INVESTIGATING THE ROLE OF TRANSPOSONS IN TEMPERATURE-INDUCED DNA DAMAGE DURING SPERMATOGENESIS

by

COLIN MAXWELL

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Diana E. Libuda, Ph.D.

Meiosis is a specialized form of cell division that sexually reproducing organisms use to generate haploid sex cells. Developing sperm are particularly sensitive to temperature fluctuations, with some studies indicating that exposure to elevated temperature increases DNA damage in spermatocytes, but not oocytes. Although temperature-induced DNA damage has been observed, the underlying molecular mechanisms remain unknown. DNA transposons are mobile genetic elements that produce double-strand DNA breaks (DSBs) when excised from the genome. Additionally, transposons can excise from the genome under heat stress. I hypothesize that heat stress causes transposon excision which may be observed as a linear relationship between transposon copy number and the quantity of DSBs in developing spermatocytes exposed to elevated temperature. To test this hypothesis, I conducted an immunofluorescence screen of wild type *Caenorhabditis elegans* strains with varying transposon copy numbers. Using deconvolution microscopy, DSBs were visualized via the recombinase RAD-51, a protein involved in the early stages of meiotic DSB repair. Quantification of RAD-51 foci was performed to determine the frequency of

temperature-induced DSB formation. My results indicate that *Tc1* transposons can excise and insert into the *genome* after heat shock. Most strains I studied exhibited significant differences in the amount of DNA damage before and after heat shock. Additionally, there seems to be an increase in variability in the amount of DNA damage between the no heat shock and heat shock conditions. Comparisons between *Tc1, Tc3*, retrotransposons, and DNA transposons, suggests that there is no correlation between transposon copy number and the amount of temperature-induced DNA damage. Taken together, these results indicate temperature-induced DNA damage in spermatocytes has multiple mechanisms, with excision of *Tc1* transposons as one possible mechanism.

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Introduction

DNA damage and repair during meiosis

The faithful inheritance of the genome from adult to offspring is critical to the survival of a species. Organisms that reproduce sexually use a specialized form of cellular division called meiosis to generate haploid sex cells like sperm or egg cells from a diploid parent cell (Roeder, 1997). Meiosis includes one round DNA replication followed by two rounds of cellular division (Figure 1). Each round of division can be broken down further into four phases: prophase, metaphase, anaphase, and telophase. Research in the Libuda Lab focuses on the first round of division (meiosis I), and more specifically on prophase I. During prophase I, homologous chromosomes pair with each other which allows for homologous recombination, the exchange of genetic material

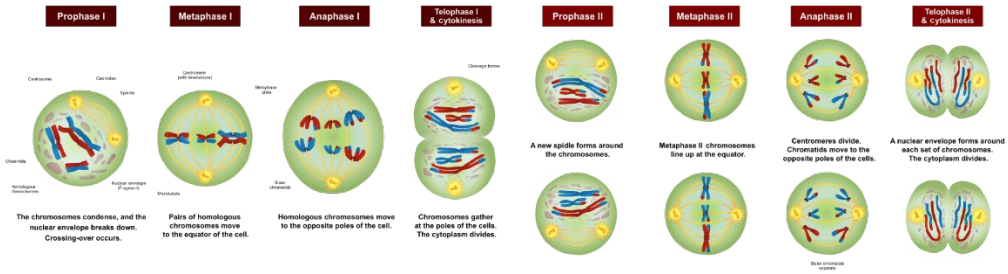


Figure 1: Overview of the stages of meiosis

Meiosis is characterized by one round of DNA replication followed by two rounds of cellular division. There are four main stages of meiosis: Prophase, Metaphase, Anaphase, and Telophase. These phases occur twice, once for each meiotic division. During Meiosis I, homologous chromosomes separate. During Meiosis II, sister chromatids segregate, resulting in sex cells with half the number of chromosomes as the original parent cell. My research focuses on events that occur during Prophase I of Meiosis I. (Figure adapted from Campbell Biology)

between chromosomes, to occur. Recombination serves to create physical connections between homologous chromosomes which are critical for proper chromosome segregation. Additionally, the recombination process also generates genomic diversity between gametes by allowing for the exchange of genetic information between chromosomes. Despite genomic stability being imperative for successful reproduction in a species, meiotic recombination is initiated by the production of a large number of double-strand DNA breaks (DSBs), which are made by the highly conserved endonuclease SPO-11 (Figure 2). These breaks are produced in excess and must be carefully repaired to ensure faithful inheritance of the genome. Mis-repair, or failure to repair, of DSBs can lead to cell death and genomic instability, which may result in birth defects and infertility (Mahaney et al., 2010; Page and Hawley, 2003).

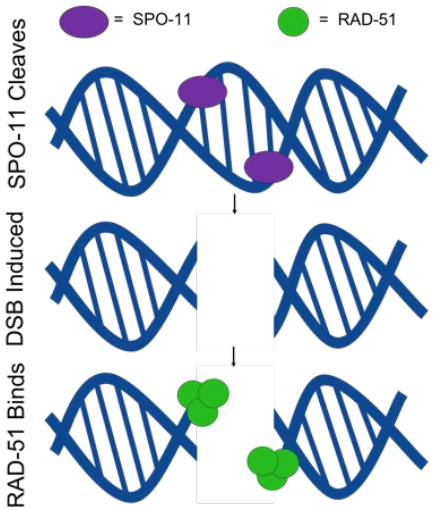


Figure 2: Simplified schematic of double-strand break induction

Double-strand DNA breaks (DSBs) are made by the highly conserved endonuclease SPO-11. Sometimes after these breaks are induced in early pachytene, RAD-51 binds to the site of the DSB to mark it for repair by other proteins. RAD-51 ultimately acts as a marker of DNA damage.

Temperature-induced DNA damage during spermatogenesis

Spermatogenesis involves the generation of haploid spermatids from diploid progenitor cells using the process of meiosis. While many tissues are affected by extreme temperature changes, developing sperm in the testes are particularly sensitive to small fluctuations in temperature, with spermatogenesis requiring a narrow isotherm of 2-7°C below core body temperature (Perez-Crespo et al., 2008). It is the only developmental process to have this requirement.

In humans and other mammalian models, gonadal exposure to elevated temperature has been shown to produce impaired fertility (Banks et al., 2005; Kim et al., 2013; Perez-Crespo et al., 2008; Thonneau et al., 1998). Furthermore, in mammalian models, elevated testicular temperature has been shown to produce increased DNA damage, decreased sperm counts, and decreased motility (Perez-Crespo et al., 2008). While the relationship between heat exposure, male infertility, and DNA damage is apparent, the underlying molecular mechanisms remain unknown. My research investigates how exposure to increased temperature may lead to DNA damage specifically in spermatocytes.

Caenorhabditis elegans: a model for research

To answer our research question, we exploit key features of the model organism *Caenorhabditis elegans* (Figure 3). *C. elegans* are a microscopic nematode commonly found in soil and rotting fruit. They are easy to maintain in a laboratory setting, have a quick life cycle, and are amenable to genetic manipulation. *C. elegans* exist in two sexual forms: hermaphrodites and males (Figure 3A). Hermaphrodites, which produce their own sperm and eggs, are capable of self-fertilization and make up the majority of

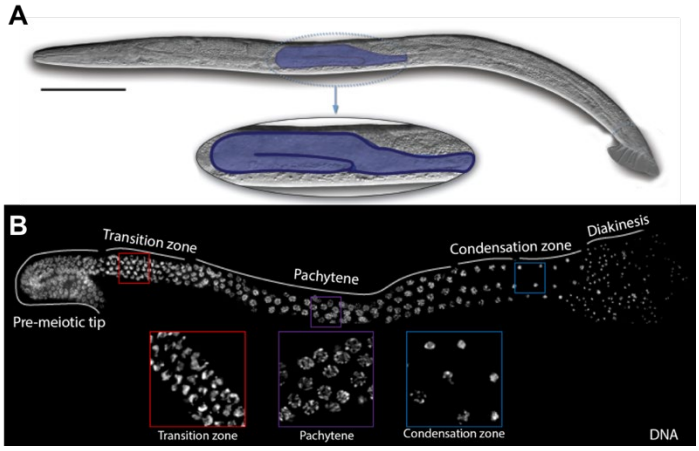


Figure 3: Diagram of a male *Caenorhabditis elegans*

A. Image of an adult male C. elegans. The gonad is highlighted in the middle and enlarged. Scale bar indicates 0.1 mm (Figure adapted from Wormatlas). **B.** Image of an extruded male gonad stained for DNA. Stages of meiosis can be identified by morphological features as shown in enlarged boxes (transition zone, pachytene, condensation zone).

the population. Males occur at a frequency less than 0.1% in a population of wild type strains (Hodgkin and Doniach, 1997; Ward and Carrel, 1979). Since both hermaphrodites and males generate sperm, spermatogenesis and oogenesis can be studied simultaneously within the hermaphrodite gonad, or spermatogenesis can be studied independently in the male gonad. This is particularly valuable for my research in that we have the ability to parse our spermatocyte-specific effects from sex-specific effects. *C. elegans* is exceptionally well-suited for the study of meiosis. The gonad is large, occupying a majority of the body cavity, and is organized in a spatial-temporal gradient (Figure 3B). Sex cells originate in the distal tip of the gonad and develop though subsequent stages of meiosis as the cells move through the gonad. The various stages of prophase I, including the pre-meiotic zone, transition zone, pachytene, diplotene, and diakinesis, can be easily differentiated by morphological differences

between gametocyte chromosomes (Figure 3B). This "pipeline" of meiosis allows for visualization and comparison of all stages of meiosis simultaneously. Moreover, *C. elegans* is transparent and is useful for experiments that employ fluorescent markers that tag proteins present during meiosis to be visualized under a microscope.

