

INVESTIGATING HEAT SHOCK INDUCED DOUBLE
STRAND BREAKS IN *CAENORHABDITIS ELEGANS*
SPERMATOCYTES

by

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Diana E. Libuda, Ph.D.

Meiosis is a specialized form of cell division that occurs in sexually reproducing organisms to generate haploid gametes, such as sperm and eggs. Double strand DNA breaks (DSBs) are a form of DNA damage and are intentionally induced in cells during meiosis. It is necessary that these breaks are properly repaired because improper repair can cause infertility, birth defects, miscarriages, or cancer. Unlike other tissues in the body, sperm are known to be sensitive to small fluctuations in temperature; the inability of sperm to thermoregulate in response to high temperature is known to cause male infertility. Previous research in *Caenorhabditis elegans* has shown that heat shock of adult males results in a dramatic increase in DSBs in the developing sperm of the germline. This phenotype is specific to spermatocytes and independent of *spo-11*, the enzyme that induces DSBs. This paper examines the effect of a spectrum of heat shock temperatures on the induction of DSBs in *C. elegans* spermatocytes. Adult male worms were heat shocked and the gonads were dissected and stained with antibody to mark DSBs. The results show a relatively uniform quantity of breaks from 25-33°C with a threshold increase in the number of breaks happening at 34°C. Additional DSBs were

induced at heat shock temperatures above 34°C. These results indicate that spermatocytes are extremely sensitive to a slight fluctuation in temperature and that deviation from a narrow isotherm leads to the induction of an abundance of DNA damage. This work has helped to increase our understanding of how small temperature fluctuations contribute to the induction of DNA damage in spermatocytes, which can ultimately lead to male infertility.

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Introduction

Meiosis

All living species must be able to reproduce in order to survive; however, the means by which they do so is unique among species. While some organisms are able to reproduce asexually, by making a near copy of themselves, many individuals reproduce sexually. Sexual reproduction is extremely complicated because it requires each parent to have specialized sex cells, known as gametes, which contain the genome that will be transmitted on to the next generation. Prior to fertilization, the germ cells of an organism undergo the process of meiosis, which is a specialized form of cell division that results in the creation of haploid gametes.

Meiosis begins with DNA replication followed by two separate rounds of cell division, resulting in four daughter cells that contain a haploid genome (Figure 1). Before meiosis occurs organisms are diploid, meaning they have two sets of homologous chromosomes, which are chromosome pairs of the same size that possess genes for the same characteristic at corresponding loci. One homologous chromosome is inherited from the father and one the other is from the mother. These homologous chromosomes are replicated to form sister chromatids. The proper segregation of homologous chromosomes during meiosis I and sister chromatids during meiosis II are necessary to ensure proper genome inheritance.

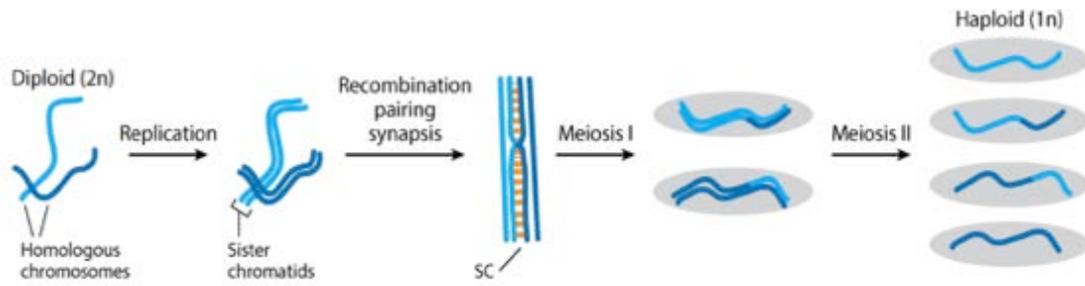


Figure 1: Overview of Steps Before and During Meiosis

Prior to cell division an organism is diploid and has 2 homologous chromosomes, which are replicated to result in sister chromatids. These chromatids align and cross over to form the synaptonemal complex, which is the structure that is formed between the chromosomes. During meiosis I the two sets of homologs are separated and in meiosis II the sister chromatids are separated, resulting in four haploid daughter cells.

Image from *Keeney et. al.* 2014.⁴

Meiosis can be broken down into multiple distinct steps, with each stage playing a different and important role in cell division. During the first stage of meiosis, known as prophase I, the synaptonemal complex is formed between homologous chromosomes and crossover recombination events are formed between DNA molecules (Pattabiraman *et al.* 2017). Both events primarily function to maintain the cell's genomic integrity and promote proper chromosome segregation during cell division. Prophase I can further be broken down into 5 sub-steps: leptotene, zygotene, pachytene, diplotene, and diakinesis. Each of these stages plays a unique role in promoting proper chromosome segregation and preparing the cell for upcoming meiotic divisions.

Double Strand DNA Breaks

During prophase I, double strand DNA breaks (DSBs) are induced. A DSB is a break in the phosphate backbone of both strands of a DNA double helix, resulting in two separate segments of DNA. These breaks are deliberately induced because they allow the cells to form crossovers, which are necessary for proper chromosome

segregation (Yu *et al.* 2016). There are numerous complex mechanisms underlying the induction and repair of DSBs that are in place to ensure that DSBs are made at the correct time and place, and in the correct amount. It is necessary that DSBs are accurately repaired in cells because improper repair can lead to a vast array of birth defects and health problems, including miscarriages, infertility, and cancer (Bhattacharjee *et al.* 2016).

DSB Induction and Repair

Both the induction and repair of DSBs require the cooperation and coordination of a vast array of proteins. To begin, DSBs are formed by SPO-11, which is a conserved topoisomerase relative that cleaves DNA (Dernberg *et al.* 1998). This enzyme works by creating a covalent protein-DNA intermediate structure and then cleaving this intermediate to release SPO-11 along with a short oligonucleotide (Keeney *et al.* 2014).

The removal of SPO-11 results in open DNA ends with single stranded 3' overhangs that allow the loading of a protein called RAD-51 onto the single stranded DNA, therefore forming a nucleoprotein filament (Gartner *et al.* 2000). This protein was originally isolated in *S. cerevisiae* and it is homologous to the bacterial RecA protein (Alpi *et al.* 2003). RAD-51 is involved in meiotic recombination, specifically functioning to promote recombinational repair and DNA strand exchange (Takanami *et al.* 1998). In wild type cells, RAD-51 localizes to distinct foci along DNA in the transition zone (leptotene/zygotene), increases in abundance in early pachytene, and disappears by late pachytene (Figure 2).

