UNIVERSITY OF CALIFORNIA SANTA CRUZ

Regulators of Meiotic Recombination and Checkpoint Control in *C. elegans*

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Alice Ye

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The Dissertation of Alice Ye is approved:

_____

Professor Needhi Bhalla, chair

_____

Professor Rohinton Kamakaka

_____

Professor Susan Strome

_____

Tyrus Miller

Vice Provost and Dean of Graduate Studies

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Abstract

Alice Ye

Regulators of Meiotic Recombination and Checkpoint Control in *C. elegans*

All sexually reproducing organisms rely on the events of meiosis, the cell division that produces gametes such as sperm and eggs. In order to ensure a proper chromosome complement in successive generations and to promote genetic diversity, meiotic chromosomes will align with their counterparts, synapse, and undergo recombination, the means of genetic exchange. Failure to accurately complete these steps during meiosis results in devastating consequences for progeny.

The mechanisms that execute and monitor the genetic interactions in meiosis are an exciting area of study in the field. In particular, understanding the importance of chromatin localization and dissecting the interactions of chromatin and various associated meiotic proteins are critical to our understanding of meiosis. Additionally, checkpoints exist that monitor proper completion of meiotic events, but the control of these checkpoints is not fully understood.

Using a variety of cytological and genetic techniques in *C. elegans*, the work here addresses the importance of perinuclear localization of chromatin during meiosis. A class of nuclear membrane-associated proteins called the LEM proteins

are involved in perinuclear tethering of chromatin. The three LEM proteins in *C. elegans* have differential effects on meiotic fidelity. Loss of one of these proteins, LEM-2, causes impaired repair and processing of double-stranded DNA breaks, which are necessary for recombination. It also induces meiotic errors that result in elevated levels of cell death in the germline. Loss of another one of these proteins, EMR-1, also affects meiotic DNA repair but less severely than LEM-2. Finally, LEM-3 appears to be required for a meiotic checkpoint that monitors synapsis.

This thesis also provides insight into the regulatory mechanisms that trigger germ cell death when errors in meiosis activate checkpoints. A conserved pro-apoptotic protein, EGL-1, is necessary for the two checkpoints to induce apoptosis, but its requirement for each checkpoint depends on the manner in which they are activated. Experiments also reveal that CED-13, a largely uncharacterized protein with homology to EGL-1, also has a role in meiotic checkpoints. Moreover, my studies indicate that recombination negatively regulates *egl-1* mediated germline apoptosis, potentially as a mechanism to promote repair over removal of defective meiotic nuclei.

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Background and Model System

Meiosis

Meiosis is a fundamental process in all sexually reproducing organisms, whereby a germ cell undergoes one round of DNA replication and then two rounds of division to produce haploid gametes. Errors in meiosis can cause mutations and chromosomal aberrations that have severely deleterious consequences for progeny. These include the production of aneuploid gametes, leading to Down, Turner, and Klinefelter Syndromes, as well as an increased disposition to cancer (Hassold and Hunt 2001; Hoeijmakers 2009). There is also evidence that schizophrenia is associated with copy number deletions of specific loci and that alterations in repeat copy number have been related to meiotic errors in aging males (Malaspina 2001).

To ensure proper segregation and accurate genetic exchange, homologous chromosomes proceed through a series of interactions during prophase of meiosis I (see Figure 1). Many of the mechanisms that ensure that each chromosome finds its homolog and establishes the correct linkages are still being uncovered, but the temporal progression through the requisite interactions has been described to varying detail in many systems.

At the onset of meiosis, replicated chromosomes search for their respective homologs and pair, as reviewed in (Bhalla and Dernburg 2008). In most organisms studied, chromosome ends at this time are closely associated with the interior of the

nuclear envelope (Zickler and Kleckner 1998). Homologs then undergo synapsis, in which a ladder-like proteinaceous structure loads between homologs to stabilize their association and facilitate recombination. This structure consists of a lateral or axial element that loads along the axis of each homolog and a central element that bridges the two homologs (Bhalla and Dernburg 2008). Meiotic recombination begins with the introduction of double strand breaks (DSBs) by the conserved endonuclease SPO-11, and the subsequent repair and resolution of some of these DSBs into crossovers (Giroux, Dresser et al. 1989; Keeney and Neale 2006). Crossovers (COs) are the sites of DNA exchange. They nucleate the localization of a number of CO-associated proteins, including ZHP-3 (Bhalla, Wynne et al. 2008) and COSA-1 (Yokoo, Zawadzki, et al. 2012), and provide the physical linkages that hold homologous chromosomes together and allow them to correctly orient to opposite poles of the Meiosis I spindle for later divisions.

Meiosis and the *C. elegans* germline

Meiosis in the *C. elegans* hermaphrodite is unique in a number of ways. Pairing, synapsis, and recombination are highly interdependent in many systems, such that the initiation of recombination is necessary to stabilize pairing and synapsis (Bhalla and Dernburg 2008). Single-stranded DNA (ssDNA) ends produced by the double-strand breaks at the beginning of recombination facilitate the homology search (Pittman, Cobb et al. 1998; Yoshida, Kondoh et al. 1998). These events are uncoupled during meiosis in the worm germline. Pairing and synapsis occur at the onset of meiosis. Both can be stabilized in the absence of recombination, and chromosomes can pair even when unable to synapse (MacQueen, Colaiacovo et al.

2002). This is possible because pairing is mediated by specialized *cis*-acting sites at the ends of chromosomes called Pairing Centers (PCs) that, similarly to the telomere ends of chromosomes in some other systems, are associated with the nuclear envelope (Sato et al 2009). PCs also contain various PC-associated proteins that are necessary for homologous pairing, each of which is specific for the PC motifs of one or two chromosomes, (Phillips and Dernburg 2006).