Using immunofluorescence to visualize DNA damage

To examine DSB formation during spermatogenesis in *C. elegans*, we use a technique known as immunofluorescence (Figure 4A). This technique can specifically mark proteins of interest with fluorescent tags that can be visualized under a fluorescent microscope. Immunofluorescence uses antibodies produced by an organisms' immune system that target foreign molecules and bind to them. Our experiment uses indirect immunofluorescence which employs the use of two antibodies: a primary antibody and

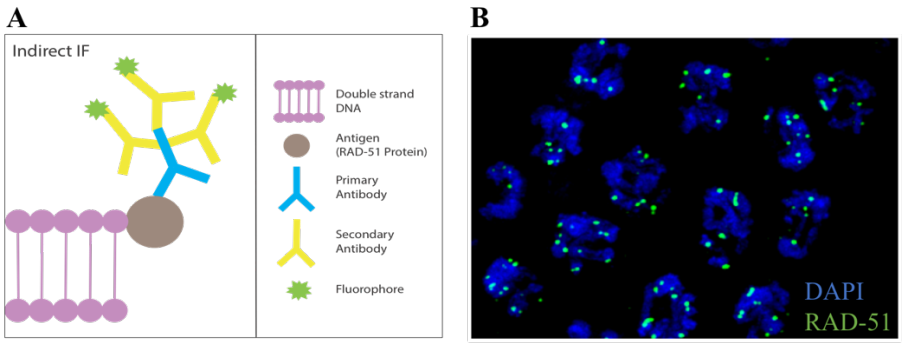


Figure 4: Using antibodies for immunofluorescence

A. Schematic of immunofluorescence. Primary antibodies are used to specifically target a protein. Secondary antibodies, conjugated with a fluorophore, bind to the primary antibody. Immunofluorescence can be used to visualize RAD-51, which binds at sites of DNA damage. (Figure adapted from Kaycee Schoellhorn) **B.** Example immunofluorescence image of developing spermatocytes from a male gonad. Each blue cluster represents the DNA in the nucleus of a developing spermatocyte. RAD-51 foci mark DNA damage in green.

a secondary antibody. Primary antibodies bind to the target protein while secondary antibodies, which are conjugated with a fluorophore, bind to the primary antibody. When the fluorophore is excited with a specific wavelength of energy, the compound emits a specific wavelength of light. Our experiments utilize a primary antibody raised against RAD-51, a marker of DSBs (Figure 2). An example of this type of immunofluorescence image of developing spermatocytes with RAD-51 marked DSBs is shown in (Figure 4B).

Temperature-induced DNA damage in *C. elegans*

In both male and hermaphrodite germlines maintained at 20°C, baseline levels of DSBs are produced during the normal course of meiotic progression that are SPO-11 dependent and are apparent during prophase I (Figure 5A-B). The region of RAD-51 foci is consistent, beginning in early pachytene when DSBs are produced, and ending

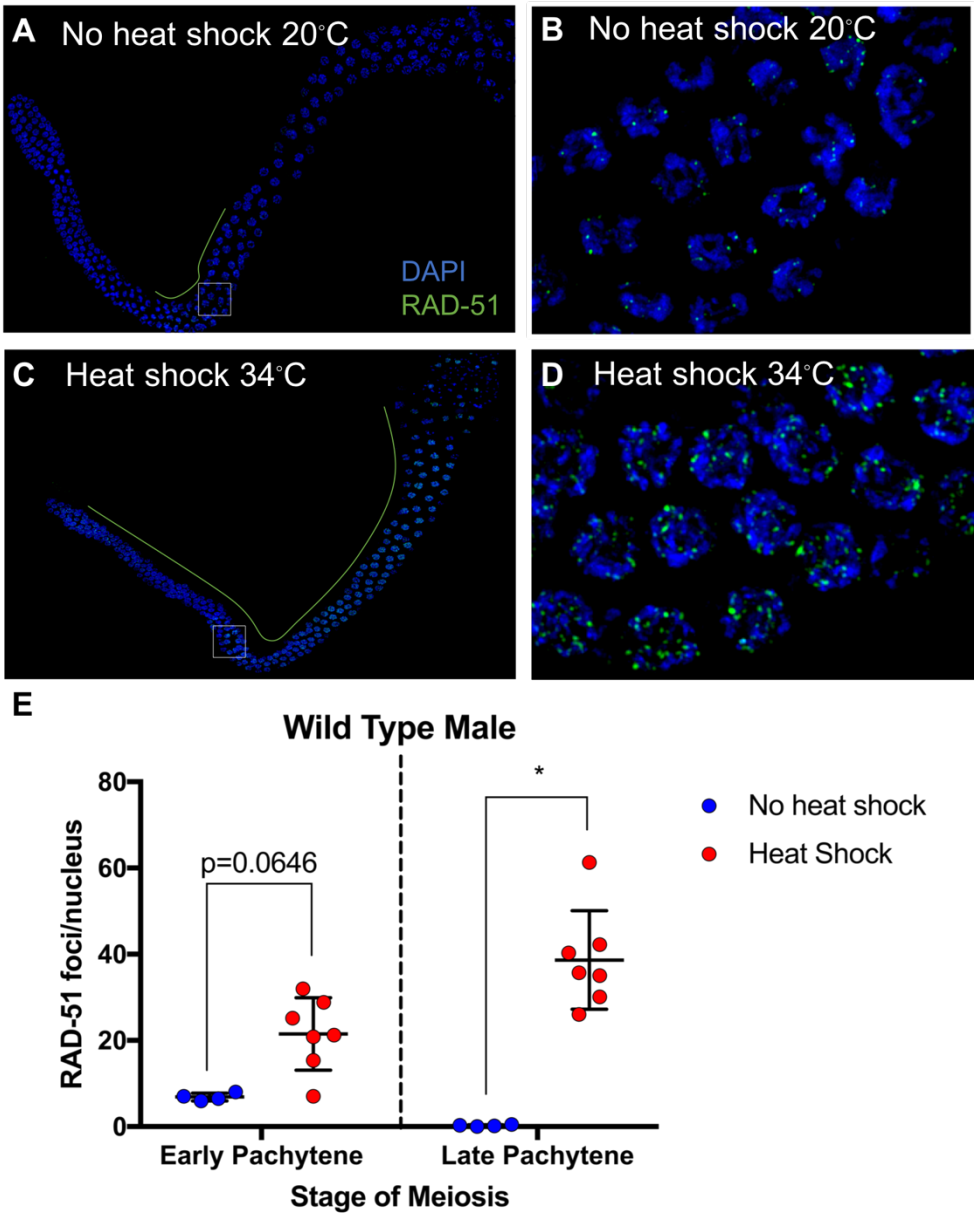


Figure 5: Temperature-induced DNA damage in *C. elegans*

A. Extruded and fixed male gonad maintained at 20°C. Green line denotes RAD-51 zone. **B.** Enlarged area of early pachytene at 20°C. **C.** Extruded and fixed male gonad heat-shocked for 2 hours at 34°C. Green line denotes RAD-51 zone. **D.** Enlarged area of early pachytene at 34°C. **E.** Quantification of N2 wild type RAD-51 foci per nucleus before and after heat shock in early and late pachytene. Error bars denote SD. Asterisk indicates significance of $p<0.0001$ with Bonferroni's multiple comparisons test.

by late pachytene as these programmed DSBs are repaired. Previous work done by Dr. Libuda has demonstrated that, similarly to humans, *C. elegans* spermatocytes also produce increased DNA damage upon exposure to elevated temperature. Exposing wild-type worms to an elevated temperature of 34°C (strains are usually maintained at 20°C) for 2 hours both increases the number of DSBs observed and produces DSBs much later in meiosis I than is usually observed (Figure 5C-D). We have also demonstrated that these heat-stress induced DSBs are specific to spermatocytes. Using young adult hermaphrodites, where oogenesis and spermatogenesis are occurring simultaneously, we found that only the spermatocytes demonstrated an increase in DSB formation upon heat shock (Figure 6A-B). Furthermore, temperature-induced DNA damage is SPO-11 independent. Using a *spo-11* null mutant, which has no SPO-11 protein and thus produces no programmed DSBs, it was found that exposing the worms to elevated temperatures still produced an increase in DSB formation comparable to wild type worms (Figure 7). Work by Kaycee Schoellhorn, a previous Honors College student, described how temperature induced DNA damage in wild-type spermatocytes occurs at or above the threshold temperature of 34°C (Figure 8). She performed the heat shock assay at a range of temperatures from 25-38°C and found no effect from 25-33°C; however, at 34°C there was a marked increase in DSBs as well an extension of the zone in which RAD-51 was found in the gonad. The degree and extent of temperature-induced DNA damage continued to increase linearly at 36°C and 38°C. Taken together, we have established that temperature induced DNA damage in *C. elegans* is a spermatocyte-specific phenotype that occurs independent of SPO-11 and requires a

temperature threshold. While temperature-induced DNA damage is readily observable in *C. elegans*, the mechanism in which this damage is occurring is still unknown.