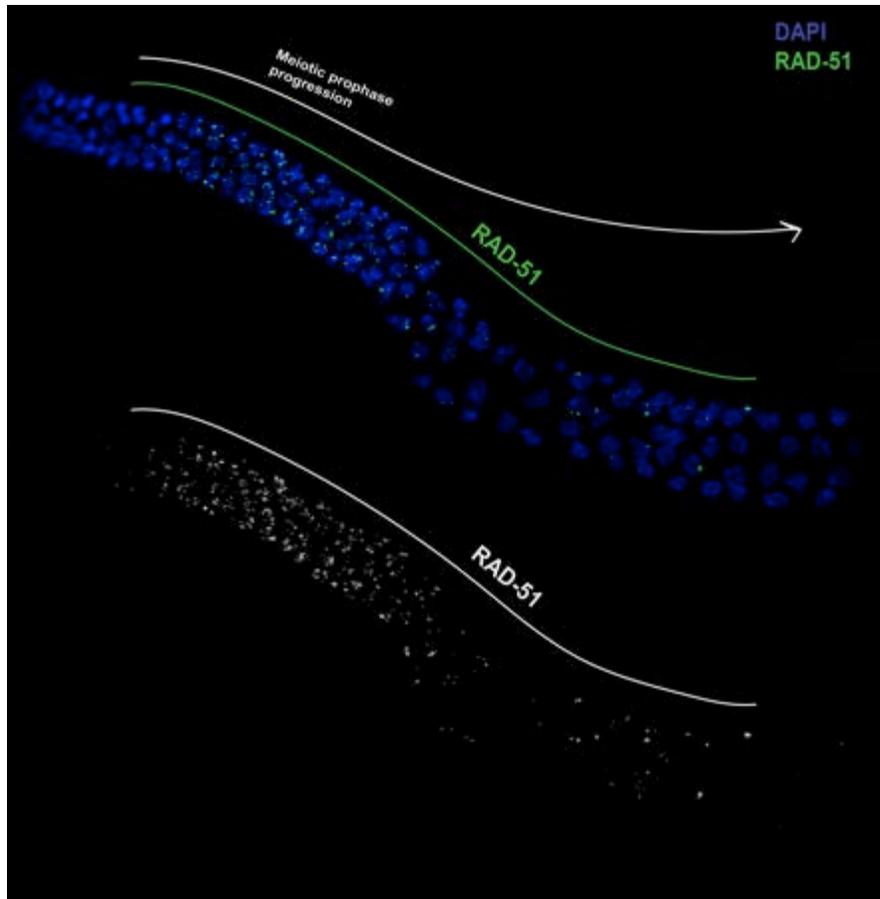


Figure 2: Wild Type RAD-51 Localization

RAD-51 is present in the gonads starting in the transition zone and persisting through early/mid pachytene. In this image gonad is pictured from early transition zone through mid/late pachytene.

This wild type localization of RAD-51 can be dramatically different in worms that are mutant for genes involved in DSB formation and repair because the pattern in which DSBs are formed is drastically changed. For example, in worms lacking a functional *spo-11* gene, there are no RAD-51 foci present in the gonad because the DSBs are not induced (Alpi *et al.* 2003). Given its localization at DSBs soon after DSB formation, RAD-51 is used as a marker for early DSB repair stages because it permits visualization of the location and quantity of DSBs in the gonad.

DSB repair is an extremely complex process due to the many different pathways that can be utilized to repair the induced DSBs. The first decision that the cell needs to make when faced with a DSB is whether to perform interhomolog or intersister repair. Intersister repair involves the repair of a DSB between sister chromatids, whereas interhomolog repair uses the homologous chromosome to repair damage. Furthermore, interhomolog repair can be in the form of a crossover or non-crossover. Non-crossovers result in the replacement of one DNA sequence with a homologous one whereas crossovers involve the physical crossing over of the chromosome arms, resulting in a structure known as the chiasma. In meiotic cells, at least one DSB per chromosome pair must be resolved as a crossover to ensure the proper segregation of chromosomes at the first meiotic division. There is still very little known as to how cells choose which repair pathway to use in the event of DSBs. Previous research has found that germ cells have specialized modes of DSB repair depending on the stage of meiosis that they are in, and each mode is dependent on different proteins, including RAD-51 (Hayashi *et al.* 2007).

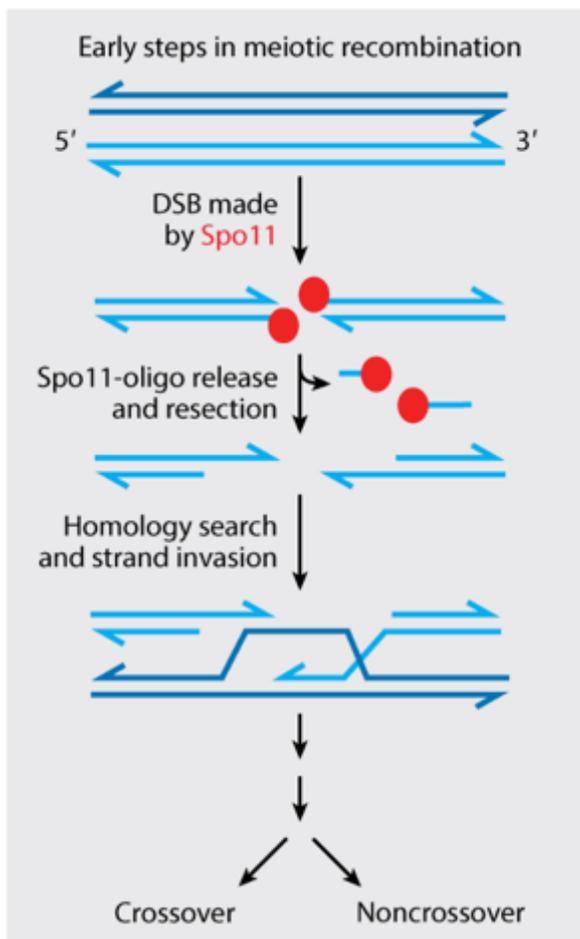


Figure 3: General Overview of DSB Induction and Interhomolog Repair Choice

In prophase I DNA double strand breaks are induced by the SPO-11 enzyme. This enzyme creates an intermediate with the DNA and is then cleaved off to result in DSBs. These breaks can be repaired by either the sister chromatid (same color line) or the homologous chromosome (different color lines). This image illustrates interhomolog repair and once the strand has invaded the homologous chromosome it can either form a crossover or noncrossover to repair the DNA. Image from *Keeney et. al.* 2014.

Role of Heat Shock in DSB Induction

Although it may seem like SPO-11 is the only factor that can induce DSBs, there are other environmental factors that are able to cause these breaks. Unpublished data from Dr. Diana Libuda illustrates an increase in frequency of DSBs in spermatocytes that are heat shocked at 34° C. This phenotype is solely expressed in

spermatocytes, which includes both males and young adult hermaphrodites that are still undergoing spermatogenesis. Adult hermaphrodites do not exhibit the change in frequency of DSBs with regard to heat shock, confirming that this is a spermatocyte specific response. To further investigate this phenomenon, Libuda examined worms with different genetic backgrounds to see if increased temperatures affected them. First, *spo-11* mutant males and hermaphrodites were tested for heat shocked induced DSBs. The *spo-11* mutant means that the worms were lacking a functional *spo-11* gene and therefore could not produce the protein that typically induces DSBs. Interestingly, it was shown that in the hermaphrodites and non-heat shocked males there were no DSBs induced. In contrast, the male heat shocked worms exhibited a large quantity of DSBs after they were exposed to the increased temperatures (Figure 4). This is interesting because it shows that there is another factor that is somehow causing the induction of the DSBs. The observed heat shock phenotype is SPO-11 independent, meaning it occurs both in the presence and absence of SPO-11.

The next genotype examined was worms with decreased transposon copy numbers compared to wild type (CB4856). Transposons are mobile elements of DNA that move throughout the genome. The first transposable element that was discovered in *C. elegans* is called Tc1, and its copy number is strain dependent: the wild type N2 strain contains around 30 Tc1 copies (Van Luenen *et al.* 1997). Libuda observed that in worms with decreased Tc1 copy numbers, compared to the Bristol N2 strain, there was no increase in double strand breaks following heat shock (Figure 5). Males with normal transposon copy number had a high frequency of DSBs following heat shock, whereas in the mutant worms there were very few breaks observed. This data indicates that the

formation of temperature sensitive DSBs is dependent on transposon copy number. These findings ultimately led to the hypothesis that in spermatocytes transposons are mobilized following heat shock at 34° C, which may lead to increased DNA damage and therefore infertility.

