutations that behave effectively wild-type at one temperature (the permissive temperature) and only exhibit their mutant phenotype when placed at a second, restrictive temperature. In this case, *mel-15*(*it7ts*) has dramatically reduced progeny viability when grown at 24° (restrictive temperature) compared to normal progeny viability when grown at 15° (permissive temperature) (see Figure 1A in the original paper).

The researchers then took advantage of the biology of the hermaphrodite germ line to ask whether the *mel-15*(*it7ts*) allele was playing a role during spermatogenesis, oogenesis, or both to affect embryonic viability (see Figure 1B in the original paper). They performed "shift-up" experiments, where animals were kept at the permissive temperature of 15° during the L3 larval stage when spermatogenesis is occurring and then shifted to the restrictive temperature of 24° during the adult developmental stage when oogenesis occurs. A "shift-up" experiment, as described, would cause the temperature-sensitive (*ts*) allele to have its mutant effect only during oogenesis. They also performed the reciprocal "shift-down" experiments. This is where animals are kept at the restrictive temperature of 24° during spermatogenesis (L3–L4 stage) and then moved to the permissive temperature of 15° during oogenesis (adult stage). In this "shift-down" experiment, the temperature-sensitive allele would have its mutant effect only during spermatogenesis. When progeny viability assays were performed after each of these experiments, hermaphrodites that had completed spermatogenesis at 24° had progeny with reduced embryonic viability compared to those that completed spermatogenesis at 15°, thus indicating an issue during spermatogenesis (Jaramillo-Lambert *et al.* 2016). The researchers further confirmed that the *it7* allele was Pel by taking *it7* mutant males raised entirely at the restrictive temperature (24°) and mating them to wild-type hermaphrodites. In this case, all progeny were Emb, indicating that the defect in *it7* animals is with the sperm.

The next step was to identify the genetic location of the *mel-15*(*it7*) allele. In the original genetic screen that generated the *it7* allele, the alleles were named based on their phenotypic characterization and not mapped to specific genomic locations. Thus, the gene affected by this mutation was not known. Utilizing Hawaiian SNP-based mapping and WGS, Jaramillo-Lambert and colleagues identified the exact location of the genetic variation in the *it7* allele. They discovered a genomic alteration in the *top-2* gene that led to an amino acid change at position 828 in the protein (arginine 828 to a cysteine). A complementation test with a *top-2* deletion allele was used to confirm that the *mel-15*(*it7ts*) mutation was in fact in the *top-2* gene. In this assay, two different recessive mutants are mated to generate a *trans*-heterozygote. The phenotype of the *trans*-heterozygote is then observed to see if the two mutations complement (restore wild-type phenotype and thus are in two separate genes) or fail to complement (continue to exhibit the mutant phenotype and thus are mutations in the same gene) [reviewed in Yook (2005)].