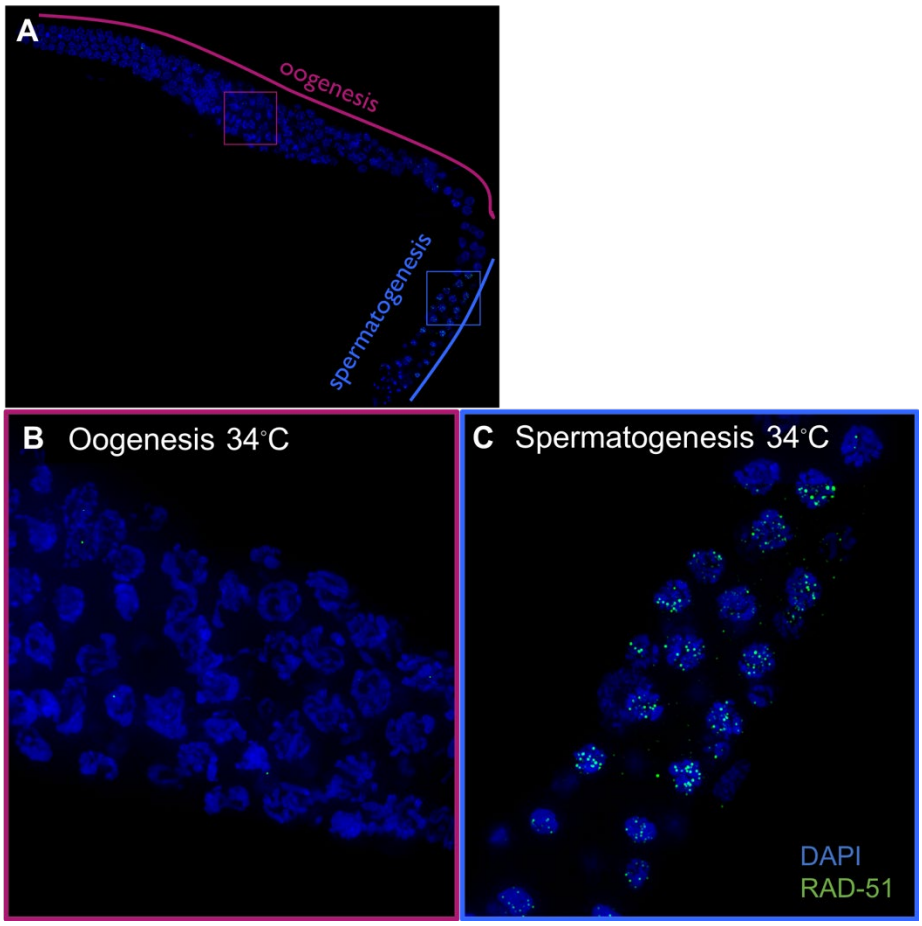


Figure 6: Temperature-induced DNA damage is spermatocyte specific

A. Image of an extruded and fixed *spo-11* hermaphrodite gonad undergoing both spermatogenesis and oogenesis. **B.** Early pachytene image of *spo-11* hermaphrodite gonad during oogenesis after 34°C heat shock for 2 hours. **B.** Early pachytene image of *spo-11* hermaphrodite gonad during spermatogenesis after 34°C for heat shock for 2 hours.

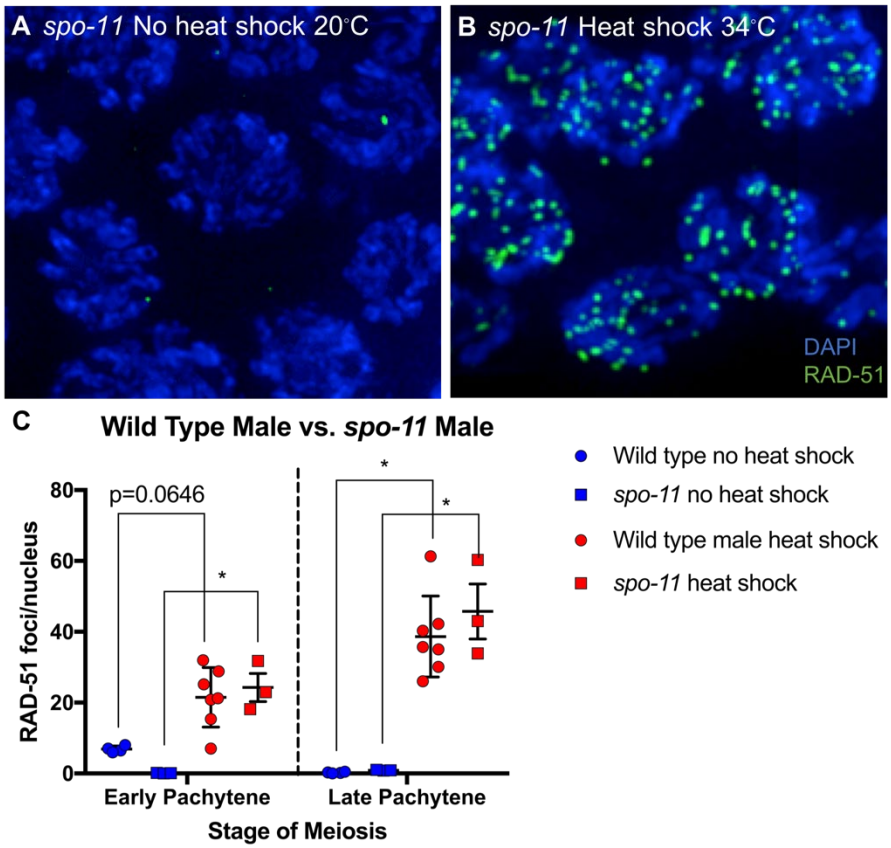


Figure 7: Temperature-induced DNA damage is independent of SPO-11

A. Enlarged image of an extruded and fixed male gonad *spo-11* null mutant maintained at 20°C. Image is of early pachytene. **B.** Enlarged image of an extruded and fixed male gonad *spo-11* null mutant maintained at 34°C for 2 hours. Image is of early pachytene. **C.** Quantification comparison of N2 wild type and *spo-11* RAD-51 foci per nucleus in early and late pachytene. Error bars denote SD. Asterisk indicates significance of $p<0.0001$ with Bonferroni's multiple comparisons test.

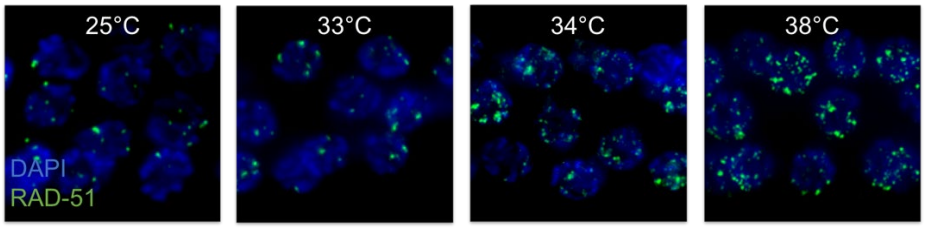


Figure 8: Temperature-induced DNA damage displays a threshold temperature

Enlarged male gonad images of early pachytene. Worms were exposed to 25°C, 33°C, 34°C, and 38°C for two hours. (Image adapted from Kaycee Schoellhorn)

A role for transposon activity in temperature-induced DNA damage during spermatogenesis

Transposons, also called transposable elements, are discrete units of DNA that are capable of moving around the genome (Figure 9). This mobility is attained either by a copy-and-paste mechanism (Class I retrotransposons) or by a cut-and-paste mechanism (Class II DNA transposons). Notably, excision of class II transposons results in a DSB at the excision site. Approximately 12% of the C. elegans genome consists of transposons and their genetic derivatives (Bessereau, 2006). Of the transposons in *C. elegans*, *Tc1* and *Tc3* transposons, which are class II DNA transposons, are the most active and most well-characterized. *Tc1* transposons are 1.7 kilobases long and code for a *Tc1*-specific transposase protein that mediates its transposition within the genome (Bessereau, 2006; Liao et al., 1983; Rosenzweig et al., 1983). *Tc3* transposons are 2.5 kilobases long and also code for a *Tc3*-specific transposase (Collins et al., 1989a). Transposase is the only factor that is required to mediate transposition of *Tc1* and *Tc3* transposons (Vos et al., 1996). Furthermore, 54 base pair inverted repeats at each end define *Tc1* insertion sites and 70 base pair inverted repeats define *Tc3* insertion sites (Collins et al., 1989a; Ketting et al., 1997). *Tc1* and *Tc3* transposon copy number in the genome also varies between strains of *C. elegans*. For example, approximately 20±5 copies of *Tc1* transposons are present in the genome of the most commonly used wild type *C. elegans* strain, the Bristol N2 strain (Fischer et al., 2003), and approximately 17 copies of *Tc1* are present in the genome of the Hawaiian CB4856 strain (Laricchia et al., 2017). Important for my work, transposons have been shown to be mobilized under stress conditions such as nutrient

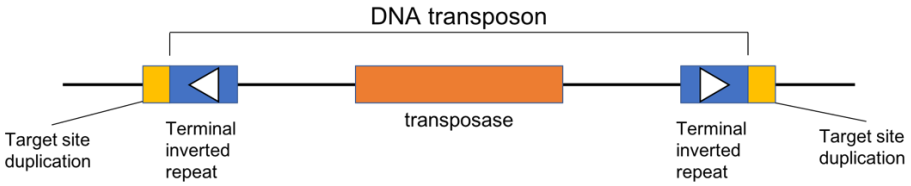


Figure 9: Overview of DNA transposable element

Schematic of a transposable element of class II like *Tc1* and *Tc3* (DNA transposons). DNA transposons are characterized by a gene encoding a transposase enzyme (in orange) that allows for transposition within the genome. The ends of the transposon have terminal inverted repeats (in blue) that contain the target site for integration. Target site duplication sequences (in yellow) are not part of the transposon, but rather a portion of the genome that is copied when transposons insert into the genome.

deficiency and temperature (Capy et al., 2000), although their activity is usually suppressed in the germline (Sijen and Plasterk, 2003).