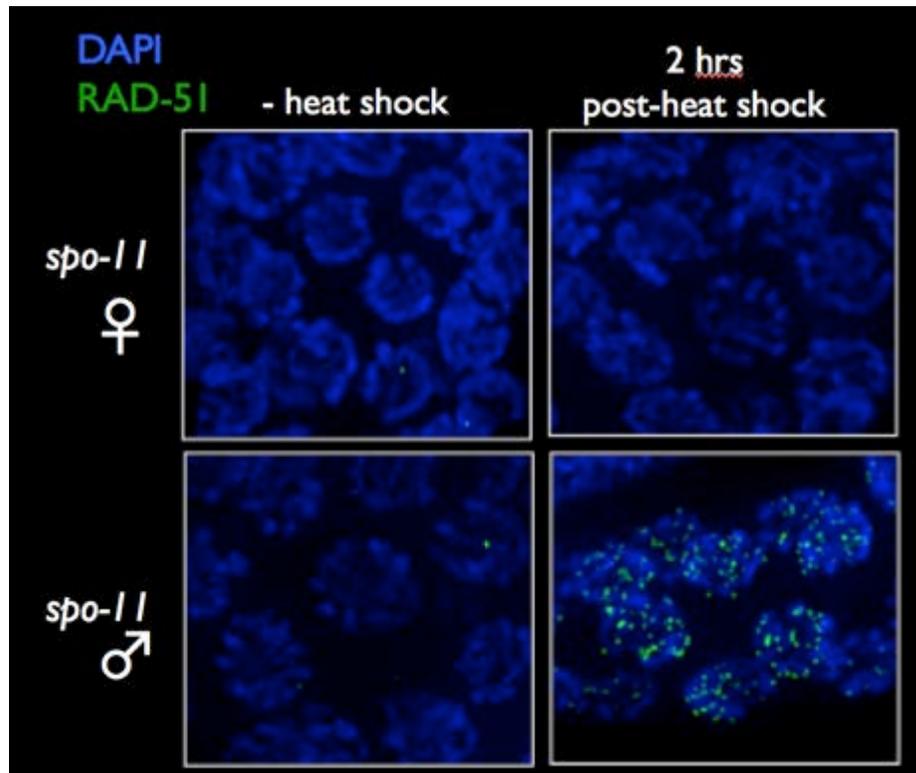


Figure 4: *spo-11* Mutant Gonads

spo-11 mutant male and hermaphrodite nuclei stained with DAPI (Blue) and RAD-51 (Green). There are no DSBs induced in the hermaphrodites, nor the male that wasn't heat shocked. In the heat shocked male there is a dramatic induction of DSBs shown by the presence of RAD51 foci. Images taken by D. Libuda.

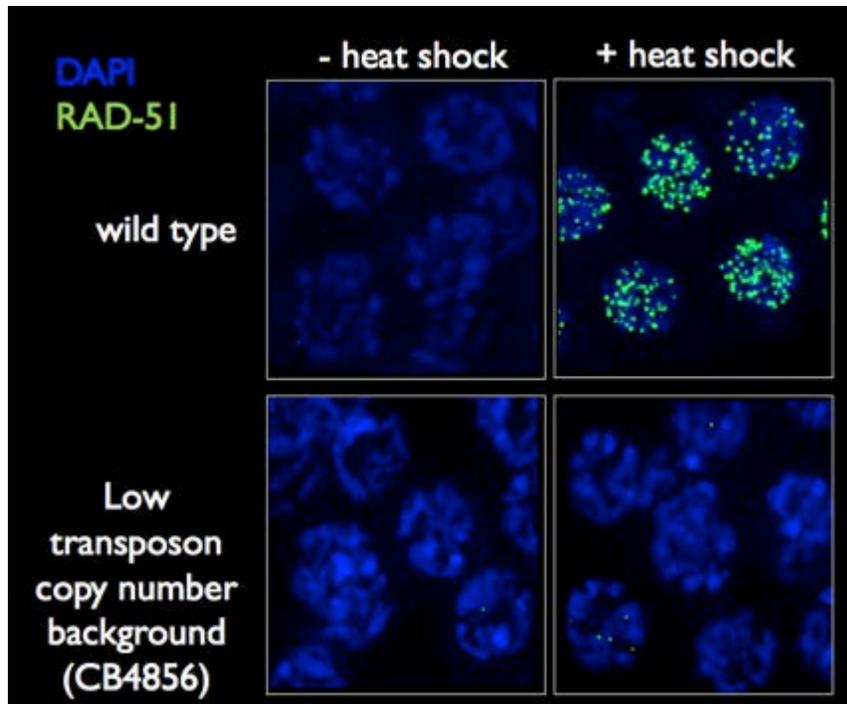


Figure 5: Male Gonads with Normal and Low Transposon Copy Number

Both wild type and low transposon number worms were heat shocked to examine the induction of DSBs. The wild type males had no RAD-51 foci when they weren't heat shocked however a high quantity of foci appeared following heat shock, as previously described. The males that had low transposon copy numbers did not have RAD-51 foci, independent of heat shock. Images taken by D. Libuda.

Introduction to *C. elegans*

The nematode *Caenorhabditis elegans* was utilized as a model organism for the conduction of this research project. *C. elegans* are microscopic round worms that exist in two sexual forms: self-fertilizing hermaphrodites and males (Corsi *et al.* 2015). The hermaphrodites are able to self-fertilize because the gonad forms an ovotestis that first produces sperm and then produces oocytes once the worms become young adults; they are essentially females that produce sperm for a short period of time before producing oocytes (Alpi *et al.* 2003). Both hermaphrodites and males have five autosomal chromosomes, however they differ in the fact that the hermaphrodites have two X

chromosomes (XX) whereas the males only have one (XO). There are numerous features of *C. elegans* that make them advantageous to understanding DNA repair during meiosis. Firstly, *C. elegans* are very small organisms, about 1 mm in length, which makes them easy to care for and maintain in a lab setting. Additionally, they have a relatively quick life cycle, meaning they are fast to reproduce, allowing the study of multiple generations in a short time period. Lastly, their cells are organized in a temporal/spatial gradient along the germline, which allows for the visualization of all the stages of meiosis in a single gonad (Figure 6) (Garcia-Muse *et al.* 2007). This aspect of *C. elegans* morphology is important to this research project because it allowed visualization of all the cells in the gonad and therefore examination of the effect of heat shock on spermatogenic cells.