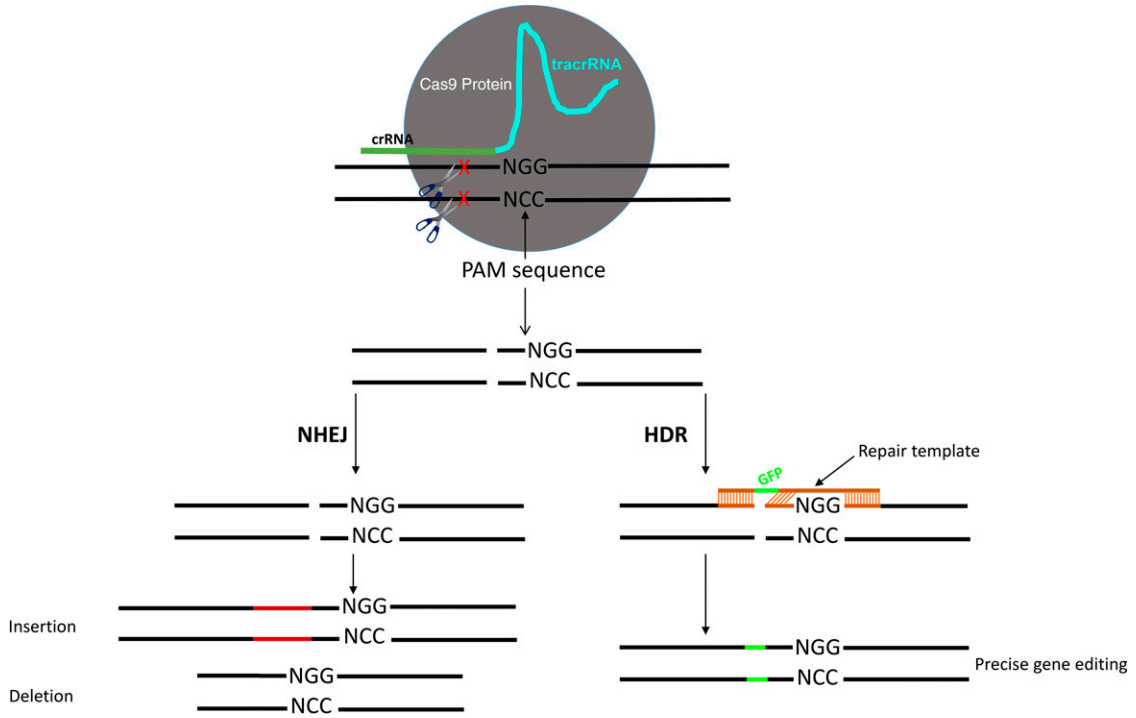

Figure 4 Overview of CRISPR/CAS-9 endogenous genome editing. The DNA sequence to be edited is recognized by the crRNA portion of the sgRNA. The tracrRNA portion of sgRNA brings the Cas9 endonuclease enzyme. Cas9 must bind to a PAM site before initiating a DSB upstream of the PAM site. The DSB can then be repaired by error-prone NHEJ or by error-free HDR utilizing a specifically designed repair template. CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR RNA; DSB, double-strand break; HDR, homology-directed repair; NHEJ, nonhomologous end joining; PAM, *protospacer-adjacent motif*; sgRNA, single-guide RNA; tracrRNA, *trans*-activating CRISPR RNA.

The researchers turned to the endogenous genome-editing technique CRISPR/Cas9 to generate two genome modifications that further confirmed *mel-15*(*it7ts*) as a mutation in the *top-2* gene. They made a "reverted line," where they took the original *mel-15*(*it7ts*) line and changed the mutant nucleotide back to the wild-type nucleotide, thus restoring the proper amino acid at position 828. This reverted line was embryonic viable at both the permissive and restrictive temperatures (see Figure 2B in the original paper). They also "recreated" the single-nucleotide change observed in the *mel-15*(*it7ts*) strain in N2 wild-type animals. They were able to generate multiple recreated lines, and each line had significant decreases in embryonic viability at the restrictive temperature. Both these pieces of evidence, when combined with the WGS of *mel-15*(*it7ts*), supports the notion that the *it7ts* allele is actually in the *top-2* gene, and should hence forth be called *top-2*(*it7ts*).

Phenotyping the top-2(ts) mutant in both males and hermaphrodites

Jaramillo-Lambert *et al.* (2016) set out to examine the causative reason as to why *top-2*(*it7ts*) sperm that develop at the restrictive temperature result in Emb progeny. They utilized a combination of live imaging of fluorescently-tagged worm strains and Hoechst staining combined with differential interference contrast (DIC) microscopy to characterize any sperm issues. The transparency of *C. elegans* allows for easy visualization of fluorescent protein reporters in living animals (Corsi *et al.* 2015). Fluorescent reporters are proteins that have previously been identified as emitting a detectable light after being exposed to some trigger. One of the best-known fluorescent reporters is GFP, which glows green when exposed to visible blue light (light of a wavelength of ~475 nm). GFP is a naturally occurring protein found in jellyfish that scientists have adopted for usage as a fluorescent reporter. Other fluorescent reporters, such as mCherry, respond and glow under light of different wavelengths, thereby allowing the usage of multiple fluorescent reporters in one sample.