My project investigates the role of transposons in temperature-induced DNA damage and begins to elucidate the mechanisms underlying temperature-induced DNA damage specifically during spermatogenesis. The majority of this project involves performing our heat shock and immunofluorescence experiments to quantify RAD-51 foci per nucleus for multiple wild type *C. elegans* strains with varying transposon copy numbers to determine whether transposon type and/or copy number correlates with the amount of temperature-induced DNA damage produced during spermatogenesis. I hypothesize that if transposons are required for spermatocyte-specific heat shock response, then *C. elegans* strains that have low transposon copy numbers will have reduced numbers of double-strand DNA breaks while *C. elegans* strains that have high copy numbers of transposons will have increased numbers of double-strand DNA breaks.

Methods

C. elegans strains and maintenance

We maintained worms at 15°C and 20°C. *C. elegans* were kept on nematode growth medium (NGM) agarose plates with a lawn of *E. coli* for food. We used the OP50 *E. coli* strain, a uracil-deficient mutant used to prevent overgrowth of the bacterial lawn (Brenner, 1974). Other worm maintenance procedures were done as described in Brenner, 1974. Additionally, a small amount of garlic extract was applied on the edges of the agar plates for strains that had males that tended to wander and desiccate on the sides of the petri dishes (Chatterjee et al., 2013). We produced sufficient males for experiments by plating at least two males for every one hermaphrodite.

The strains N2, CB4856, RW7012, VC1924, NL3643, CB4851 and RW7000 were obtained from our lab stocks. Male mating stocks were maintained for all strains except for the Bergerac strains CB4851 and RW7000. Previous research on the Bergerac strains indicated difficulty in generating a male stock, with some suggesting the Bergerac strain produces infertile males (Fatt and Dougherty, 1963; Vertino et al., 2011; Wood et al., 1980). Other wild type strains (CX11314, DL238, ED3017, EG4724, JT11398, JU775, MY16, LKC34, QX1791, and XZ1515) were generously provided by Erik Andersen's lab at Northwestern and the transposon profile of each strain was characterized in Laricchia et al., 2017. Transposon data used in this study for the Andersen strains is located in Supplemental Figure 1.

Media and solutions

Detailed composition of solutions used in this study are located in Supplemental Table 2. Of note, garlic extract was prepared as described in Chatterjee et al., 2013. Garlic was shown to act as a chemorepellent for *C. elegans*. Garlic extract was applied to the sides of petri dishes to prevent males from desiccating on the sides of the dishes when mate-searching for hermaphrodites.

Immunofluorescence staining of heat-shocked gonads

Approximately 80 L4 males of a given strain were maintained at 15°C for 24 hours on NGM plates inoculated with the *E. coli* strain OP50. 40 of these males were incubated at a temperature of 34°C for two hours. The remaining 40 males were maintained at 20°C for two hours as a control. After two hours, the worms in the 34°C incubator were incubated at 20°C for one hour to allow for recovery. Heat-shocked worms that were not allowed to recover had difficulty extruding gonads during dissections.

The heat-shocked worms after the recovery period were transferred and dissected under a dissecting microscope on a cover slip with 30µL of 1x egg buffer and Tween20 solution. Extruded gonads were fixed to a separate coverslip for 5 minutes using 15µL of 2% paraformaldehyde solution. The slide was immersed in liquid nitrogen and allowed to freeze for at least 1 minute. A razor blade was used to quickly tear off the coverslip from the slide, removing the cuticle from the worm. The slide was immersed in -20°C 100% methanol for 1 minute. Afterwards, the slide was immersed 3-4 times in a jar of 1x PBST to wash off excess methanol and soaked in a separate 1x

PBST jar for 5 minutes. Two additional 5-minute washes in 1x PBST were performed. This process was repeated for each strain and experimental condition.

After all the worms had been dissected, fixed, and washed, the slides were blocked in 50mL of 0.7% BSA solution for 1 hour. After the BSA block, 50 µL of primary rabbit antibody targeting RAD-51 and diluted in 1x PBST (Rabbit anti-RAD-51s, 1:500) was aliquoted on to each slide and covered in parafilm. The slides were incubated overnight in a humidifying chamber. Then, the slides were washed in 1x PBST for 10 minutes, 3 times. A 50 µL aliquot of secondary goat anti-rabbit antibody diluted in 1x PBST (1:200 Alexa Fluor™ 488 goat anti-rabbit IgG, Invitrogen) was placed on to each slide and covered with parafilm. The slides were incubated for 2 hours at room temperature in a humidifying chamber in the dark. The slide was washed 3 times in 1x PBST, 10 minutes each, in the dark. A 50 µL aliquot of 2µg/mL H_2O of DAPI was placed on the slides and incubated with a parafilm coverslip for 5 minutes. I washed the slide one final time in 1x PBST for another 5 minutes. The slides were mounted using 15 µL of Vectashield with a coverslip and sealed with nail polish.

Fluorescent microscopy and deconvolution

Slides were imaged using the Applied Precision DeltaVision Elite High-Resolution Microscope. Images were taken with 512x512 pixel dimensions using a 60x objective and an additional 1.5x optivar lens. Oil immersion was used with a 0.514 diffraction index. Exposure times for DAPI and GFP were automatically set by the softWoRx software at the brightest point on the gonad for each filter. The transmission percentage was manually set so that the exposure times for DAPI were between 0.010-0.030ms and the exposure times for GFP were between 0.050-0.10ms. Images were

acquired as Z-stacks at 0.2μm intervals and deconvolved using the softWoRx software using a conservative ratio over 15 cycles. The deconvolved images were cropped with border roll off and projected as maximum intensity images. The processed images were exported as 24-bit RGB TIFFs. Imaging began at the pre-meiotic tip through the rest of the phases of meiosis, leaving at least a 3 gonad overlap between images. At least one full gonad image was taken for each strain. Some subsequent images were only taken of early and late pachytene to expedite the imaging process.

RAD-51 foci quantification

Quantification of RAD-51 foci was done using the Imaris Microscopy Imaging Analysis Software. 3D images from the Delta Vision were used to perform the quantification analysis. Representative images of early pachytene and late pachytene were taken for each gonad. Early pachytene was considered the image after transition zone nuclei were ending. Late pachytene was considered the image before condensation zone nuclei became apparent. RAD-51 focus counts and nucleus counts were generated automatically in the Imaris software with parameters. RAD-51 foci were generated in the 525 nm wavelength channel with an estimated XY diameter of 0.10 μm, an estimated Z diameter of 1.37 μm, automatic background subtraction, and automatic quality check. Early pachytene nuclei were generated in the 435 nm wavelength channel with 0.137 μm smoothing, 5.00 μm as the largest diameter, automatic threshold for background subtraction, 2.00 μm seed diameter, automatic quality check, and a volume between 8.00 μm^3 and 50.0 μm^3. Late pachytene nuclei were generated with 0.300 μm smoothing, 5.00 μm as the largest diameter, automatic threshold for background

subtraction, 4.00 μm seed points diameter, automatic quality check, and a volume between 15.0 μm^3 and 75.0 μm^3.

Image processing

Maximum intensity projection images were manually stitched together and color-adjusted using Adobe Photoshop. Images were merged onto one canvas and manually positioned so that nuclei between images overlapped. Blue and green color levels for DAPI and GFP respectively were manually adjusted to reduce background noise. Final images were saved as flattened images.

Tracking *unc*-22 transposition events using polymerase chain reaction

The following strains were used to assess whether *Tc1* is inserted into or excised from *unc-22(st136*) upon heat-shock: RW7000 (wild-type mutator strain), RW7012 (*Tc1* insertion at *st136*), VC1924 (point mutation in *unc-22*). Approximately 24 hr post-L4 stage adult worms, both male and hermaphrodite, were exposed to heat-shock at 34°C for 2 hours, or left at 20°C. Within an hour of heat-shock genomic DNA was isolated from individual worms in lysis buffer. Samples were flash frozen in dry ice and ethanol then thawed in a 65°C water bath three times, then digested with proteinase K at 65°C for 1 hr. Proteinase K was inactivated by heating the samples to 95°C for 15 minutes. PCR was performed to determine whether *Tc1* was in *st136* (Figure 10). Primers flanking *st136* (Supplemental Table 3) were used, with a smaller product size of 644 indicating an absence of *Tc1*, and a larger product size of 2254 indicating the presence of Tc1 in *st136*.