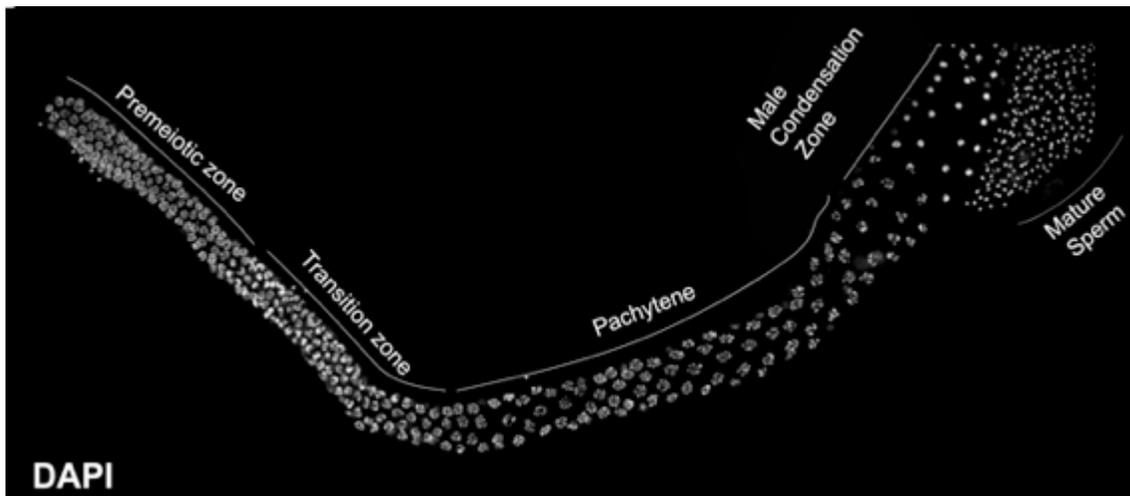


Figure 6: Organization of *C. elegans* Germline

A male gonad is stained with DAPI, which marks DNA. The nuclei of the gonad are organized in a spatial/temporal gradient, which allows for the visualization of nuclei in each stage of meiotic prophase I.

Experimental Questions

In this study, I aimed to answer two specific questions regarding heat shock induced DSBs in *C. elegans* spermatocytes. Both research goals of this project involved examining heat shock from a gradient of temperatures in order to determine how the spermatocytes were affected. The goal of this research was to fully characterize the heat shock phenotype that was previously discovered in males.

Question 1

The first question that I aimed to answer was: At what temperatures are non-endogenous double strand DNA breaks induced in spermatocytes? By examining a gradient of temperatures, I was able to determine at which temperatures DSBs are induced in spermatocytes and compare these with each other. It was hard to hypothesize the effect of the different temperatures on DSB induction due to the lack of previous knowledge; however, I hypothesized that there would likely be a linear increase in the quantity of RAD-51 foci.

Question 2

My second research question was: In males, which stages of meiotic prophase are specifically sensitive to DSBs induced by heat shock? This is the section where using *C. elegans* for research becomes extremely advantageous because in my experiments, I was able to visualize each of the stages of meiotic prophase I and therefore deduce which ones are specifically sensitive to heat shock. It is beneficial to understand which stages are particularly susceptible to the DSBs to further understand the mechanisms acting behind this phenotype. I believed that as the temperature increased, the length of the RAD-51 zone would also increase at a similar rate.

Introduction to Experimental Techniques: Immunofluorescence

In order to examine the induction of DSBs within the gonad, immunofluorescence (IF) experiments with antibodies raised against RAD-51 were performed. Antibodies are proteins that are produced by the immune system to target foreign proteins known as antigens and mark them for destruction. In a lab setting we use antibodies generated from various organisms to locate and identify proteins that we want to study further. The antibodies that are used are conjugated with a fluorophore, which is a fluorescent chemical compound that emits light upon excitation by a specific wavelength of light. There are two different classes of immunofluorescence techniques that are frequently performed in a lab setting: direct and indirect (Figure 7) (Direct vs Indirect 2017). The principal difference between these two techniques is that direct IFs utilize a primary antibody that is already tagged with a fluorophore, whereas indirect IFs use a primary antibody first to identify the antigen and then a secondary antibody tagged with the fluorophore to identify the primary antibody. Although direct IFs have fewer steps there are many reasons that indirect IFs are more advantageous to my research. First, indirect IFs tend to be more sensitive than direct IFs, meaning that the signal emitted from indirect experiments is typically stronger. This is because in direct IFs only one primary antibody molecule, and therefore one fluorophore, is capable of binding the antigen. In contrast, indirect IFs allow multiple secondary antibodies, and their conjugated fluorophores, to bind the primary antibody and therefore the antigen, causing a brighter signal. Additionally, indirect IFs are more flexible and less expensive because there is an extremely limited availability of commercially produced directly conjugated primary antibodies.

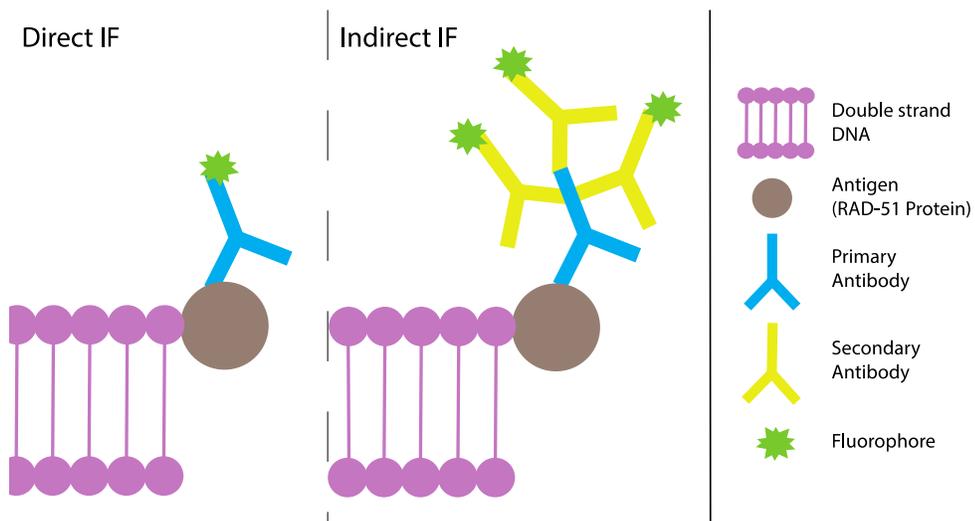


Figure 7: Direct and Indirect Methods of Immunofluorescence

Both direct and indirect methods use antibodies to target a specific antigen in cells. The difference between the two techniques is that direct IFs only require a primary antibody tagged with a fluorophore to bind the antigen, whereas indirect IFs require a primary antibody to bind the antigen and a secondary antibody tagged with a fluorophore to bind the primary antibody. In this research the antigen targeted was the RAD-51 protein, which binds onto DNA double strand breaks.

Methods

Worm strains and maintenance

Worms were maintained at 20° Celsius under standard conditions, as described by Brenner (1974). The Bristol N2 wild type strain was used for all experiments. Males were generated by setting up mating plates and crossing three males with one hermaphrodite.

Immunofluorescence

L4 (larval stage 4) males were picked 19-21 hours prior to heat shock and kept at 20° C. The worms were then heat shocked for two hours at one of the following temperatures: 25°, 27°, 29°, 31°, 33°, 34°, 36°, or 38° C, with a control group remaining in the 20° incubator. Following heat shock the worms were left to rest at 20° C for one hour, which allowed the worm to contract and therefore allow for easier removal of the gonads.