A benefit of strains containing fluorescent reporters is that one can observe reporter expression patterns in real time in living animals, including the movement of labeled gene products and changes in subcellular localization. Jaramillo-Lambert and colleagues crossed an animal that had three different proteins tagged with fluorescent reporters into the *top-2*(*it7ts*) mutant. The first protein tagged was Histone H2B, one of the histone proteins involved in forming chromatin and an indicator of where DNA is. Histone H2B was tagged with the red fluorescent reporter mCherry. Then, both tubulin (a component of microtubules and a spindle marker) and a nuclear pore complex protein (NPP-1, a component of the nuclear envelope) were tagged with GFP. In this animal [*top-2*(*it7ts*); mCherry::H2B; NPP-1::GFP; GFP::Tubulin], the researchers could then visualize the state of the DNA, nuclear envelope, and spindles in real time under both permissive and restrictive

conditions. Through these live imaging experiments, the researchers made the important conclusion: that newly fertilized *top-2*(*it7ts*) one-cell embryos completely lack paternal DNA.

Armed with the knowledge that there was a lack of paternal DNA, the researchers looked at the process of spermatogenesis to characterize the various stages of sperm development after a shift to the restrictive temperature. Combining Hoechst staining and DIC imaging of isolated *top-2*(*it7ts*) mutant sperm raised at the restrictive temperature, the researchers identified a very specific chromosome defect. Hoechst is a nuclear acid stain that binds to DNA and emits a blue fluorescence when excited with UV laser light. Importantly, Hoechst can be used in living animals because this molecule is able to pass through the cell membrane. DIC microscopy is a specific microscopic technique that enhances the contrast in unstained, transparent samples (Murphy *et al.* 2017). It is DIC that enables the authors to clearly visualize the transparent sperm when the sperm have been dissected from male gonads onto a glass slide, and the Hoechst that enables them to see the state of the chromosomes.

In addition to meiotic chromosome defects, the authors also identified early mitotic S-phase defects in the germ lines of hermaphrodites and male mutants shifted to the restrictive temperature [see Figure 5 in Jaramillo-Lambert *et al.* (2016)]. They thus needed to address whether the defects they saw later on in spermatogenesis (the meiotic chromosome segregation issues) were a result of these early S-phase mitotic or premeiotic defects, or if they were due solely to issues in male meiosis. To accomplish this, the researchers took advantage of the biology of the male germ line and the previously published knowledge that it takes between 20 and 24 hr for male germ cells to progress from S-phase to meiosis (Jaramillo-Lambert *et al.* 2007). If one shifts an animal to the restrictive temperature for only 2 hr, that is not enough time for a germ cell to progress through S-phase and meiosis. Rather, germ cells undergoing mitosis would be affected, and also those undergoing meiosis at the time the temperature shift occurred. Ultimately, they determined that the mutant meiotic chromosome-segregation defect of *top-2*(*it7ts*) males was observed very quickly after a shift to the restrictive temperature.

Localization of TOP-2 protein expression via immunofluorescence microscopy

Knowing that the *top-2*(*it7ts*) mutation affects the male meiotic divisions, the authors were interested in determining if the TOP-2 protein was expressed in the germ line and its specific localization pattern. To address this, one would typically perform an antibody immunofluorescence-staining protocol utilizing an antibody against their protein of interest. Very briefly, an antibody is a naturally produced protein in the body that is generated by the body's own immune system. Antibodies are generated directly in response to harmful, foreign substances (referred to as antigens) being identified by the immune system. The binding of an antibody to its target antigen actives macrophages to destroy the foreign substance, and hence serves a protective or immunological function.

Scientists have adapted the concept of antibodies for use in research applications and have perfected many different ways of generating antibodies to their specific protein of interest. The most commonly utilized method is to inject your protein of interest, or a portion of the polypeptide sequence of your protein of interest, into a host animal, such as a mouse, rat, or rabbit. This generates an immune response in the host animal and the generation of antibodies against that foreign protein. In research, this type of antibody is referred to as a primary antibody since it recognizes a specific antigen or polypeptide sequence. There are also secondary antibodies, or antibodies that are capable of recognizing the common conserved regions of primary antibodies generated in a specific host species (for instance recognizing a primary antibody made in a mouse host *vs.* a primary made in a rabbit host). Typically, secondary antibodies are fused to a fluorophore or fluorescent marker (such as GFP or the Alexa Fluor family of dyes), thereby allowing detection of the secondary antibody upon fluorescence microscopy. Since the secondary antibody binds to the primary antibody, one can infer the specific localization of the primary antibody by visualizing where the secondary antibody localizes.