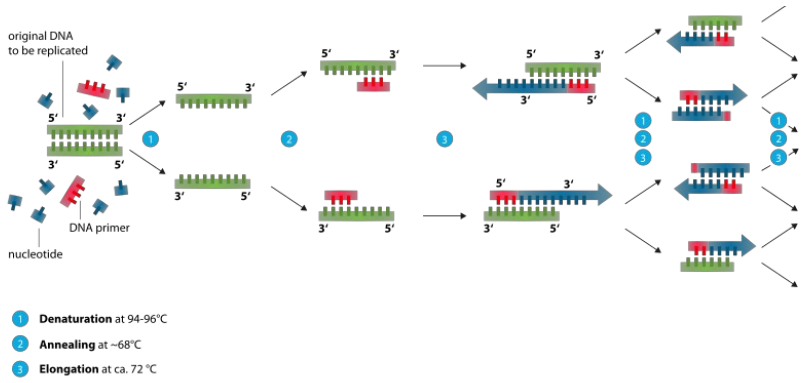


Figure 10: Overview of polymerase chain reaction

A schematic of the polymerase chain reaction (PCR). PCR is used to amplify the number of copies of a particular sequence. Two primers that flank the region of interest are designed. A reaction mixture is created with the designed primers, the DNA of interest, DNA polymerase, and nucleotides to create new strands of DNA. This reaction mixture is placed in a machine that runs at three different temperatures. First, the DNA is denatured to separate the two strands of DNA. Second, the primers are annealed to the DNA of interest. Third, the DNA polymerase binds to the primers and creates another strand of DNA. This process is repeated. (Figure adapted from Microbe Online)

Results

Evidence of *Tc1* excision and insertion in *unc-22* after heat shock

To examine whether elevated temperature was sufficient for transposons to excise and insert into the genome, we performed a polymerase chain reaction (PCR) experiment using a known *Tc1* transposon insertion site in the *unc-22* gene (Fischer et al., 2003). Primers were designed within the *unc-22* gene, flanking the st136 allele, to specifically amplify the insertion site (Supplemental Table 1). The *unc-22* DNA fragments were separated on a gel by size to determine whether or not the DNA sequences contained *Tc1*. An *unc-22* DNA sequence without a *Tc1* insertion was expected to be 644 base pairs in length whereas an *unc-22* DNA sequence with a *Tc1* insertion was expected to be 2254 base pairs in length.

The experiment utilized three different strains of *C. elegans* that have different *unc-22* profiles (Figure 11). We used a RW7000 hermaphrodite that has a wildtype copy of *unc-22* to determine if transposons could insert into the gene in hermaphrodites. We used a VC1924 male twitcher mutant that has a point mutation in *unc-22* to determine if transposons can insert into the gene in male worms. An RW7012 male has a *Tc1* transposon insertion inside *unc-22* and was used to determine if transposons can excise from the gene upon heat shock in male worms. We compared the results of whole worm lysates followed by PCR of worms maintained at 20°C to worms that had been heat shocked for 2 hours at 34°C (Figure 11). The RW7012 male after heat shock had significantly less of the *unc-22* with the *Tc1* insertion, and more of the *unc-22* with no *Tc1* insertion (Figure 11A). Moreover, the VC1924 male indicated presence of the

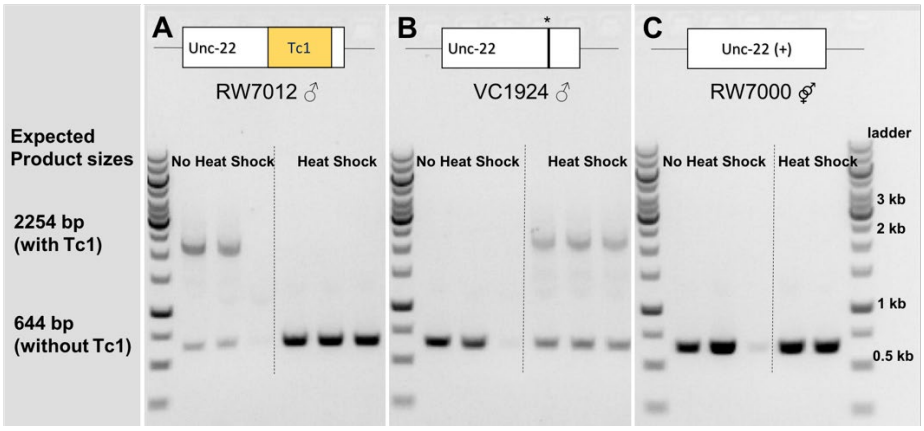


Figure 11: Transposons excise and insert into the genome upon heat shock

Polymerase chain reaction experiment amplifying the *unc-22* gene. Whole worm lysates were done with worms maintained at 20°C and with worms that had been heat shocked for 2 hours at 34°C. Bands with larger base pair sizes indicate *Tc1* insertion whereas bands with smaller base pairs sizes indicate an absence of *Tc1*. **A.** RW7012 male worm. This strain has an insertion in unc-22. **B.** VC1924 male worm. This strain has a point mutation in unc-22 and displays a twitcher phenotype. **C.** RW7000 hermaphrodite worm. This strain has a wild type unc-22 gene.

unc-22 gene with *Tc1* insertion after heat shock (Figure 11B). In contrast to these two results, the RW7000 hermaphrodite saw no change in the amount of *unc-22* with no *Tc1* insertion after heat shock (Figure 11C). Taken together, these results indicate that elevated temperature is sufficient for transposons to excise and insert into the *C. elegans* genome and that these transposition events seem to be specific to males.

C. elegans strains with different transposon copy numbers upon heat shock exhibit a high variability and higher amount of DNA damage in spermatocytes

We hypothesized that transposon copy number could modulate the amount of temperature-induced DNA damage that occurred in a developing spermatocyte. To test this hypothesis, strains with varying transposon profiles were generously donated by the Erik Andersen Lab at Northwestern. For my work, I decided to focus on *Tc1* and *Tc3*

transposon copy number as these transposon types are known to be the most active transposons in the *C. elegans* genome (Collins et al., 1989b; Eide and Anderson, 1988). The Andersen Lab characterized the transposons in many wild type strains and our lab was given a small subset of these strains to analyze using our immunofluorescence RAD-51 quantification experiment (Supplemental Table 1).

Our pipeline for our experiment started with our immunofluorescence experiment that targets and allows for visualization of RAD-51 for worms maintained at 20°C and at 34°C for two hours. These slides were imaged using deconvolution microscopy. The RAD-51 foci number and the number of spermatocyte nuclei were quantified. After analysis, we calculated the number of RAD-51 foci per nucleus of developing spermatocyte. We used images of early pachytene and late pachytene. We focus on the late pachytene image analyses to avoid differences in the number of SPO-11 mediated DSBs induced during early pachytene between strains.

Our results indicate a wide array of responses in the various strain backgrounds (Figure 12). Looking at the results of each strain individually, CB4856, EG4724, JU775, and N2 all exhibited statistically significant differences of RAD-51 foci per nucleus in late pachytene between no heat shock and heat shock ($p<0.05$). In contrast, CX11314, DL238, JT11398, QX1791, and XZ1515 did not display a statistically significant difference in their RAD-51 foci per nucleus between no heat shock and heat shock in late pachytene ($p>0.05$) LKC34 and MY16 were borderline statistically significant between the no heat shock and heat shock condition ($p=0.08$, $p=0.07$). ED3017 was excluded from statistical analysis due to only having one image for late pachytene. All of the strains tested (besides ED3017) displayed a much higher

variability between worms of the same strain in the amount of DNA damage upon heat shock as compared to no heat shock. Where no heat shock DNA damage numbers were consistently low and restricted in all strains, heat shock DNA damage numbers varied considerably. Notably, XZ1515 and CX11314 did not see a statistically significant increase in the number of RAD-51 foci per nucleus after heat shock in late pachytene. N2 and JU775 were the only two strains that had statistically significant differences in early pachytene between no heat shock and heat shock ($p<0.05$). Like late pachytene, the heat shock condition in early pachytene seems to display an increased variability between worms of the same strain in the amount of RAD-51 foci per nucleus as compared to the no heat shock condition. Taken together, these results indicate that some strains exhibit significant differences in the amount of RAD-51 foci per nucleus upon heat shock, and most strains exhibit increased variability of the number of RAD-51 foci per nucleus between worms of the same strain.