Once the hour of rest was complete, the worms were transferred to a coverslip with 1x egg buffer and a needle was used to dissect the gonads out of the worms. The gonads were then added to a 2% paraformaldehyde solution and a slide was placed onto the coverslip, resulting in the gonads being sandwiched between the slide and the coverslip. After 5 minutes in the paraformaldehyde, the coverslip was “cracked off” by freezing the slide in liquid nitrogen and using a razor blade to remove the coverslip. The slides were then placed in methanol for 1 minute, rinsed in PBST, and left to block in a solution of BSA in PBST. Following the BSA block were two separate incubations with the antibodies: first an overnight incubation with primary antibody and second a 2-hour

incubation with secondary antibody. The primary antibody that was used was a 1:500 dilution of Rabbit anti-RAD51 and the secondary antibody was a 1:200 dilution of Goat anti-rabbit (488). Lastly, the DNA was stained with DAPI and a coverslip was placed on the slide, making them ready for microscopy. IFs for each temperature were repeated in triplicate to prevent false results. Each individual step in an IF plays a unique roll, and figure 8 illustrates an overview of each of these stages.

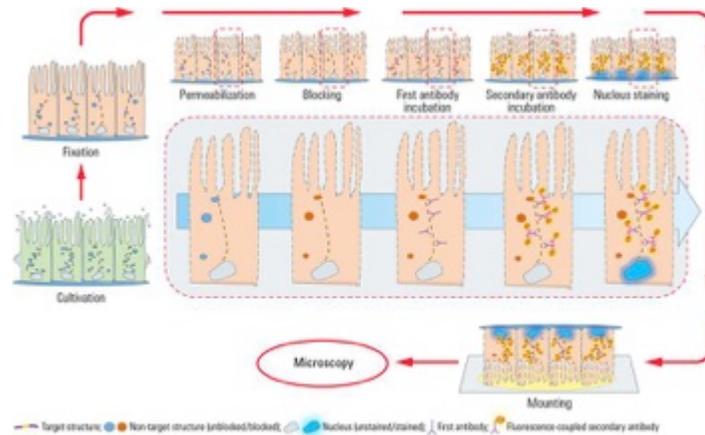


Figure 8: Illustration of the Steps of Indirect Immunofluorescence

This figure illustrates each steps of an indirect IF performed on epithelial cells growing on a coverslip. In this image the cells are cultivated because they grow on the slide, whereas the experiment outlined in this paper involved dissecting *C. elegans* gonads. Following dissection the cells are fixed with paraformaldehyde solution, therefore killing them. Next the coverslip is removed by freezing the slide cracking off the frozen coverslip, which helps to permeabilize the membrane and therefore allows antibodies to penetrate. The block with the BSA and PBST solution reduces unspecific binding of antibodies to non-target structures. Next the incubation with the primary antibody takes place, which specifically recognizes target proteins. In a second incubation the secondary antibody tagged with a fluorophore is added, and this binds the primary antibody and therefore allows visualization of the target protein. Following incubation with antibodies DAPI is added to stain the DNA and the slide is mounted with vectashield to help with preservation. Image from Hoff, 2015.

Fluorescent Imaging

After the gonads were dissected and stained with antibody, the slides were imaged using fluorescence microscopy. A Leica fluorescent microscope and LASX software were used to take all the images of the gonads, using both 405nm (for DAPI) and GFP (for RAD-51) filter sets. To prepare for microscopy, the exposure and brightness were set to minimum values to prevent the bleaching of slides. Additionally, the IF GFP DAPI setting on the microscope was used, which allowed for the visualization of both DNA and DSBs simultaneously. Once the microscope was set up, the slides were placed on the stage and screened at 10X magnification for intact gonads. When a gonad was found, the microscope objective was changed to 63X for imaging because it has the highest numerical aperture, and therefore the best resolution. Prior to imaging, the brightness and exposure settings were re-adjusted for the best image quality. Exposure settings were determined at the brightest section of the gonad: the pre-meiotic tip for DAPI and early pachytene for RAD-51. Images were taken using the XYZ setting, so that a Z-stack of multiple images through the Z-plane are acquired to obtain three-dimensional information throughout the nucleus. The Z-stack allows for the projection of every layer of the selected area of the gonad into one image. Z-stacks were set to specifically image the top layer of the nuclei in each of the gonads. Gonads were imaged starting at the pre-meiotic tip and working through to the mature sperm. Three gonads were imaged from each slide created, allowing for a wide data set. Once the images were taken they were exported as maximum projections, meaning every layer of the Z-stack was condensed into one two-dimensional image.

Image Processing

The maximum projection images were pieced together and adjusted using Adobe Photoshop. All images that were taken were of small segments of the gonads; they had to be pieced together, similar to a puzzle, in order to form the image of one whole gonad. Additionally, the colors were adjusted using the levels function in Photoshop. This was done to reduce the background and clean up many of the images. Once the images were tiled and adjusted they were assembled into figures using Adobe Illustrator.

Results

This research project examined the role of a range of heat shock temperatures on the induction of DNA double strand breaks in *C. elegans* spermatocytes. I tested eight different temperatures ranging from 25°-38° C and performed each heat shock experiment three times to confirm results. By performing these heat shock experiments, I was able to fully characterize the unique spermatocyte specific phenotype that is exhibited following heat shock.

Increase in RAD-51 Foci is Spermatocyte Specific

To confirm the spermatocyte specificity of the observed heat shock phenotype, both adult male and hermaphrodite worms were heat shocked at 34°, the gonads were removed and stained following the immunofluorescence procedure. Additionally, a group of adult males were not heat shocked and instead kept at 20° C leading up to the dissection, acting as a control group for the experiments. The males that were not heat shocked and the hermaphrodites that were heat shocked both had similar quantities and spatial distribution of RAD-51 foci through meiotic prophase. In contrast, there was a marked increase in the quantity and change in localization of RAD-51 foci in the heat shocked males (Figure 9). These findings confirm that the phenotype of increased RAD-51 foci, and therefore DSBs, is specific to spermatocytes in wild type worms. Following the confirmation of this phenotype, I examined temperatures other than 34°C to see how they affected the quantity of DSBs.