At the time of publication there were no known *C. elegans* TOP-2 antibodies [a *C. elegans* TOP-2 antibody has recently been published by Ladouceur *et al.* (2017)], so the authors elected to instead use CRISPR/Cas9 technology to generate an endogenously 3xFLAG-tagged TOP-2 strain (*top-2*::*3xflag*). FLAG is an eight-amino acid polypeptide tag (peptide sequence DYKDDDDK) that can be added to either the N- or C-terminus of a protein (Einhauer and Jungbauer 2001). Due to its small size, it is thought to minimize the effect that these additional amino acids might have on the normal function, secretion, and transport of the protein of interest. Importantly, there exist many commercially available anti-FLAG primary antibodies that recognize the FLAG peptide sequence and can be utilized in immunofluorescence protocols. Gonads from the *top-2*::*3xflag* strain were dissected, fixed, stained with the primary anti-FLAG antibody followed by the appropriate secondary antibody fused to a fluorescent tag, and then subjected to fluorescence microscopy. In this manner, the authors were able to successfully visualize the expression localization of the TOP-2 protein both in male and hermaphrodite *C. elegans* gonads.

Suggestions for Classroom Use

This Primer provides background information on the *C. elegans* genetics, meiosis, and experimental techniques needed to assist a beginner student in comprehending the experiments and results presented in Jaramillo-Lambert *et al.* (2016). The original article and this accompanying Primer would be appropriate for an entry-level Genetics course or an advanced course in Molecular Techniques. The detailed discussion of meiosis in this Primer article and the visible meiotic defects presented in the Jaramillo-Lambert *et al.* (2016) article would serve as a great complement to course discussion on meiosis. Additionally, in both the scientific article and this Primer, there are introductions to a multitude of common molecular techniques such as antibody staining, RNAi assays, and CRISPR/Cas9 genome editing. Detailed familiarity with these

techniques will improve a student's ability to critically analyze and discuss other modern peer-reviewed scientific articles. In particular, knowledge of CRISPR is becoming a necessity in the scientific world as usage of this technique is becoming widespread in research and clinical settings. As there are many ethical implications arising regarding the potential usage of CRISPR in humans, an instructor could also use this article to delve into a scientific ethics discussion with their class on the pros and cons of CRISPR.

Finally, as *C. elegans* is an extremely amenable model system, an instructor could add lab course investigations to complement the reading of this article. RNAi libraries exist that contain RNAi-feeding vectors to a majority of the genes in the *C. elegans* genome. Students could use the freely available database WormBase (www.wormbase.org) to identify a gene of interest that they wish to knockdown via RNAi feeding, and then characterize the phenotype(s) observed after reduction of their gene. There are detailed protocols available online to assist an instructor with such experiments and an excellent website (www.wormclassroom.org) with predesigned and tested protocols (Eliceiri and Lu 2017; Ahringer 2006).