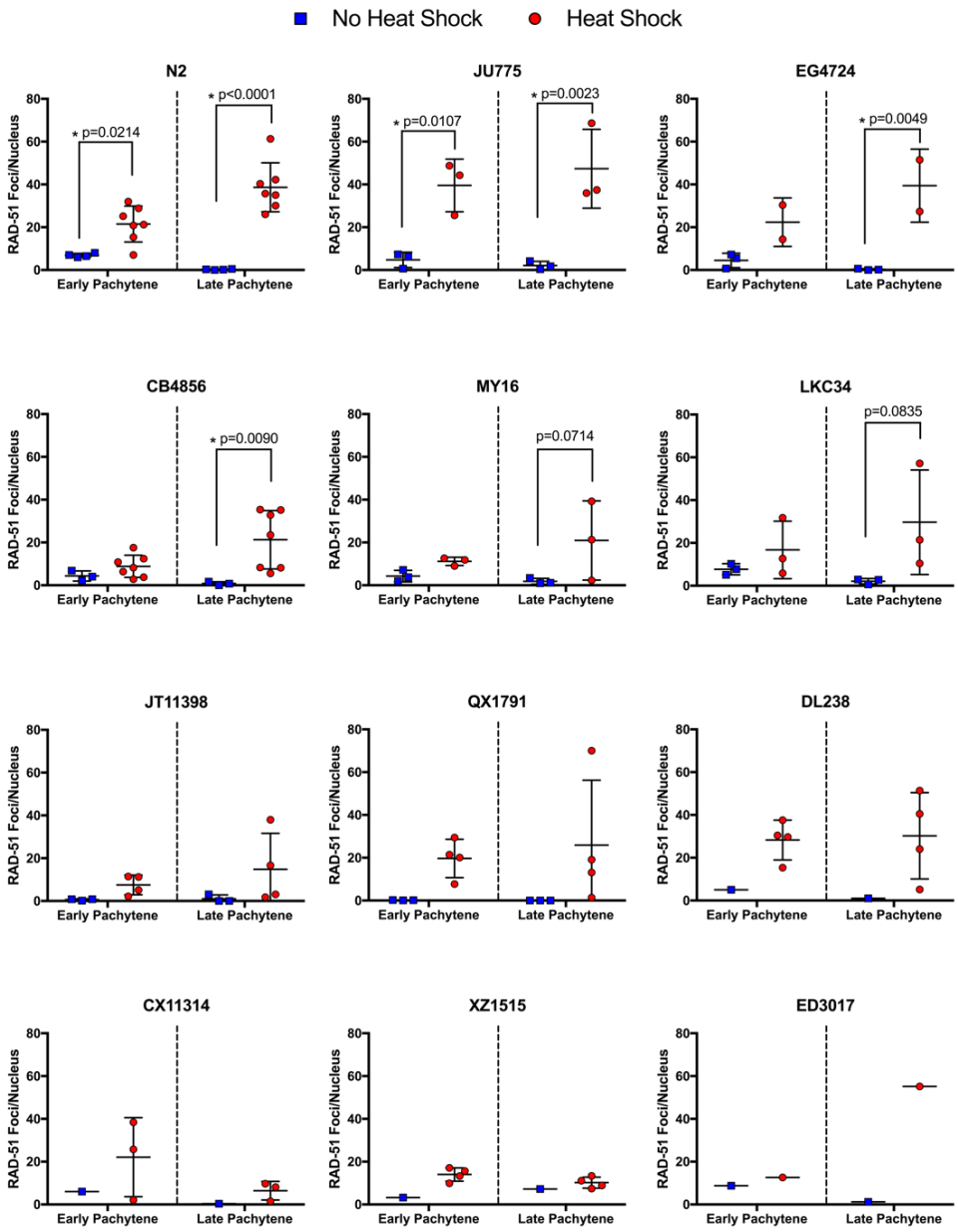


Figure 12: RAD-51 foci per nucleus number of wild type strains with different transposon copy numbers before and after heat shock

An immunofluorescence experiment targeting RAD-51 was performed on wild type strains with different transposon copy numbers. RAD-51 foci per nucleus quantification was performed in the Imaris software. 2-way ANOVA with Sidak's multiple comparisons test. Significance was set as $p<0.05$.

We also directly compared each strain to the most universal and well-characterized wild type N2 strain (Figure 12). Of the strains analyzed, CB4856, CX11314, JU775, MY16, JT11398, and XZ1515 displayed statistically significant differences in the heat shock phenotypes compared to that of N2 ($p<0.05$). Most of these strains exhibited lower amounts of RAD-51 foci per nucleus after heat shock than that of N2. Moreover, CX11314 and XZ1515 had a consistent number of RAD-51 foci per nucleus after heat shock between worms in late pachytene whereas N2 displayed a considerable increase in variability in the amount of DNA damage between worms in the same area. Of the strains that showed statistically significant differences, only JU775 displayed more RAD-51 foci per nucleus after heat shock but only during early pachytene ($p<0.05$), which could either indicate differences in basal levels of DSBs, greater response to heat shock, or both. Taken together, these comparisons to N2 indicate that there are some significant differences between wild type strains. However, it seems that these differences are not universal between all wild type strains, suggesting that a strain's genetic background plays a role in the amount of temperature-induced DNA damage.

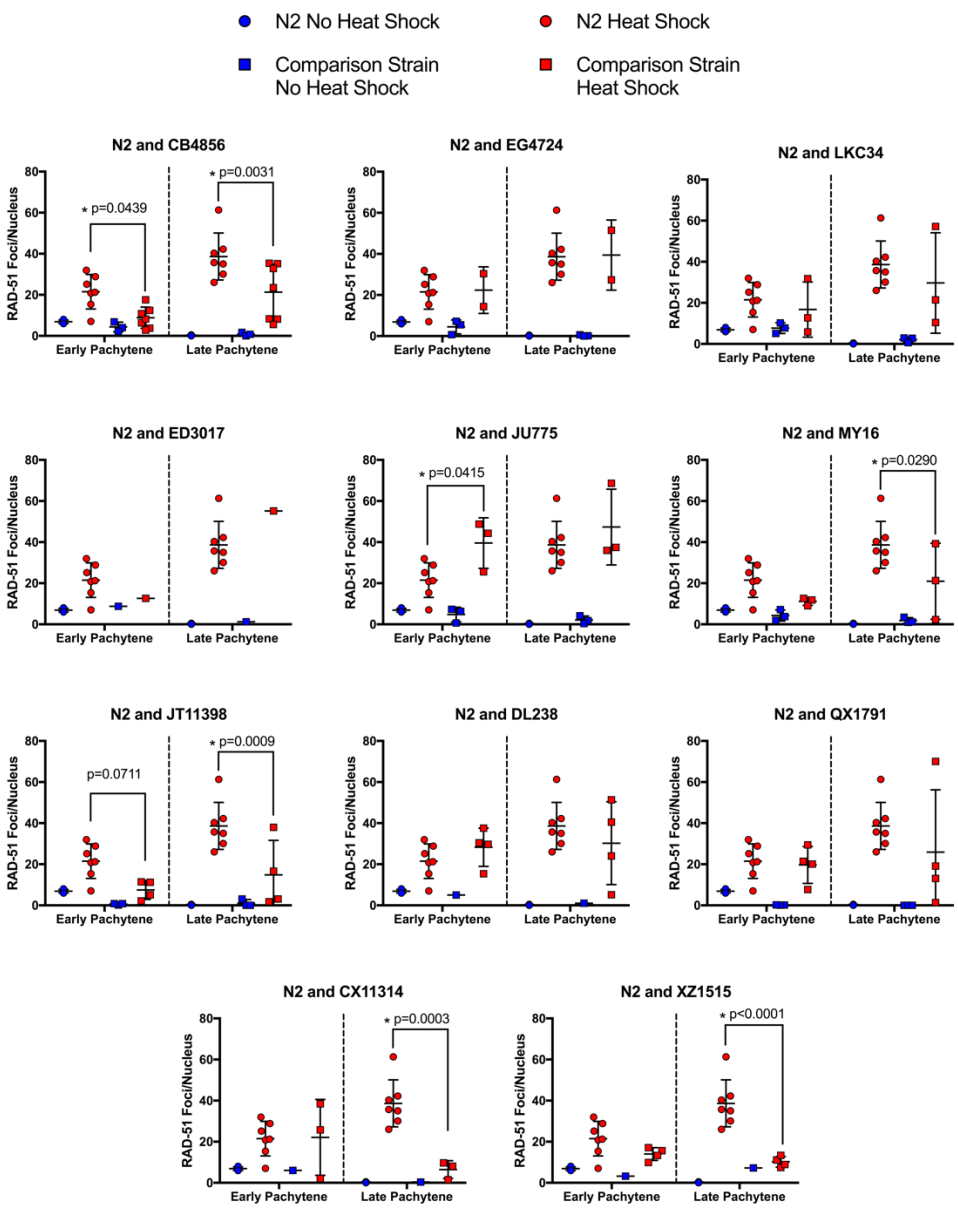


Figure 13: N2 comparison of RAD-51 foci per nucleus number of wild type strains with different transposon copy numbers before and after heat shock

An immunofluorescence experiment targeting RAD-51 was performed on wild type strains with different transposon copy numbers. RAD-51 foci per nucleus quantification was performed in the Imaris software. Wild type strains were compared to the commonly used wild type N2 strain. 2-way ANOVA with Tukey's multiple comparison test for each comparison to N2. Significance was set as $p<0.05$.

Temperature-induced DNA damage during spermatogenesis is independent of transposon copy number

The RAD-51 quantification data from the Andersen lab strains with varying transposon copy numbers were also visualized using a scatter plot to correlate transposon copy number with the amount of temperature-induced DNA damage after heat shock. With the heat shock condition in late pachytene, the data show that there is no trend between RAD-51 foci per nucleus and *Tc1*, *Tc3*, all retrotransposons, nor all DNA transposons (Figure 14A-D). While there does seem to be a slight positive linear trend in *Tc3* transposon copy number versus RAD-51 foci per nucleus in late pachytene, the trendline does not match well with the variability in the data. Heat shock in early pachytene also shows no obvious trends between *Tc1*, *Tc3*, all retrotransposons, nor all DNA transposons (Supplemental Figure 1A-D). Altogether, these results indicate that transposon copy number in the genome does not correlate with the amount of temperature-induced DNA damage in spermatocytes. Moreover, while we have shown direct evidence of heat shock promoting transposition events in the genome, our results indicate that temperature-induced DNA damage has more than one mechanism, with transposons as one possible mechanism.