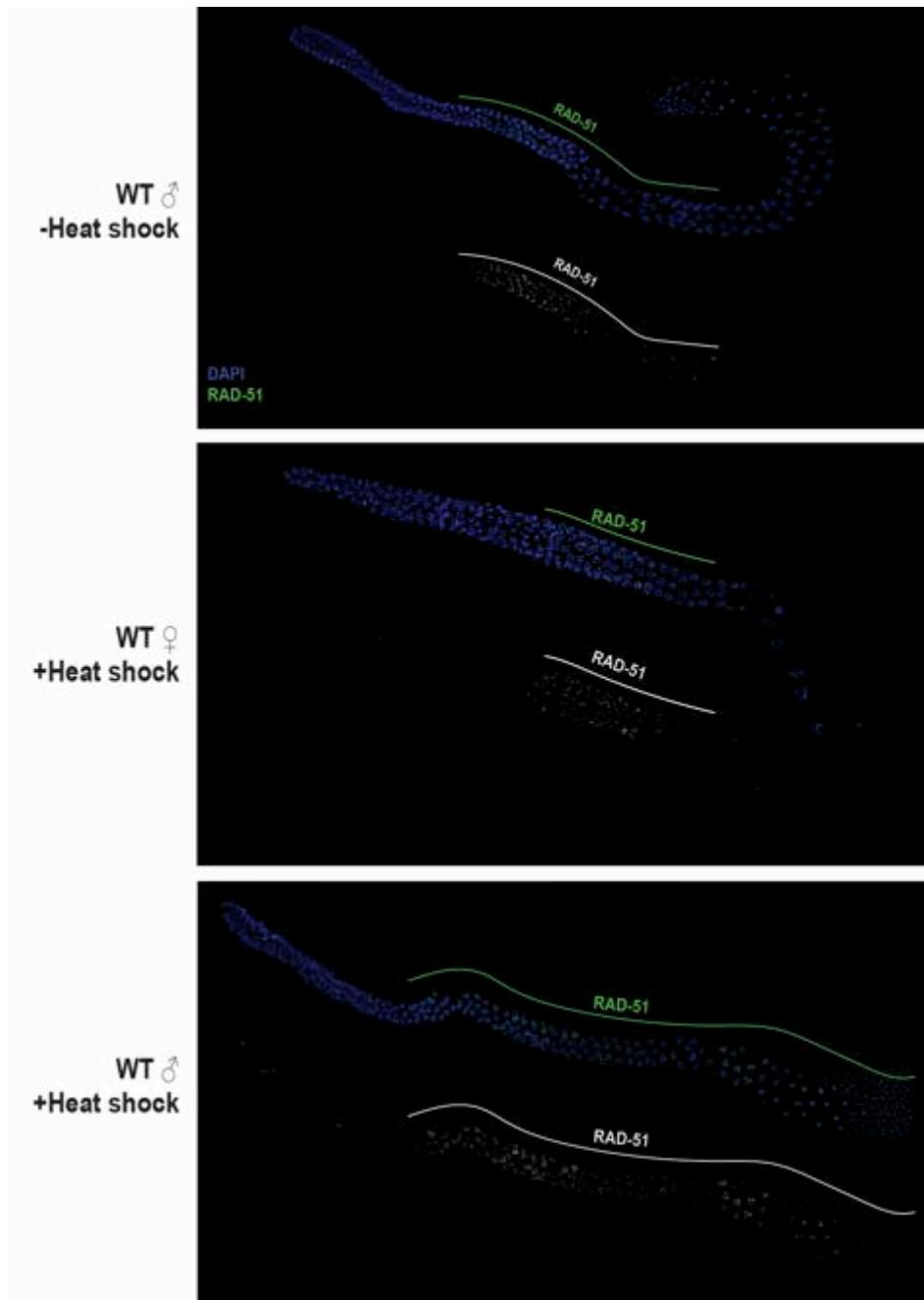


Figure 9: Heat Shock Phenotype is Spermatocyte Specific

Wild type male and hermaphrodite worms were heat shocked at 34°C and immunofluorescence for RAD-51 was performed. Wild type males (without heat shock) and heat shocked hermaphrodites both exhibit a wild type RAD-51 zone, with foci persisting from the transition zone through mid pachytene. In contrast, heat shocked males appear to have a dramatically increased RAD-51 zone, with foci persisting through the male condensation zone.

Quantity of RAD-51 Foci Increases with Temperature

To examine the role of heat shock on the induction of non-endogenous DSBs in spermatocytes, wild type young adult worms were heat shocked at one of the aforementioned temperatures and their gonads were removed and stained following the immunofluorescence protocol. Following immunofluorescence, the gonads were imaged using fluorescence microscopy and trends in DSB induction were analyzed.

Worms that were heat shocked from 25-33° C exhibited relatively wild type quantities of RAD-51 foci. In contrast, worms that were heat shocked at 34° C showed a threshold increase in the number of RAD-51 foci throughout the gonad. Following this threshold, there appears to be a slight increase in RAD-51 foci in worms that were heat shocked at both 36° C and 38° C; however, this increase is far more subtle and nowhere near as dramatic as the 33-34°C threshold.

Figure 10 presents representative nuclei in early pachytene from a selection of the heat shock temperatures. Images were taken in early pachytene because in wild type worms this zone has the highest concentration of RAD-51 foci. The images from both 25°C and 33°C have similar numbers of RAD-51 foci in each nucleus, appearing to average around 4 foci. There is a marked increase in these numbers in the worms that were heat shocked at 34°C, with around 10 foci in the majority of the cells in early pachytene. Lastly, in the worms that were heat shocked at 38°C there was another increase in foci during late pachytene. Most of the nuclei in these worms seem to have more than 10 RAD-51 foci, with most having upwards of 15-20. Taken together, this data shows that there is a threshold increase of quantity of DSBs at 34°C, with a gradual linear increase in foci up through 38°C.

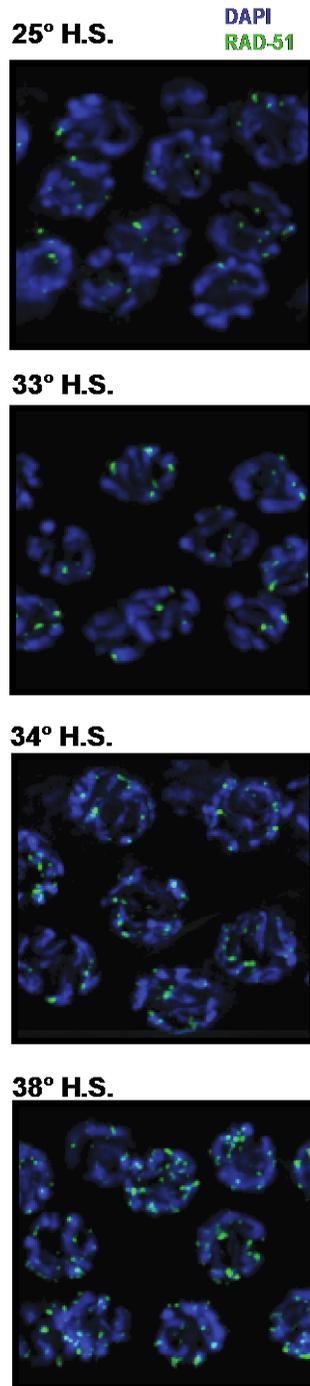


Figure 10: Representative Early Pachytene Nuclei from Heat Shocked Males

These images show representative nuclei in early pachytene following the noted heat shock temperatures. The frequency of DSBs seems to remain relatively uniform from 25-33° C, with a threshold increase at 34° C and further increase in foci at 38° C. DNA is stained in blue and RAD-51 in green.

Length of RAD-51 Zone Increases with Temperature

To examine the specific stages of meiosis that are sensitive to heat shock induced DSBs, images of whole gonads from heat-shocked males were compared. Worms that were heat shocked at temperatures from 25°-33° Celsius displayed RAD-51 zones that were relatively wild type in length, beginning in the transition zone and disappearing by mid/late pachytene. Worms that were heat shocked at 34°C showed a marked increase in the length of the RAD-51 zone. These gonads also had RAD-51 foci starting in the transition zone; however, these foci persisted through late pachytene and the male condensation zone. Both 36°C and 38°C heat shock resulted in a gradual increase in the zones that expressed RAD-51 foci. The 38°C heat shock produced the most dramatic effect, with significant numbers of RAD-51 foci present throughout the entire gonad, including the pre-meiotic zone and the mature sperm.

Figure 11 shows images of whole gonads from representative temperatures. The top two images (25°C and 33°C heat shock) both exhibit rather similar length RAD-51 zones, with the majority of the foci localized in early pachytene. There is a dramatic difference once 34°C heat shock is administered because RAD-51 foci are present throughout the entire meiotic region of the gonad. Lastly, the final image in the sequence shows 38°C heat shock, which resulted in RAD-51 foci throughout the entire pre-meiotic region, meiotic region, and the mature sperm. In contrast to the restriction of DSBs to a small region in male germ lines heat shocked at 34°C and below, this result shows that heat shock at 38 °C induces DSBs are induced in every germ cell in adult male germline.