Questions for Review and Discussion

1. In Jaramillo-Lambert *et al.* (2016), the authors identified a temperature-sensitive mutant in the *top-2* gene. Define this term. How might a temperature-sensitive mutation be working? How would the results differ if this particular mutation was nonfunctional at all temperatures?
2. How did the fact that the *top-2*(*it7ts*) allele was a temperature-sensitive allele allow the researchers to reach their conclusions regarding the specific involvement of TOP-2 in male gametogenesis (spermatogenesis) *vs.* female gametogenesis (oogenesis)?
3. Why does the *trans*-heterozygote generated between *mel-15*(*it7ts*) and the *top-2* deletion [*top-2*(*ok1930*Δ)] support the assertion that *mel-15*(*it7ts*) is a mutation in the *top-2* gene [Figure 2A of Jaramillo-Lambert *et al.* (2016)]? What embryonic lethality result would the researchers have observed for the *trans*-heterozygotes if *mel-15*(*it7ts*) was not in the *top-2* gene?
4. In Figure 2A of Jaramillo-Lambert *et al.* (2016), what does the reduced embryonic lethality at 15° in the *unc-4*(*e120*) *mel-15*(*it7*)/*top-2*(*ok1930*Δ) mutant compared to the original homozygous *unc-4*(*e120*) *mel-15*(*it7*) imply?
5. The authors propose that the recreated *top*-2 point mutation strains are not as severe as the original *mel-15*(*it7ts*) allele due to the nature by which the original allele was generated (via EMS mutagenesis). EMS mutagenesis can result in other mutations in the background of the strain, and thus there might be an enhancer mutation that causes a more severe embryonic lethality phenotype when combined with *mel-15*(*it7ts*). Can you propose other reasons why these three new recreated *top-2* alleles do not exhibit as severe embryonic lethality as the original *mel-15*(*it7ts*)?
6. Why do you think the researchers used so many different methods (for example, complementation testing, WGS, and CRISPR mutation recreating) before concluding that *mel-15*(*it7ts*) was a mutation in the *top-2* gene?
7. Many researchers are now utilizing CRISPR/Cas9 genome-editing technology to address their need to generate specific genetic modifications in their gene of interest. However, CRISPR also has some potential negative aspects that researchers need to be aware of when utilizing it. What are some potential side effects that one should be concerned with when performing a CRISPR/Cas9 experiment?
8. Despite the main foundation of the article revolving around the *top-2* gene, the authors also generated mutations in the *cin-4* gene via CRISPR/Cas9 editing technology. Why were the authors concerned about the *cin-4* gene? What did they hope to address by generating a mutant in *cin-4*?
9. In Figure 6 of Jaramillo-Lambert *et al.* (2016), the authors conducted DAPI staining of sperm at various time points after the wild-type or *top-2*(*it7ts*) males were shifted to the higher, restrictive temperature. What is the significance of these various time points? How did this experiment help the researchers to make their final conclusion that TOP-2 plays a role during the male meiotic divisions?
10. Why do you think the researchers tagged TOP-2 with 3xFLAG and not a fluorescent tag such as GFP? What would be the benefits and disadvantages of tagging with GFP? What are the benefits and disadvantages of tagging with 3xFLAG?
11. Factoring in the previously known role for type II DNA topoisomerases in alleviating topological chromosome stresses, what do you hypothesize is the specific role for TOP-2 in *C. elegans* male meiosis?
12. Based on the TOP-2 localization and *top-2*(*it7ts*) mutant phenotype, the authors propose that TOP-2 has an essential function in chromosome condensation and karyosome formation during spermatogenesis. How could you test whether TOP-2 is involved in proper karyosome formation?

Literature Cited

Addgene, 2016 CRISPR 101: *A Desktop Resource Created and Compiled by Addgene*, pp. 1–125. Addgene.

Ahringer, J., 2006 *Reverse Genetics* (April 6, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.47.1, http://www.wormbook.org.

Chu, D. S., and D. C. Shakes, 2013 Spermatogenesis. Adv. Exp. Med. Biol. 757: 171–203.

Corsi, A. K., B. Wightman, and M. Chalfie, 2015 A transparent window into biology: a primer on *Caenorhabditis elegans*. Genetics 200: 387–407.

Dickinson, D. J., and B. Goldstein, 2016 CRISPR-based methods for *Caenorhabditis elegans* genome engineering. Genetics 202: 885–901.

Dickinson, D. J., J. D. Ward, D. J. Reiner, and B. Goldstein, 2013 Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. Nat. Methods 10: 1028–1034.

Doitsidou, M., R. J. Poole, S. Sarin, H. Bigelow, and O. Hobert, 2010 *C. elegans* mutant identification with a one-step whole-genome-sequencing and SNP mapping strategy. PLoS One 5: e15435.

Doudna, J. A., and E. Charpentier, 2014 Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346: 1258096.

Einhauer, A., and A. Jungbauer, 2001 The FLAG peptide, a versatile fusion tag for the purification of recombinant proteins. J. Biochem. Biophys. Methods 49: 455–465.

Eliceiri, K. W., and F. Lu, 2017 Available at: www.wormclassroom.org. Accessed: September 1, 2017.

Hall, D. H., V. P. Winfrey, G. Blaeuer, L. H. Hoffman, T. Furuta *et al.*, 1999 Ultrastructural features of the adult hermaphrodite gonad of Caenorhabditis elegans: relations between the germ line and soma. Dev. Biol. 212: 101–123.