Although pairing and synapsis are independent of recombination in the worm germline, proper recombination requires synapsed chromosomes. Worms that load lateral elements of the synaptonemal complex but not the central elements in between homologs fail to accomplish interhomolog recombination (Colaiacovo, MacQueen et al. 2003). The crossover landscape is also distinctive: typically each of the six pairs of homologs will incur just one classical CO, which will be marked by the presence of specific CO promoting proteins (Bhalla, Wynne, et. al. 2008, Yokoo, et. al 2012).

Further, meiotic nuclei in the worm germline are laid out on a spatio-temporal gradient, such that the different phases of meiosis I are distinguishable by nuclear morphology and arrayed in order from the distal to proximal ends of the gonad (see Figure 2). The distal end of the worm germline develops throughout the larval stages of the worm (L1 – L4), growing towards the vulva at the proximal end (Kimble and Crittenden 2005). Oogenesis in the hermaphrodite germline begins in early adulthood, when nuclei dividing off the distal tip of the gonad first progress through rounds of mitotic division as they travel towards the proximal end (Kimble and Crittenden 2005). In the transition zone, these nuclei enter meiosis I (Kimble and

White 1981). In wild-type worms, homologs will pair and synapse in this region and the nuclei take on a distinctive “lopsided” morphology; chromosomes will cluster to one side of the nucleus, a phenomenon which is thought to facilitate the homology search and is loosely reminiscent of chromosome morphology during this phase in other organisms (Zickler and Kleckner 1998). Nuclei then enter pachytene and chromosomes become more dispersed. Pachytene occupies a large area within the germline and is the phase in which recombination occurs and COs are formed. This arrangement of nuclei allows us to use cytological techniques to observe an approximate timecourse of meiotic prophase for many of our investigations.

Meiotic checkpoints

To safeguard against errors in meiotic events, checkpoints exist to monitor their completion (Chin and Villeneuve 2001; Gartner, MacQueen et al. 2004; Bhalla and Dernburg 2005). In the *C.elegans* germline, there are two known meiotic checkpoints. Failure to satisfy either or both of these checkpoints leads to elevated cell death in the hermaphrodite gonad, visible at the end of pachytene. The meiotic DNA damage checkpoint (sometimes termed the meiotic recombination checkpoint) responds to unresolved DSBs in late pachytene (Gartner, Milstein et al. 2000) and involves conserved components of the DNA damage response, such as the checkpoint components *hus*-1 (Hofmann, Milstein et al. 2002) and the worm ortholog of p53, *cep-1* (Schumacher, Hofmann et al. 2001, Derry, et. al. 2001). The synapsis checkpoint verifies that chromosomes have synapsed and is dependent on unsynapsed chromosomes having an active PC (Bhalla and Dernburg 2005).

Figure 1

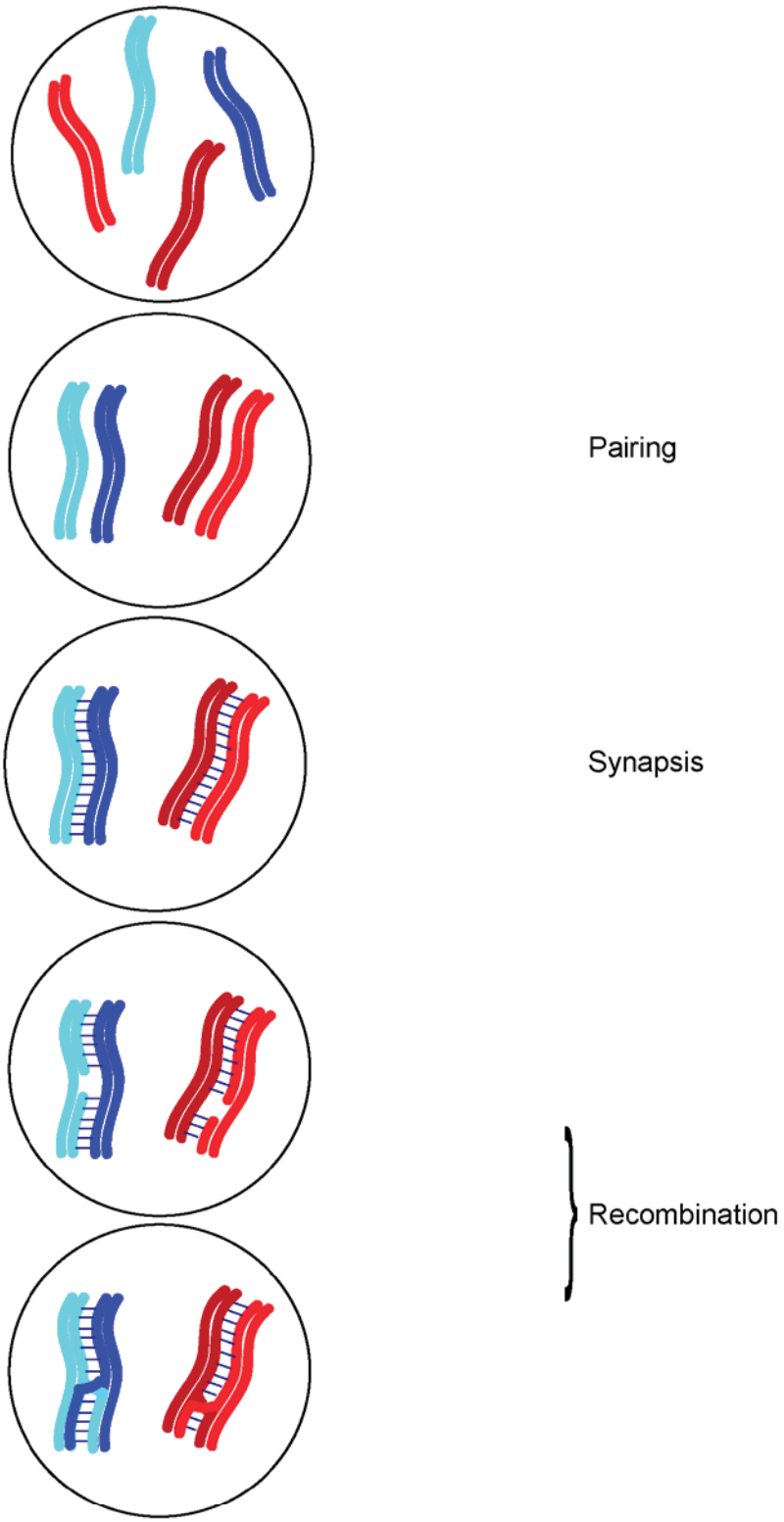

Figure 1: Events in meiotic prophase. Homologs under a series of steps to ensure genetic exchange and direct proper chromosome segregation.