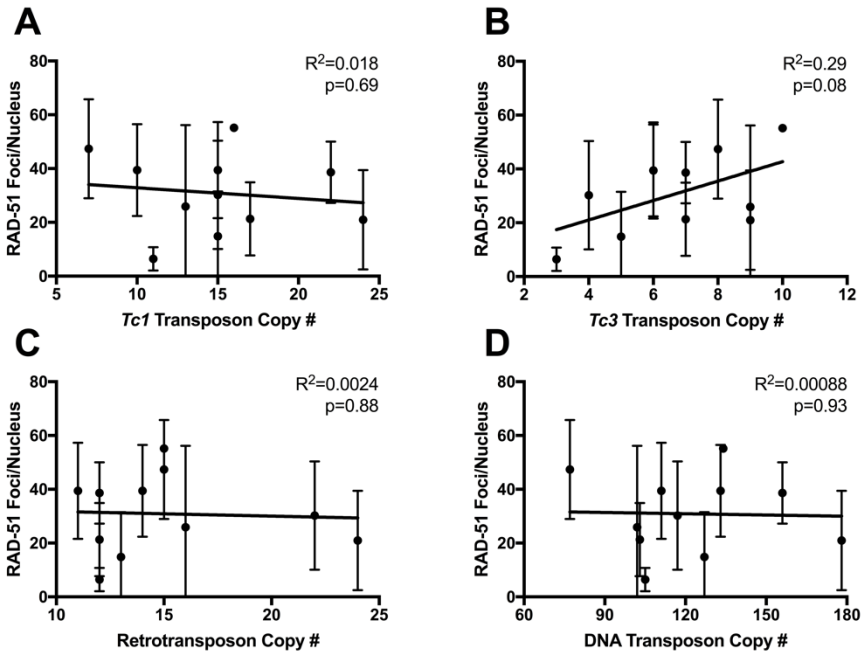


Figure 14: Temperature-induced DNA damage is independent of transposon copy number in late pachytene

Strains with varying transposon copy numbers were heat shocked for 2 hours at 34°C. Immunofluorescences images were taken at late pachytene of dissected gonads. Each image was quantified for amount of RAD-51 and number of nuclei using Imaris software. The number of RAD-51 foci per nucleus was calculated. Graph represents mean RAD-51 foci per nucleus with error bars denoting SD. Transposon data was taken from Laricchia et al., 2017. **A.** Tc1 transposon copy number. **B.** Tc3 transposon copy number. **C.** Retrotransposon copy number. **D.** Total DNA transposon copy number.

Discussion

Transposons play a role in temperature-induced DNA damage in spermatogenesis

Or data suggests that *Tc1* transposons in *C. elegans* are capable of excising and inserting into the genome after exposure to transient elevated temperature. As hypothesized, male worm candidates displayed *Tc1* transposon movement after heat exposure. In contrast, the hermaphrodite candidate not undergoing spermatogenesis at the time of the experiment, showed no movement of *Tc1* transposons. As we used whole worm lysates in our experiment that include both the soma and germline, we must consider that these transposition events could be occurring in the soma rather than in the germline. However, if these transposition events were occurring in the soma, we would have expected to see transposition events in the RW7000 hermaphrodites if this were the case. Though it is possible that some unknown factor in the RW7000 hermaphrodite background is contributing to the silencing of transposition events in the *unc-22* gene. These PCR experiments that track transposition events in *unc-22*, or any other location of a known transposon insertion site, could easily be repeated using only dissected gonads to verify that these transposition events are specifically occurring in the germline and specifically in spermatocytes.

Not all of the strains studied with different transposon copy numbers exhibited a statistically significant difference in RAD-51 foci per nucleus between no heat shock and heat shock. While it is true that perhaps the means were not statistically significant, many of the strains exhibited a difference in the constriction of the number of RAD-51 foci. This increase in spread suggests that heat shock deregulates some meiotic process,

making inconsistent DSBs to be made in the genome, whether that be in early or late pachytene. Additionally, the amount of variability in temperature-induced DNA damage that was seen between worms of the same strain background was substantial. Interestingly, XZ1515 does not seem to exhibit a considerable increase in the RAD-51 foci per nucleus in either early nor late pachytene nor does there seem to be a considerable increase in the variability of RAD-51 foci per nucleus. Unfortunately, it was impossible to compare XZ1515 with transposon copy number as this strain was not characterized by the Andersen Lab. Comparing these strains individually between no heat shock and heat shock indicates that most of these strains display a difference, whether that be in mean DNA damage or increase in variability, but the extent of the difference is not consistent across all of the wild type strains.

It is not surprising that many of the strains did not exhibit significant differences in RAD-51 foci count compared to the N2 heat shock group. Of the strains characterized, *Tc1* transposon copy number only varied by at most 17 copies and *Tc3* transposon copy number only varied by at most 7 copies. The small difference in the amounts of these transposons in the genome may contribute to the little amounts of mean variation we exhibited between strains. I had planned to work with the *C. elegans* Bergerac strains CB4851 and RW7000, both of which have over 300 copies of *Tc1* inserted into the genome to investigate whether large differences in transposon copy number affected the amount of temperature-induced DNA damage. Unfortunately, these strains were found to produce infertile males and insufficient numbers of males were generated for our immunofluorescence assay to be productive. It may be easier to

discern significant differences between strains using strains that have fold-differences in their transposon copy numbers.

Putative role of small RNA pathways in temperature-induced DNA damage

Organisms strive to maintain genomic integrity of their germline cells. Transposition events have the potential consequence of causing mutations in the genome if transposons are inserted or excised. Organisms such as *C. elegans* have complex mechanisms that maintain general genomic integrity and suppress transposition events during oogenesis and spermatogenesis. These pathways that protect the genome utilize small RNAs, which are short non-coding RNAs that are capable of differentiating between self and non-self nucleic acids. Small RNAs include micro RNAs (miRNA), small interfering RNA (siRNA), and Piwi-interacting RNAs (piRNA) (Kim et al., 2009). These small RNA pathways are controlled by regulatory proteins in the Argonaute family that act to specifically target foreign RNA sequences. Moreover in *C. elegans*, small RNA pathways contribute directly to silencing transposition of transposons in the germline (Aravin et al., 2007; Houwing et al., 2007; Theurkauf, 2011). There are also distinct differences in Argonaute protein roles between oogenesis and spermatogenesis (Campbell and Updike, 2015; Conine et al., 2013). Furthermore, many Argonaute protein null mutants in *C. elegans* are temperature sensitive, displaying decreased fertility when exposed to higher temperatures (Conine et al., 2010; Wang and Reinke, 2008). Taken together, these small RNAs are compelling regulators of transposable elements in the genome, and it is possible that their abrogation may allow for their transposase-mediated excision and insertion into the genome.

Elevated temperature may allow for but not guarantee transposon movement

Our hypothesis operated under the assumption that if transposition events of transposons is the mechanism in which temperature-induced DNA damage occurs, more transposons in the genome would result in more DSBs after heat shock. We also assumed that heat directly causes excision and insertion of transposons. However, it is possible that heat indirectly causes transposition events to occur. There is significant evidence that small RNA pathways play a significant role in transposon silencing in the genome. If these upstream effectors like small RNAs were to be abrogated, transposons could move around the genome freely given transposase is readily accessible. In addition to upstream regulators, it has been shown that transposon sites in the genome are sometimes repressed by histone methylation and changes in chromatin structure (Lippman et al., 2003; Wolfswinkel, 2005). Preliminary evidence in our lab indicates that histone 3 lysine 9 dimethylation (H3K9me3) is lost in developing spermatocytes after heat shock. This provides another possible mechanism of heat shock indirectly causing transposition events in the genome; if a region of DNA with a transposon insertion or transposon site were to be demethylated after heat shock, then the likelihood that a transposon was to insert or excise in the genome increases. Taken together, I hypothesize that heat shock abrogates transposon regulators which then allows for transposons that are normally silenced in the germline to move around the genome. This theory may explain some of the highly variable results that were displayed between worms of the same strain (Figure 13).

Future directions

Temperature-induced DNA damage seems to be a multifaceted phenomenon, and the Libuda Lab will be examining many different potential regulators. There seems to be a relationship between temperature and transposon mobilization. Thus, we will be investigating the role of upstream regulators of transposition events such as small RNA pathways and histone modifications to determine their role in temperature-induced DNA damage. Of note, since my strains used in my immunofluorescence experiments all had unique genetic backgrounds, any number of transposon regulators may be different between the strains. In the case of XZ1515, which showed little difference in the amount of DNA damage before and after heat shock, it is worth investigating what in that strain's genetic background makes it seemingly impervious to temperature-induced DNA damage.

The Bergerac strains RW7000 and CB4851 both have astounding copy numbers of *Tc1* transposons, each having over 300 copies. Our initial plan was to perform our heat shock experiment to investigate whether large differences in the number of transposons affected the amount of temperature-induced DNA damage, but insufficient males were generated for experiments due to male infertility in the Bergerac line (see Methods). To circumvent infertility, our lab will be utilizing RNA interference (RNAi) to knock-down a known protein where null mutants have increased incidence of males HIM-8. Theoretically a knock-down of this protein will also generate more males than usual to use directly in a heat shock experiment, bypassing the infertility of the Bergerac strain entirely. Additionally, we may examine young (L4) Bergerac

hermaphrodites that are undergoing both spermatogenesis and oogenesis to investigate whether we see an increase in temperature-induced DNA damage.