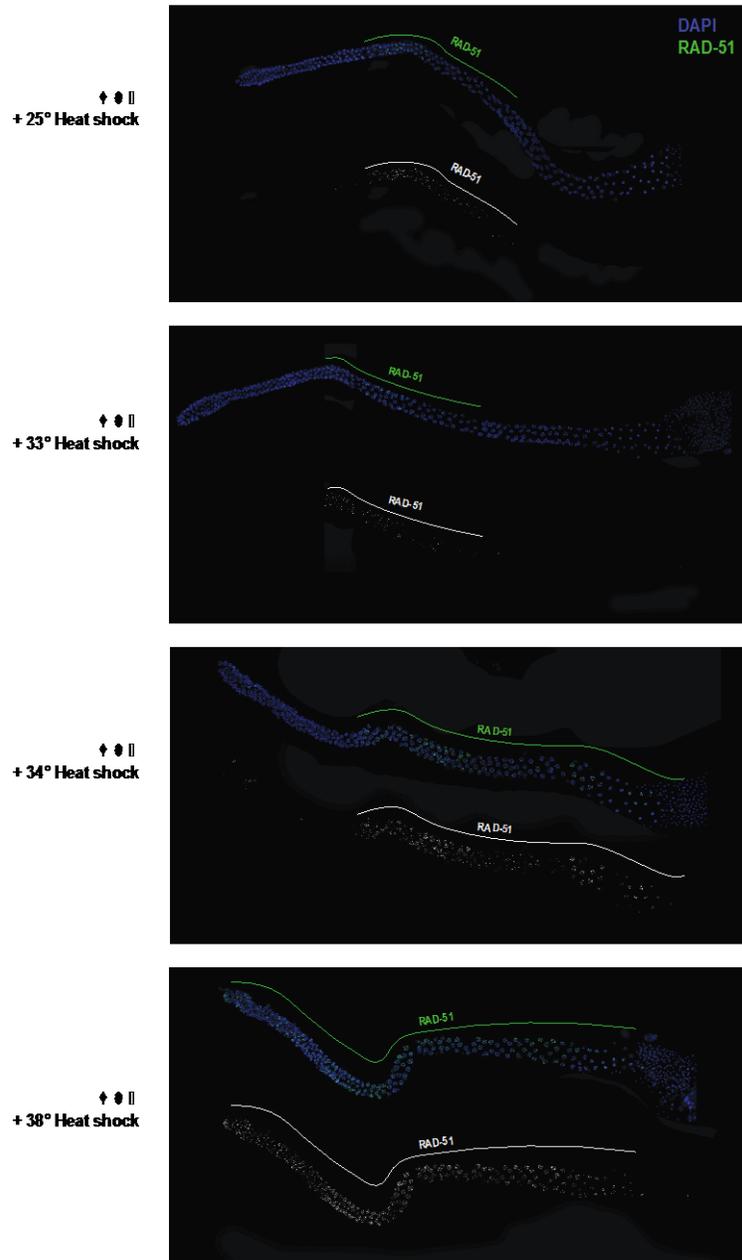


Figure 11: Full Gonad Images Following Heat Shock

This image shows full gonads from representative heat shock temperatures. From 25°-33°C, the zone with RAD-51 foci stays relatively constant (ranging from transition zone to early pachytene). At 34°C, the zone dramatically increases to range from the early transition zone through the male condensation zone. At 38°C, there is RAD-51 throughout the entire gonad.

Discussion

High Temperatures Result in Increased Levels of DSB Formation

This research allowed me to characterize the role of a spectrum of heat shock temperatures on the induction of DSBs in *C. elegans* spermatocytes. In order to conduct these studies, adult male *C. elegans* were heat shocked and their gonads were stained with an antibody that marks the RAD-51 protein, serving as an indicator of DSB formation. Originally, I hypothesized that there would be a gradual increase in quantity and length of RAD-51 presence in the gonad from 25-38°C, however this was not the case. My results indicate that there is a threshold temperature of 34°C that results in a dramatically increased quantity of RAD-51 foci, along with an extended RAD-51 zone. Temperatures below 34°C showed no dramatic changes in levels of RAD-51 foci, compared to the non-heat shock controls. Lastly, temperatures above 34°C (specifically 36°C and 38°C) showed a gradual increase in the quantity of RAD-51 foci, with the most foci in the worms that were heat shocked at 38°C. In addition to a high quantity of DSBs, these worms also exhibited RAD-51 foci throughout the entire gonad, including the cells that were not undergoing meiosis. This observation means that surpassing a threshold temperature causes a continual increase in the amount of DSBs that are induced. A qualitative overview of the trends in RAD-51 foci induction observed in this study is outlined in Table 1.

Heat Shock Temperature	Number of RAD51 foci (compared to WT)	Length of RAD51 zone (compared to WT)
25° C	Normal	Normal
27° C	Normal	Normal
29° C	Normal	Normal
31° C	Normal	Normal
33° C	Normal	Normal
34° C	↑	↑
36° C	↑↑	↑↑
38° C	↑↑↑	↑↑↑

Table 1: General Overview of Trends in Temperature Sensitive DSB Induction

This table shows the overall trends that were observed in my experiments. Up to 33°C, there appeared to be a normal RAD-51 concentration relative to non heat shocked controls; however, there was a threshold temperature at 34°C that led to a dramatically increased RAD-51 zone. Following the threshold, a gradual increase of foci was observed up to 38°C. These measurements in this table are all qualitative and compared to WT numbers.

Role of Transposons in Temperature Sensitive DSB Induction

The results that I observed in these studies show that there is a threshold temperature of 34°C that promotes the induction of DSBs in *C. elegans* spermatocytes. This phenotype has been previously shown to be *spo-11* independent and transposon dependent; therefore the observed results are most likely caused by increased transposon excision, solely in the male germline. These findings suggest that there is a mechanism that is being either activated or inhibited at this temperature that results in the increased rate of transposon excision. Many proteins and small RNA pathways are involved in transposon mobility, and one of these elements could be up/down regulated as the result of increased temperatures. A specific example is of a pathway that is

potentially involved in this phenotype involves piRNA, which stands for PIWI interacting RNAs. These distinct RNA molecules interact with PIWI proteins, which are involved in regulation of stem cells and cell division maintenance in germ cells (Hyun *et al.* 2017). piRNAs are also known to protect genomes from instability by repressing transposon mobility via transcriptional gene silencing; therefore, they are a likely contender for causing the increased DSB phenotype in males (Khanduja *et al.* 2016). An increased temperature could in turn disrupt these small RNA pathways that typically suppress transposon mobility in the germline. In several organisms, studies have shown that Argonaute proteins that form complexes with these small RNAs can cause transcriptional silencing in the germline. Some of the roles of Argonaute proteins involve cleavage of transposable elements and the recruitment of factors that mediate transcriptional and post-translational silencing (Toth *et al.* 2015). Inhibition of the piRNA pathway results in the overexpression of transposons, compromised genome structure, and ultimately germ cell death and infertility (Toth *et al.* 2015). These proteins are therefore one of many targets that could be functioning to cause the increase in DNA damage within male germlines that experience heat shock.

Significance of Research

Double strand DNA breaks (DSBs), although necessary for proper chromosome segregation and maintenance of genomic integrity, have the potential be very dangerous to cells. The improper repair of these DSBs can lead to numerous health problems including cancer, miscarriages, and birth defects. Additionally, when there are too many DSBs in a cell it may commit apoptosis, which is essentially cell suicide. In males

specifically, a problem associated with increased DSB induction and apoptosis is infertility.