Figure 2

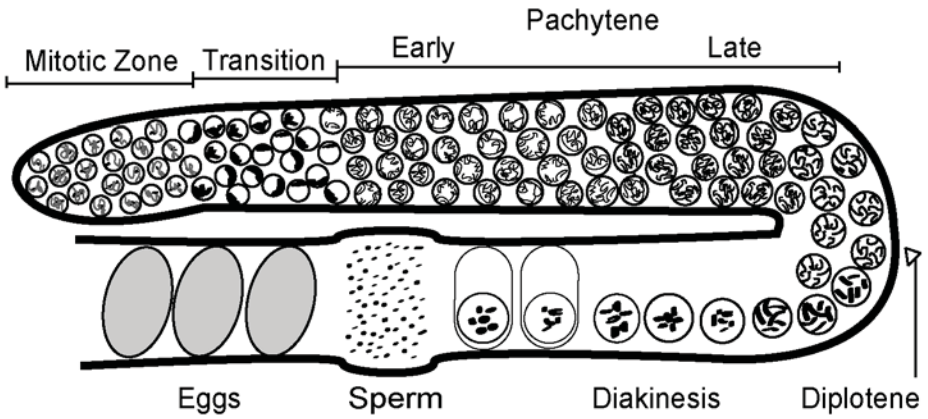

Figure 2. Diagram of *C. elegans* hermaphrodite gonad. Meiosis begins in the transition zone.

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SECTION I

Nuclear Architecture in Meiosis:

The LEM Proteins

Introduction: Section I

The Nuclear Envelope

In eukaryotic cells, the nucleus is demarcated from the cytoplasm by the nuclear envelope (NE). The nuclear envelope comprises a double membrane, nuclear pore complexes and integral membrane proteins (Gruenbaum, Margalit et al. 2005). The outer nuclear membrane connects to cellular cytoskeleton and endoplasmic reticulum, while the inner nuclear membrane is lined by a network of proteins collectively termed the lamina, which performs a wide variety of functions in the cell (Stuurman, Heins et al. 1998)

The lamina is composed of filament proteins called lamins and associated membrane-bound and nucleoplasmic proteins. Animal cells encode two classes of lamin proteins, A- and B-type lamins, both of which are intermediate filament proteins (Aebi, Cohn et al. 1986). Although most nuclear lamin filaments line the inner nuclear membrane, there are also lamin 'scaffolds' that stretch into the interior of the nucleus (Naetar and Foisner 2009). The lamins provide structural support and shape to the NE, and play an essential role in binding chromatin-binding factors to help organize chromatin within the nuclear space (Liu, Rolef Ben-Shahar et al. 2000). The lamina is also involved in bridging some of these chromatin complexes to partners on the outer

nuclear membrane, providing a connection to the cytoskeleton (Starr and Han 2002) (Gruenbaum, Margalit et al. 2005).

Other members of the nuclear lamina include the lamin-associated proteins, some of the most important of which are LBR (lamin B receptor) and the LEM (LAP2/emerin/MAN1) domain-containing proteins. LBR binds lamin and heterochromatin and is thought to contribute to chromatin silencing (Olins, Rhodes et al.). The LEM domain proteins share a common ~40-amino-acid LEM domain that mediates binding to a common, conserved chromatin-bridging factor BAF (Wagner and Krohne 2007). There are both integral membrane and nucleoplasmic LEM family proteins, all of which bind BAF (barrier to autointegration factor) and lamin *in vitro*. Additionally, other binding partners have been found for many LEM proteins (Wagner, Schmitt et al. 2004). LEM proteins LAP2β and emerin bind transcription factors and are thought to regulate gene expression, and emerin also binds actin (Gruenbaum, Margalit et al. 2005). MAN1, a LEM family member with two transmembrane domains and a C-terminal tail that is thought to bind DNA directly, has also been shown to bind Smad proteins that mediate signalling in the TGF-β family (Konde, Bourgeois et al. ; Lin, Morrison et al. 2005).

Lamina mutations lead to a number of diseases collectively known as laminopathies (Gruenbaum, Margalit et al. 2005). These include premature aging syndromes, other progressive diseases, and dystrophies of various tissues. Hutchinson–Gilford progeria syndrome (HGPS), a premature aging disease, is caused by a mutation in the gene encoding human A-type lamin, *LMNA* (Eriksson, Brown et al. 2003). Emery–Dreifuss muscular dystrophy is a progressive muscle-

wasting disease and can be caused by a mutation in either the LEM protein emerin or in *LMNA* (Morris 2001; Mounkes, Kozlov et al. 2003). Buschke–Ollendorf, a connective tissue disorder mainly affecting bone and skin, is caused by a mutation in the gene for MAN1 (Lee, Gruenbaum et al. 2000; Hellemans, Preobrazhenska et al. 2004; Pinto, Wilmington et al. 2008). The molecular interactions that give rise to these disorders are mostly unclear, although the somatic phenotypes have been well described (Mounkes, Kozlov et al. 2003).