My final experiment in lab will utilize our knowledge of *Tc1* and *Tc3* sequences to track transposon movement throughout the genome before and after heat shock. To do this, I will perform a reverse polymerase chain reaction (iPCR) experiment. Unlike a normal PCR, iPCR amplifies the regions flanking a known sequence. I will be amplifying the regions flanking *Tc1* and *Tc3* transposons before and after heat shock, and comparing differences using high throughput MiSeq® sequencing technology. Additionally, I will only be using dissected gonads, rather than whole worm lysates, to pinpoint this phenomenon in germ cells and not somatic cells. If these sequences before and after heat shock appear similar, this may suggest that transposons do not move upon heat shock and do not play a role in temperature-induced DNA damage. If these sequences are different before and after heat shock, this may suggest heat promotes transposon mobilization; and in addition, our results we show common locations where transposons excise from and move to in the genome.

Conclusion

The mechanism of temperature-induced DNA damage remains unclear. While we have demonstrated that transposons are capable of moving around the genome after heat shock, it is unclear how temperature allows for transposition events to occur in the *C. elegans* genome. Additionally, it seems that temperature-induced DNA damage is independent of transposon copy number, but the highly variable data coupled with the small range of transposon copy numbers in the strains that we studied drives us to gather more data to recapitulate the results shown in this study. There are multiple

possibilities that result in the variability of the results, including temperature indirectly affecting transposition events through small RNA abrogation. Or perhaps we captured actual variability in the amount of temperature-induced DNA damage in wild type *C. elegans* strains.

A comprehensive understanding of the mechanism of temperature-induced DNA damage during spermatogenesis will help to elucidate the relationships between chromatin structure, small RNA pathways, transposons, and other undiscovered affecters. Moreover, our study indicates that multiple mechanisms aside from transposition of transposable elements may be responsible for DNA damage after heat shock specifically in spermatocytes. This study lays the initial foundation of the role of transposable elements and demonstrates an initial understanding of the phenotype in a wide array of wild type *C. elegans* strains. Ultimately, my study could help identify potential therapies, regiments, or lifestyle changes that could alleviate male infertility in humans.

Glossary

Anaphase: Phase of meiosis characterized by the movement of chromosomes to opposite poles in the cell.

Antibody: Protein produced by an organisms' immune system that targets and binds to foreign proteins.

Diploid: Containing two sets of chromosomes, one from each parent.

Double-strand DNA break (DSB): DNA damage in which both strands of the DNA double helix are broken.

DNA: Biomacromolecule that carries genetic information from generation to generation.

Fluorophore: A fluorescent chemical compound typically used to visualize compounds of interest.

Gamete: Sex cells with half the number of chromosomes as a body cell. More commonly referred to as sperm and egg cells.

Genotype: The genetic makeup of an individual.

Germline: A population of cells in an organism that are used primarily to pass on genetic information to offspring. Sperm and egg are two examples of cells that are part of the germline.

Haploid: Having a single set of unpaired chromosomes.

Homologous Chromosomes: A set of one maternal chromosome and one paternal chromosome that pair up when the cell undergoes meiosis.

Homologous Recombination: The exchange of genetic information between chromosomes that promotes genetic diversity as well as promotes proper chromosome segregation.

Immunofluorescence: An experimental technique that utilizes fluorescently-tagged antibodies to target proteins of interest to visualize under a fluorescent microscope.

Meiosis: A specialized form of cell division that produces genetically diverse sex cells (sperm and egg) with half of the number of chromosomes as a body cell.

Metaphase: Phase of meiosis characterized by the lining up of chromosomes at the center of the cell.

Nucleus: A dense compartment in the cell that contains the cell's genetic material.

Oogenesis: The development of germ cells to eggs.

Phenotype: The observable characteristics of an individual.

Point Mutation: A genetic mutation that only affects one nucleotide base in a sequence of DNA.

Polymerase Chain Reaction (PCR): An experimental technique used to create multiple copies of a segment of DNA. This technique relies on primers that flank the sequence of interest that allow for DNA polymerase to bind and synthesize new DNA strands.

Prophase: Phase of meiosis characterized by chromatin condensing into chromosomes, the pairing of homologous chromosomes, and homologous recombination.

RAD-51: Meiotic recombination protein that binds double-strand DNA breaks to mark them for repair. This protein is used extensively in my project as a marker for DNA damage.

RNA: Biomacromolecule that is involved in the coding, decoding, regulation, and expression of genes in the genome.

Somatic/Soma: Cells in the body, in contrast to the germline.

Spermatocyte: A developing male sex cell.

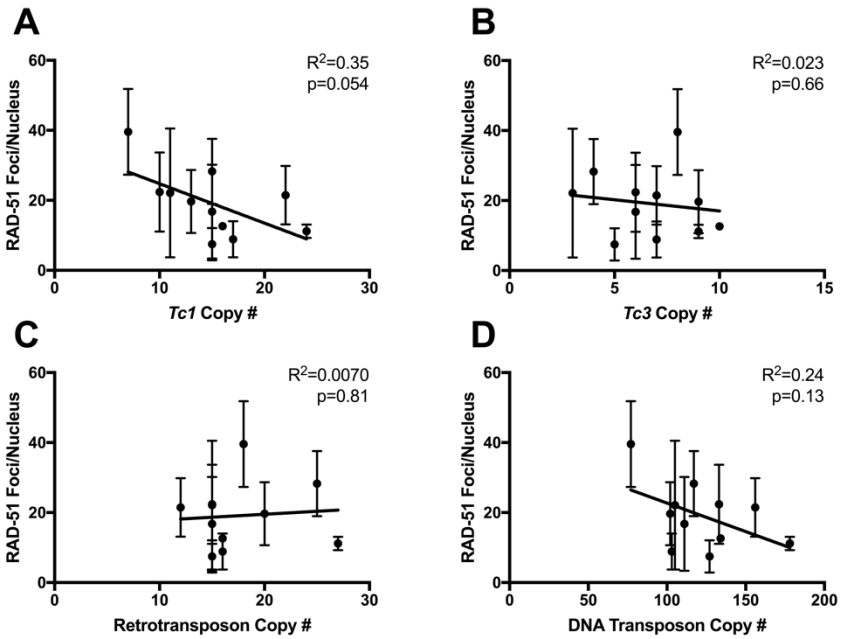
Spermatogenesis: The development of germ cells to mature sperm.

SPO-11: Endonuclease that cleaves DNA to produce double-strand DNA breaks. Without this protein, *C. elegans* cannot produce endogenous DNA breaks.

Telophase: Phase of meiosis characterized by chromosomes reaching the opposite poles of the cell and the nuclear envelope beginning to reform.

Wild Type: Organisms that are considered the norm within a population.

Supplementary Information



Supplemental Figure 1: Temperature-induced DNA damage is independent of transposon copy number in early pachytene

Strains with varying transposon copy numbers were heat shocked for 2 hours at 34°C. Immunofluorescences images were taken at early pachytene of dissected gonads. Each image was quantified for amount of RAD-51 and number of nuclei. The number of RAD-51 foci per nucleus was calculated. Graph represents mean RAD-51 foci per nucleus with error bars denoting SD. Transposon data was taken from Laricchia et al., 2017. **A.** Tc1 transposon copy number. **B.** Tc3 transposon copy number. **C.** Retrotransposon copy number. **D.** Total DNA transposon copy number.

Supplementary Table 1: Strains used in immunofluorescence experiment

Transposon insertion numbers were found by taking the total number of transposon sites for a class and subtracting by the number of active reference sites. XZ1515 was not characterized. Complete transposon data can be found at Laricchia et al. 2017.

Strain	*Tc1*	*Tc3*	DNA	Retro
CB4856	17	7	103	12
CX11314	11	3	105	12
DL238	15	4	117	22
ED3017	16	10	134	15
EG4724	10	6	133	14
JT11398	15	5	127	13
JU775	7	8	77	15
LKC34	15	6	111	11
MY16	24	9	178	24
N2	22	7	156	12
QX1791	13	9	102	16
XZ1515	?	?	?	?

Supplementary Table 2: Solutions used in this study

Mixture	Composition
10x egg buffer	1180 mM NaCl, 480 mM KCl_2, 20 mM $CaCl_2$, 20 mM $MgCl_2$, 250 mM HEPES pH 7.4
Dissection buffer	1x egg buffer, 0.1% Tween20
Paraformaldehyde fix	2% paraformaldehyde, 1x egg buffer, 0.1% Tween20
10x phosphate buffer solution (PBS)	0.1M Na_2HPO4, 0.018M KH2PO4, 1.37M NaCl, 0.027M KCl
PBST (PBS with Tween)	1x PBS, 0.1% Tween20
Blocking solution	0.7% BSA in PBST
DAPI	0.007M DAPI
LB broth	1% tryptone, 1% NaCl, 0.5% yeast extract
LB agar	1.5% agar, 1% tryptone, 1% NaCl, 0.5% yeast extract
Lysis buffer	50 mM KCl, 10 mM $Tris^{HCl}$ pH 8.2, 2.5 mM $MgCl_2$, 0.45% IGEPAL, 0.45% Tween20, 0.1 mg/mL proteinase K
Garlic extract	3.16 g of crushed garlic in 50 mL of 95% EtOH

Supplementary Table 3: Primers used in this study

Primer	Sequence
DLO683	5'- GTTTGCAGTTGGGTTCCACT -3'
DLO684	5'- CGGACTTTTGGATCTCCGTA -3'

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