Hannon, G. J., 2003 *RNAi: A Guide to Gene Silencing*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hillers, K. J., V. Jantsch, E. Martinez-Perez, and J. L. Yanowitz, 2015 Meiosis (December 22, 2015), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.178.1, http://www.wormbook.org.

Hubbard, E. J., and D. Greenstein, 2000 The *Caenorhabditis elegans* gonad: a test tube for cell and developmental biology. Dev. Dyn. 218: 2–22.

Hubbard, E. J. A., and D. Greenstein, 2005 Introduction to the germ line (September 1, 2005), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.18.1, http://www.wormbook.org.

Jaramillo-Lambert, A., M. Ellefson, A. M. Villeneuve, and J. Engebrecht, 2007 Differential timing of S phases, X chromosome replication, and meiotic prophase in the *C. elegans* germ line. Dev. Biol. 308: 206–221.

Jaramillo-Lambert, A., A. S. Fuchsman, A. S. Fabritius, H. E. Smith, and A. Golden, 2015 Rapid and efficient identification of *Caenorhabditis elegans* legacy mutations using Hawaiian SNP-based mapping and whole genome sequencing. G3 (Bethesda) 5: 1007–1019.

Jaramillo-Lambert, A., A. S. Fabritius, T. J. Hansen, H. E. Smith, and A. Golden, 2016 The identification of a novel mutant allele of topoisomerase II in *Caenorhabditis elegans* reveals a unique role in chromosome segregation during spermatogenesis. Genetics 204: 1407–1422.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. Doudna *et al.*, 2012 A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337: 816–821.

Kim, S., C. Spike, and D. Greenstein, 2013 Control of oocyte growth and meiotic maturation in *C. elegans*. Adv. Exp. Med. Biol. 757: 10.1007/978-1-4614-4015-4_10.

Ladouceur, A. M., R. Ranjan, L. Smith, T. Fadero, J. Heppert *et al.*, 2017 CENP-A and topoisomerase-II antagonistically affect chromosome length. J. Cell Biol. 216: 2645–2655.

Lander, E. S., 2016 The heroes of CRISPR. Cell 164: 18–28.

L'Hernault, S. W., 2006 Spermatogenesis (February 20, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.85.1, http://www.wormbook.org.

Lints, R., and D. H. Hall, 2009 Reproductive system, germ line. In *WormAtlas*. DOI: 10.3908/wormatlas.1.23.

Lints, R., and D. H. Hall, 2009 Reproductive system, overview. In *WormAtlas*. DOI: 10.3908/wormatlas.1.21.

Murphy, D. B., S. S. Schwartz, E. D. Salmon, K. R. Spring, M. Parry-Hill *et al.*, 2017 Differential interference contrast. Microscopy U. Available at: https://www.microscopyu.com/techniques/dic. Accessed: September 1, 2017.

Nitiss, J. L., 2009 DNA topoisomerase II and its growing repertoire of biological functions. Nat. Rev. Cancer 9: 327–337.

Nobelprize.org, 2006 Press release: the 2006 Nobel Prize in Physiology or Medicine. Available at: http://www.nobelprize.org/nobel_prizes/medicine/laureates/2006/press.html. Accessed: September 1, 2017.

Paix, A., A. Folkmann, D. Rasoloson, and G. Seydoux, 2015 High efficiency, homology-directed genome editing in Caenorhabditis elegans using CRISPR-Cas9 ribonucleoprotein complexes. Genetics 201: 47–54.

Pazdernik, N., and T. Schedl, 2013 Introduction to germ cell development in *C. elegans*. Adv. Exp. Med. Biol. 757: 1–16.

Shakes, D. C., J.-C. Wu, P. L. Sadler, K. Laprade, L. L. Moore *et al.*, 2009 Spermatogenesis-specific features of the meiotic program in *Caenorhabditis elegans*. PLoS Genet. 5: e1000611.

Shendure, J., and H. Ji, 2008 Next-generation DNA sequencing. Nat. Biotechnol. 26: 1135–1145.

Wilson, R. C., and J. A. Doudna, 2013 Molecular mechanisms of RNA interference. Annu. Rev. Biophys. 42: 217–239.

Yook, K., 2005 Complementation (October 6, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.24.1, http://www.wormbook.org.

Communicating editor: E. A. De Stasio