The Nuclear Envelope in Meiosis

Although the somatic repercussions of lamina mutations have been better studied, mutations in lamina proteins have also been found to disrupt fertility. Some *LMNA* mutations are associated with impaired fertility and a higher frequency of pregnancy complications in humans, and can impair spermatogenesis in mice (Alsheimer, Liebe et al. 2004). However, many of the specific processes that lead to these conditions are not known and are difficult to study in humans and primate model organisms. Here, the invertebrate model organisms become especially useful (Lee, Gruenbaum et al. 2000).

The involvement of LEM proteins in chromatin organization and their effects on fertility suggest several potential scenarios for the role of the lamina, and LEM proteins specifically, in meiosis (see Figure 1). The localization of specific chromatin domains to the NE could maintain a requisite pattern of gene expression necessary for proper completion of meiotic events (Joffe, Leonhardt et al. ; Guelen, Pagie et al. 2008). Alternately, proper localization of certain genomic areas to the NE may be important to prevent errant recombination or crossover placement. For example,

studies in mitosis have revealed a role for LEM domain-containing proteins in repressing recombination in heterochromatin (Mekhail, Seebacher et al. 2008).

LEM proteins in *C. elegans*

There are three LEM proteins in the worm genome. EMR-1 (emerin), LEM-2 (MAN1), and LEM-3. LEM-3 is a small protein containing a LEM domain but which has little sequence similarity with LEM proteins in other systems and was largely uncharacterized (Gruenbaum, Lee et al. 2002). EMR-1 and LEM-2 are integral transmembrane proteins of the inner nuclear membrane in all cells and function to tether chromatin. All three are nonessential when deleted alone, but simultaneous depletion of EMR-1 and LEM-2 is lethal due to catastrophic mitosis (Lee, Gruenbaum et al. 2000), demonstrating that their functions are at least partially redundant.

The research described in the following two chapters was aimed at elucidating the importance of the *C. elegans* LEM proteins to meiosis. Specifically, I investigated their impact on meiotic recombination, checkpoint control, and progeny quality. I also asked whether defects in the lamina brought upon by loss of the LEM proteins echoed premature aging defects similar to those seen in other systems.

Figure 1

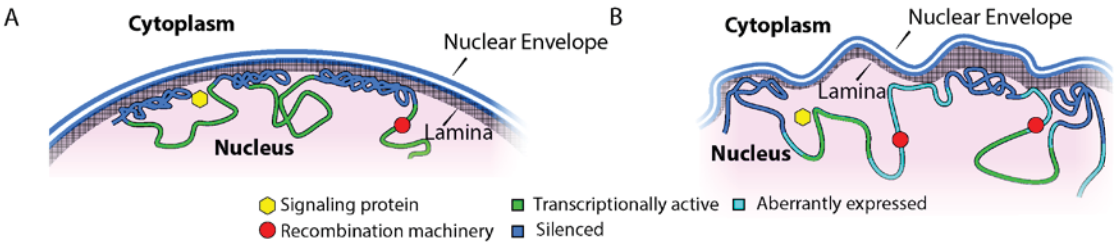


Figure 1: Role of nuclear architecture in regulation of events in meiotic nuclei: a speculative model. A) Integrity of the nuclear envelope and lamina enables maintenance of chromosomal domains in gene expression, proper signaling interactions, and access of recombination machinery to appropriate genomic regions. B) Defects in the lamina and nuclear envelope could result in loss of chromosomal domain organization, disrupted signaling, and/or inappropriate recombination events.

Chapter 1: The LEM domain proteins EMR-1 and LEM-2 in meiotic recombination

Previous work on LEM domain-containing proteins in other model systems suggested that they form a bridge between chromatin and the NE through their interactions with lamin (LMN) and barrier-to-autointegration factor (BAF1) (Gruenbaum, Margalit et al. 2005). LEM proteins have already been shown to regulate mitotic recombination, and preliminary data obtained by Matt Ragle in our lab suggested that they were also involved in regulation of meiotic recombination. Knockdown of *emr-1* and *lem-2* by RNA interference (RNAi) yielded increased apoptosis due to errors in meiotic recombination (data not shown).

We hypothesized that the loss of LEM protein function could interfere with proper recombination through altering DSB distribution or repair, or through disrupting proper crossover formation. I worked to characterize this role to reveal a mechanism of meiotic regulation at a level that is poorly understood. My experiments in this chapter demonstrate that a subset of the LEM proteins regulate meiotic recombination. Loss of LEM-2 inhibits the timely repair of double-strand breaks (DSBs) and causes errors in meiosis that elevate apoptosis. However, these defects are tolerated well enough by the worm germline that there are no gross defects in lifespan or viability of progeny.

Results

The number of apoptotic cells in meiotic prophase is used as a marker for *C. elegans* checkpoint activation in response to defects in meiosis (Gumienny, Lambie et al. 1999; Boulton, Martin et al. 2004). The level of apoptosis can be visualized using live-imaging of a transgenic worm strain that expresses a GFP-tagged CED-1, a cell death protein expressed in the gonadal sheath cells that will completely encircle apoptotic nuclei beginning in late pachytene (Figure1A). The level of apoptosis was analyzed in *emr-1* and *lem-2* null deletion mutant worms (*emr-1 [gk119]* and *lem-2 [ok1807]*, described in more detail in Materials and Methods*)*. In both mutants, apoptosis was elevated above wild-type levels (Figure 1B), suggesting that the germline was responding to errors in meiosis. In both cases, this elevation was dependent on *spo-11*, as loss of this enzyme rescued apoptotic numbers, indicating a role for the LEM proteins in meiotic recombination (Figure 1C and 1D).