Low sperm count, otherwise known as oligospermia, is one of the most common causes of male infertility. There are several factors that can lead to a low sperm count, but one of the most preventable is exposure to excessive heat. Spermatogenesis is the only developmental process that must occur at a temperature 3-4°C below the core body temperature (Sharpe *et al.* 2010). Certain stages of developing sperm, specifically cells in pachytene and early spermatids, are particularly susceptible to heat damage (Du Plessis *et al.* 2014). This data would be consistent with the results found in my experiments, because I found that at the threshold temperature of 34°C the nuclei in pachytene and the developing sperm seemed to have the most dramatic response to the heat shock. Once this temperature was reached, all the cells in these zones expressed extremely high numbers of RAD-51 foci, signifying high amounts of DNA damage. These cells that experience heat stress have previously been found to undergo programmed cell death as soon as 6 hours following exposure to heat. Sperm use multiple chemical pathways to kill themselves; some are similar to the mechanisms used by other cells in the body and some appear to be more unique (Setchell 1998). Researchers are still trying to fully understand how germ cells set their heat stress levels lower than the rest of the body's cells. Further understanding the mechanisms behind the temperature requirements of spermatogenesis may lead to the development of treatments for people who experience low sperm count due to heat stress.

Future Directions

This research provides a strong foundation and background for future research examining DSB induction in *C. elegans*. Following my experiments, a similar set of tests involving examining worms of different genotypes under heat shock temperatures could be conducted to determine the role of specific proteins in temperature-induced DSBs. My lab mentor, Nicole Codd, is currently examining the molecular mechanism of heat shock-induced DNA damage in males. She is specifically studying the effect of increased temperature in male germlines of strains that are defective in small RNA pathways, as well as strains with mutant proteins that are known to affect fertility. Currently, she is testing worms that are mutants for Argonaute proteins, including: *prg1/2*, *mut-8*, *csr-1*, and *alg-3/4*. Each of these mutant strains is defective for a unique aspect of transposon regulation. By examining specific mutants, Nicole will learn more about the factors that are involved in heat shock promoted DSB induction via transposons. Following these experiments, she will further study mutants for other proteins involved in the small RNA pathways. Furthermore, another lab member will be doing a related string of experiments; however instead of testing N2 worms, he will be looking at worms with a high and low transposon copy number. Since this phenotype was found to be transposon dependent, this will give further support that transposons are being excised upon an increase in temperature.

As temperature increases are an environmental stress condition, the temperature-induced DNA damage could be part of a general response of spermatocytes to environmental stress. To determine if this DNA damage phenotype is part of a general stress response, an assay similar to this style could be performed on wild type worms to

examine the role of different environmental conditions and stressors on the induction of DSBs. Some of these stressors could include changes in the salinity and pH of the growth medium. The results of these tests would indicate whether other environmental conditions affect DSB induction, and if the induced DSBs are also *spo-11* independent and transposon copy dependent.

As a drop-in temperature is known to cause cellular responses, a greater range of temperatures could be examined to further characterize this spectrum of results. This includes both looking at temperatures below 25°C and above 38°C. Experiments could look below 15°-20°C, which is the normal growth temperature for the worms to see if there is DNA damage that occurs when the worms get too cold. We know that there is a high concentration of DSBs at a 38°C heat shock and it would be interesting to know at what temperature the induction of DSBs plateaus. By examining a larger range of these temperatures, it would be possible to further characterize the temperature sensitive phenotype and potentially learn more about male infertility issues.

Conclusion

Through extensive research this study has successfully assembled a set of images that illustrate the effect of a gradient of heat shock temperatures on DSB induction in *C. elegans* spermatocytes. Although it was already known that 34°C heat shock results in increased DSB induction in males, this data further characterizes these findings. It appears that 34°C is a threshold temperature that causes the spontaneous induction of DSBs in spermatocytes, likely from increased rates of transposon excision. At temperatures above 34°C, the quantity of DSBs continuously increases to 38°C, meaning that there are even more transposons excised at these temperatures.

Understanding these spermatocyte specific phenotypes can give scientists a new path by which to examine male infertility. Further studies involving mutant worms will be able to classify which factors are specifically involved in this process. Ultimately, this knowledge of temperature sensitive transposon excision may help to develop treatments and therapies for males who experience temperature infertility issues.

Supplementary Information

Solution	Composition
10x egg buffer	1180 mM NaCl, 480 mM KCl ₂ , 20 mM CaCl ₂ , 20 mM MgCl ₂ , 250 mM HEPES pH7.4
Dissection buffer	1x egg buffer, 0.1% Tween20
Paraformaldehyde fix	2% paraformaldehyde, 1x egg buffer, 0.1% Tween20
10X Phosphate buffer saline (PBS)	0.1M Na ₂ HPO ₄ , 0.018M KH ₂ PO ₄ , 1.37M NaCl, 0.027M KCl
PBST	1x PBS, 0.1% Tween20
Blocking solution	0.7% BSA in PBST
2 ug/ml DAPI in water	0.007M DAPI

Table 2: The Recipes for Solutions used in this Paper

Glossary

Antibody: A specialized immune protein, produced because of the introduction of an antigen into the body. It marks the antigen for destruction.

Antigen: A toxin or other foreign substance that induces an immune response in the body, especially the production of antibodies.

BSA: Bovine Serum Albumen.

Chiasma: A point at which paired chromosomes remain in contact during the first metaphase of meiosis, and at which crossing over and exchange of genetic material occur between the strands.

Chromosome: A threadlike, gene-carrying structure found in the nucleus. Each chromosome consists of one very long DNA molecule and associated proteins.

Crossing over: The reciprocal exchange of genetic material between homologous chromosomes during synapsis of meiosis I.

Double strand break (DSB): Occurs or arises when the phosphate backbone of both strands of the DNA double helix is severed.

DAPI: A fluorescent stain that binds strongly to A-T rich regions in DNA.

Endogenous: A processes that originates from within an organism, tissue, or cell.

Fluorophore: A fluorescent chemical compound that can emit light upon light excitation.

Gamete: A mature haploid male or female germ cell that is able to unite with another of the opposite sex in sexual reproduction to form a zygote.

Germline: A series of germ cells each descended or developed from earlier cells in the series, regarded as continuing through successive generations of an organism.

Haploid cell: A cell containing only one set of chromosomes (n).

Homologous chromosomes: Chromosome pairs of the same size that possess genes for the same characteristic at corresponding loci. One homologous chromosome is inherited from the father and one the other is from the mother.

Interhomolog repair: The repair of a DSB between homologous chromosomes.

Intersister repair: The repair of a DSB between sister chromatids.

Immunofluorescence: A technique for determining the location of an antigen (or antibody) in tissues by reaction with an antibody (or antigen) labeled with a fluorescent dye.

Meiosis: A two stage type of cell division in sexually reproducing organisms that results in gametes with half the chromosome number of the original cell.

Nematode: A large phylum of worms with slender, unsegmented, cylindrical bodies, including the roundworms, threadworms, and eelworms.

Oligonucleotide: Short DNA or RNA molecules.

PBST: Phosphate buffer saline tween.

Sister chromatids: Replicated forms of a chromosome joined together by the centromere and eventually separated during meiosis II.

Topoisomerase: An enzyme that alters the supercoiled form of a DNA molecule.

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