We were also interested in whether or not disrupting chromatin tethering to the NE would hinder the checkpoints that monitor meiosis. I specifically asked if LEM-2 and EMR-1 were involved in the response to meiotic errors by introducing the mutants into the *meDf2* background. *meDf2* is a deletion of the X-chromosome Pairing Center, without which X chromosomes are unable to synapse (MacQueen, Phillips et al. 2005). Heterozygotes activate the synapsis checkpoint, and homozygotes activate the DNA damage checkpoint (Bhalla and Dernburg 2005). Loss of either EMR-1 or LEM-2 function do not inactivate the checkpoints, suggesting that perinuclear tethering through these two proteins is not required for checkpoint function in this background (Figure 2). LEM function was also not found to be required in the *syp-1* mutant (data not shown), which does not load the central

element of the synaptonemal complex and also triggers checkpoints (Bhalla and Dernburg 2005).

We then wondered if the loss of LEM tethering led to meiotic errors due to defects in the ability to make or repair double-strand breaks (DSBs). I tested for aberrations in DSB induction or repair by examining the localization of the protein RAD-51 by secondary immunofluorescence. RAD-51 binds to the ssDNA ends made at the sites of DSBs, and is visible by staining in fixed worms (Alpi, Pasierbek et al. 2003). I looked specifically for RAD-51 foci that localized to meiotic chromosomes using an antibody against HTP-3, which is an axial element component of the synaptonemal complex (Figure 3A). Typically, RAD-51 foci appear in the transition zone and disappear by late pachytene. If this standard distribution is disturbed in LEM mutants - in number, localization, or persistence through the gonad – it would argue that the LEMs influence meiotic recombination through DSBs. The initial onset of RAD-51 foci formation in LEM mutants coincided with wild-type N2 worms (Figure 3B). The LEM deletion mutants also showed peak number of RAD-51 foci similar to that in wild-type worms, but exhibited defects in the repair of DSBs (Figure 3). Levels of RAD-51 foci peaked later in the germline. This effect was more pronounced in the *lem-2* mutant than the *emr-1* mutant. In contrast to wild-type worms, the *lem-2* mutant germline nuclei still retain some unresolved DSBs at the end of pachytene, often in the form of a few individual nuclei with a large number of RAD-51 foci.

The defect in DSB repair suggested that chromosome tethering by LEM proteins was important to regulate some aspects of break processing. This could be due to the altered availability of chromatin to recombination proteins. Thus, we

thought that this might also effect the placement of recombination events, or the rate at which DSBs are repaired into crossovers.

To determine the effect on CO placement, I used genetic analysis to monitor the presence of CO formation. Single-nucleotide polymorphisms that disrupt a restriction enzyme site (snip-SNPs) have been extensively characterized in *C. elegans* (Davis, Hammarlund et al. 2005), and I chose five sites along chromosome III (Figure 4A). These sites divided the chromosome into roughly equal quarters by physical length. I generated hermaphrodites heterozygous for these snip-SNPs along the length of a chromosome and genotyped their progeny to determine the pattern of inheritance of the snip-SNPs. Typically, *C. elegans* chromosomes undergo recombination more frequently on each arm of the chromosomes and less in the center. This reflects the pattern of perinuclear tethering of the worm genome, although it is unclear whether the two phenomena are related. The *lem-2* mutant, despite having the stronger defect in DSB processing, did not disrupt this pattern, showing recombination rates similar to wild-type in all four quadrants (Figure 4B).

To test for disruption of CO formation and rate for all chromosomes, I looked cytologically at CO markers. ZHP-3 is a meiotic protein that loads onto chromosomes in meiosis and is eventually restricted to the sites of COs (Jantsch, Pasierbek et al. 2004; Bhalla, Wynne et al. 2008). COSA-1 is another protein that marks the sites of COs in late pachytene, typically 6 per nucleus (Libuda, Uzawa et al. ; Yokoo, Zawadzki et al.). Both of these proteins showed normal patterns of staining in *lem-2* and *emr-1* mutant worms (Figure 5A and B), indicating that these LEM proteins do not influence the recombination machinery that make crossovers.

We reasoned that loss of tethering alone may not be enough to increase the rate of CO formation in an organism with such strong crossover interference. I thus attempted to add a stressor to the CO regulation mechanism by introducing mutations of *lem-2* or *emr-1* into the *meDf2* background and monitoring rate of CO formation. meDf2 is a deficiency that removes the PC of the X chromosome, preventing synapsis and the formation of crossovers on the X chromosome. Loss of synapsis and recombination on one chromosome has been shown to affect the rate and location of recombination events on other chromosomes (Carlton et. al., 2006). However, asynapsis of the X chromosome in *lem-2* and *emr-1* mutants did not exacerbate the loss of crossover control in *meDf2* mutants, demonstrating that these LEM proteins are not required to for crossover control even when it is slightly relaxed. It is possible that the retention of robust CO control in the single mutants is the result of redundancy of function between EMR-1 and LEM-2. However, we were unable to test this with double depletion because of the requirement for these proteins in mitosis.

There has also recently been a class of noncanonical crossovers described in the literature that are not marked by ZHP-3 or COSA-1 (Youds, Mets et al.). It is also unlikely that these noncanonical COs are regulated by *lem-2*, as the occurrence of double-crossovers was not greater in *lem-2* mutants when assayed by snip-SNP recombination (Figure 4C).

The occurrence of unresolved DSBs at the end of pachytene made us ask if these nuclei possessed defects that would lower the fitness of the resulting progeny. Further, due to the role that the nuclear envelope as been shown to play in premature aging disorders, I tested both the lifespan of mutant worms and the

persistence or decline of reproductive fitness over time (Figure 6A). *lem-2* mutant worms have reduced progeny viability overall (Figure 6B). However, while initial experiments showed a possible increase in the severity of the viability defect with age, subsequent strain reconstruction and an additional allele of *lem-2 (tm1582*) show a steady defect (Figure 6). This defect is demonstrably due to loss of LEM-2 function, since it is rescued by introducing a *lem-2* transgenic construct tagged with GFP in the deletion mutant background. This construct also rescued a mild defect in the lifespan of the worm (Supplemental Figure 1).

Conclusion

This work demonstrates that LEM proteins regulate meiotic recombination at the level of DSB processing. The defects in DSB repair in these mutants leads to an elevation in germline apoptosis, but not to gross defects in the placement or rate of COs. Additionally, the loss of LEM-2 function leads to a decrease in viability of progeny. However, it is unclear if the reduced viability is due to the meiotic defects uncovered, to subsequent errors in mitotic divisions in the resulting embryos, or another defect as yet unknown. Further, while my initial experiments showed a worsening viability defect with parental age, further follow-ups in reconstructed (data shown in figures) mutants did not. Because of this incongruity and because there was no clear subsequent path to determine the cause of the viability defect that was within the scope of our lab, this line of inquiry was not pursued further.

Materials and Methods

Statistical Analysis

A Student's paired t-test was used for all significance analysis unless otherwise noted in the text or figures.

Strains Used

The wild-type strains used were N2 Bristol, with the Hawaiian wild-type (*CB4856)* used for snip-SNP analysis only.

emr-1 (gk119) was used for the emerin mutant and is a complex insertion/deletion on chromosome 1 removing almost the entirety of the gene.

lem-2 (ok1807) was used as the primary *lem-2* deletion; it removes the entirety of the gene on chromosome 2, as well as several hundred base pairs surrounding it.

Two additional *lem-2* mutant alleles were used. *lem-2 (tm1582)* is a smaller deletion within the *lem-2* coding region, and *lem-2 (ok1807); lem-2::gfp*, contains a GFP-tagged LEM-2 in a stably inherited extrachromosomal array.

For scoring apoptosis, the *ced-1::gfp (bcIs39)* mutant was crossed into the emerin and *lem-2* mutants, yielding *emr-1 (gk119);ced-1::gfp (bcIs39)* and *lem-2 (ok1807);ced-1::gfp (bcIs39)* and later *lem-2 (tm5862); ced-1::gfp (bcIs39).*

Those strains were further put into checkpoint activated backgrounds for analysis of apoptosis. For meiotic mutants, I used previously characterized mutants, listed below.

mnDp66; meDf2

spo-11 (ok79);ced-1::gfp (bcIs39)/ nT1[unc-?(n754) let-?]

spo-11 (ok79);syp-1(me17)ced-1::gfp (bcIs39)/ nT1[unc-?(n754) let-?]

syp-1(me17) ;ced-1::gfp (bcIs39)/ nT1[unc-?(n754) let-?]

pch-2 (tm1458); ced-1::gfp (bcIs39)

pch-2 (tm1458); syp-1 (bcIs39)/ nT1[unc-?(n754) let-?]

Worm culture and crossing

Worms were grown according to standard protocols listed in Wormbook, and cultured at 20°C unless otherwise noted. For strain crossing, either strains with a high incidence of males phenotype were used, or males were generated by heat shock at 30°C for 4-7 hours.

Live Imaging of apoptosis

Apoptosis was scored according to previous studies (Gartner, MacQueen et al. 2004). Adults or L4s were seeded to agar plates with OP50 and left to grow at 20°. If using strains requiring a balancer, balanced worms were picked. 3 or 4 days later, L4 hermaphrodites homozygous for required deletions were picked to OP50 plates in the evening and placed at 20°C. 18-20 hours later, imaging pads were made from 1.5% agarose with Levamisole. Adult worms were picked to agar pads for imaging on the DeltaVision Personal DV microscope from Applied Precision, using the 100X oil objective. The number of apoptotic nuclei in the bend of the gonad (late pachytene to diplotene) were counted and averaged, with a minimum of 20 animals scored.

Immunofluorescence of extruded gonads

Worms were fixed and stained according to standard Bhalla Lab protocol. L4 hermaphrodites were picked to OP50 plates roughly 24 hours prior to dissection. On the day of dissection, worms were dissected in Egg Buffer with .1% Tween and 3% of .5M Sodium Azide, then fixed with Formaldehyde in Egg Buffer at a concentration of 1% for 5 minutes. Some later experiments were performed dissecting with the lower concentration of 1% of the .5M Sodium Azide to get better extrusion of gonads. Samples were freeze/cracked, fixed in ice-cold Methanol (1 minute), and blocked with BSA before staining. Secondary antibodies were Cy3 (Jackson Immunochemicals) and Alexa-Fluor 488 (Invitrogen).

For RAD-51 counts, primary antibodies used were: Rb αRAD-51 @1:800; Ck αHTP-3 @1:2000; and DAPI. Slides were stored at 4°C and imaged on the DeltaVision at 100X. Image stacks through the entire gonad were taken for 3D analysis at a density of .2uM per slice. After iterative deconvolution using SoftWoRx, RAD-51 was scored by counting positive foci that colocalized with stretches of HTP-3 staining.

Generation of Hawaiian strains across selected regions

For snip-SNP analysis, *lem-2* and *emr-1* worms were crossed multiple times into the Hawaiian wild-type background (CB4856) to generate a strain with Hawaiian SNP alleles for the desired sites along chromosome III, and the desired LEM mutation. SNP alleles genotyped along ChIII and enzymes are listed in the Tables below.

Table 1. snip-SNP alleles and primers, Chromosome 3

Allele	Primer		Primer	
pkP3081	1 for_III	agcaagaatgagccgattg	1 rev_III	gtcggccgttttcaaataactg
pkP3095	22 for_III	tctcgtcaattgtcgcctg	22 rev_III	ttatttgcaatccaacggc
pkP3101	30 for_III	ccaagtgcaaactatggtgc	30 rev_III	ataaacaatttcagtgccgc
pkP3035	45 for_III	cgtaaactaccaaactcggtg	45 rev_III	ggtctactacaactatacaggc
pkP3080	75 for_III	cggtggtggtaaaagtgtaac	75 rev_III	caacattcaggctgtgctttcc

Table 2. Restriction enzymes for snip-SNP alleles, Chromosome 3

Allele	Restriction Enzyme	N2 fragment sizes	CB4856 fragment sizes
pkP3081	TaqI	[222, 149]	[195, 145, 27]
pkP3095	ApoI	[308]	[168, 140]
pkP3101	HinfI	[495]	[282, 213]
pkP3035	Eco0109I	[732]	[419, 313]
pkP3080	Hpy188III	[365, 76, 68, 35]	[241, 124, 76, 68, 35]

SNP genotyping

For the wild-type snip-SNP recombination analysis the crosses were as follows. *ced-1::gfp(bcIs39)* worms were heat shocked to generate males and then crossed to *CB4856* worms. The cross-progeny F1 hermaphrodites were verified to be GFP positive in the germline. To eliminate confusion due to self-progeny of these heterozygotes that might contain more than one recombinant chromosome, they were crossed to *mIs11* males with entirely N2 SNPs. These mutants express GFP in the pharynx, so heterozygotes are clearly marked. Thus, resulting F2 cross progeny hermaphrodites from this second cross that were used for SNP genotyping all contained one entirely N2 chromosome from the fathers and either a parental or

recombinant chromosome from the F1 mothers. The F2 worms were first allowed to produce self-progeny in liquid culture to generate more genetic material, using the method listed in this section.

For recombination analysis in the *lem-2* mutant, the mating scheme was the same. The *lem-2 (ok1807);ced-1::gfp (bcIs39)* strain was crossed into the *lem-2 (ok1807);CB4856(LGIII)* worms and the resulting cross-progeny were crossed with *mIs11* males as previous.

PCR genotyping was done using a touchdown PCR protocol. Master mixes were prepared for each 96 well plate for each individual SNP. For 100 reactions of 15uL each: 1uL each of both primers from 20uM stock, 1uL of 25mM dNTPs, .5uL of Taq polymerase, and PCR buffer. The PCR thermoprofile: 30s@95°C, followed by [15s@95°C, 30s@60°C, 45s@72°C]x10 with a decrease of 0.5°C in the annealing temperature per cycle, then by [15s@95°C, 30s@55.5°C, 45s@72°C]x35, and finally with 5min@72°C.

Reactions were run on a 2% agarose 100-well gel. Due to the close proximity in size of many of the bands, runoff of ethidium bromide during a long run was a concern. Thus, gels were run without EtBr directly in the agarose. EtBr was added to the running buffer during the run.

Microtiter worm culture for clonal expansion

Growth medium was S-complete medium (as per Wormbook Strain Maintenance Protocol 2), to which was added Nystatin [100U/mL] and Streptomycin [50ug/mL]. Between 1 and 4 days before use, the growth media was supplemented with freshly grown HB101, spun down and resuspended to a density of OD550=2.0.

Individual worms were picked to wells of a 96-well plate (Corning costar 3598) with 40uL supplemented growth medium and plates were placed in humid chamber at 20°C until resulting clonal colonies were starved (3-4 days). Healthy worms produced 50-100 progeny during this time, in various stages of larval arrest. To make lysates for PCR, 20uL of the starved liquid culture was added to 2x PCR buffer and Proteinase K was for a final [0.2mg/mL]. Lysate mixes were incubated at 65°C for 90 minutes and then inactivated at 95°C for 20 minutes. 1 uL of the resulting dirty lysate was used for PCR analysis per SNP allele.

Progeny viability

To assess reproductive viability, L4s of wild-type, *emr-1 (gk119),* and *lem-2 (ok1807)* genotypes were plated individually to thinly spread OP50 plates. Later experiments also included additional alleles of *lem-2* with the same protocol. Plates were grown at 20°C, and timepoints were marked post L4. At each timepoint, they were moved to new plates and the eggs and larvae were counted. Timepoints were at 24 hours, 36 hours, 48 hours, 60 hours, 84 hours, and 108 hours. 36 was considered Day 1, since worms were picked at approximately mid-L4 stage and would require approximately 12 hours post-L4 to progress to gravid adults. After three days, each set of plates was counted again for adults and L4s to assess survival of progeny. Viability was scored as the number of grown worms over the larvae/eggs hatched, expressed as a percentage. Averages of 12 animals per genotype are shown. Viability is frequently above 100% for the first day because some eggs/L1 larvae are not visible during initial scoring. Progeny number is the total number of surviving adult/L4 progeny from each worm.

Lifespan assay

A dozen gravid hermaphrodites of each genotype were bleached in equal parts 60% bleach and 1M sodium hydroxide on OP50 plates. After growing 3 days at 20°C, the resulting synchronized L4 progeny were used for the lifespan assay. 40 worms of each genotype were plated at 10 per plate to 4 plates and grown at 20°C. Live worms were moved and counted each day for the first 6 days. After Day 6, worms were counted every day and moved every three days. Worms that were moving or that moved in response to touch were scored as alive. Worms that died from matricide by bagged progeny were removed from final analysis.

Figure 1

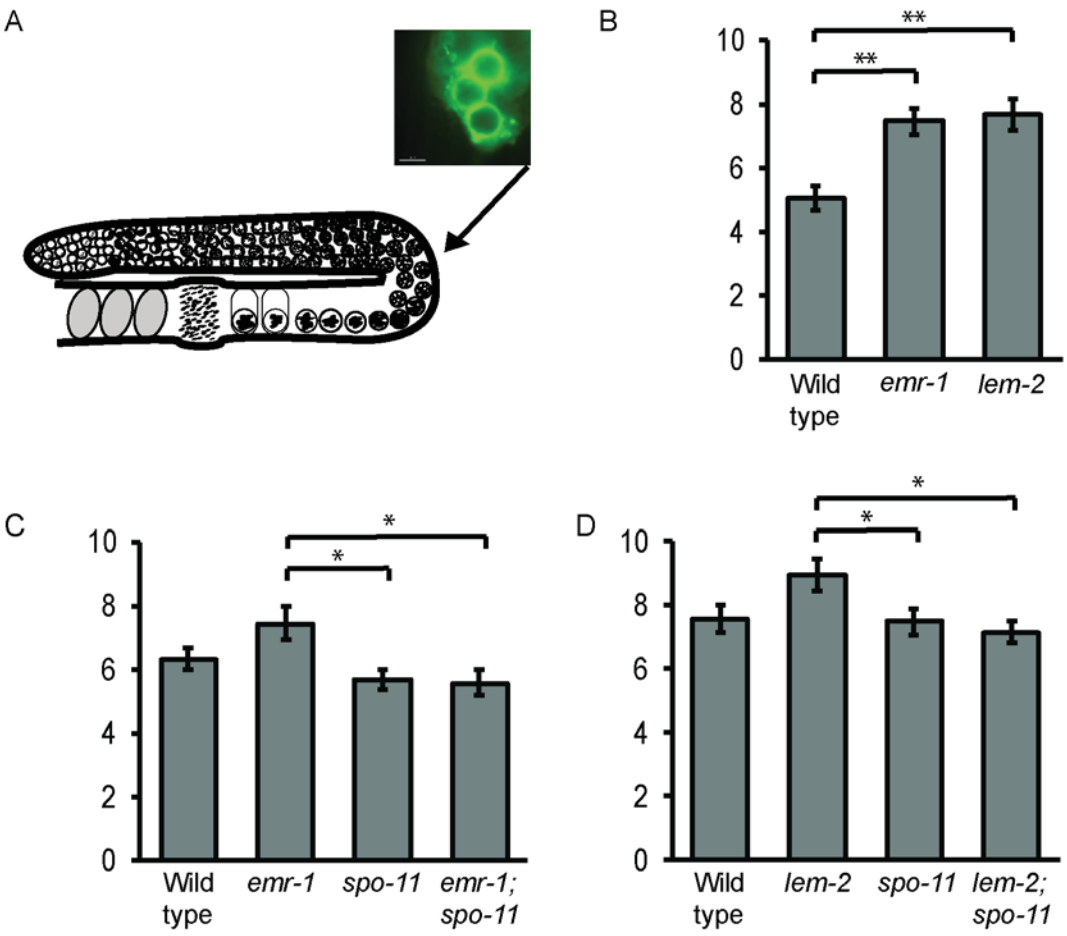


Figure 1. LEM proteins regulate meiotic recombination. A) Apoptotic nuclei are visible starting in late pachytene near the "bend" of the worm germline. B) Deletion of the LEM proteins EMR-1 and LEM-2 causes increased apoptosis, suggesting checkpoint activation in response to errors in the germline. C) This increase is lost in a *spo-11* mutant.

Figure 2

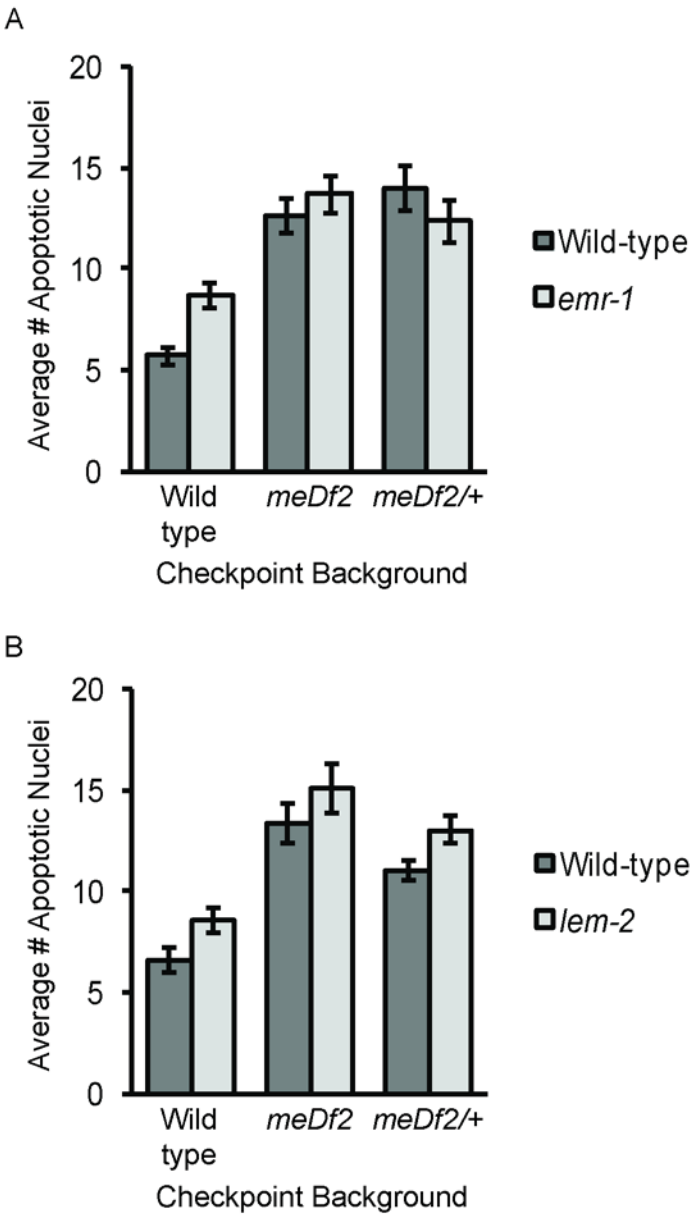


Figure 2. LEM mutants do not exhibit checkpoint defects. A) Apoptosis upon loss of EMR-1 function when either the DNA damage checkpoint (*meDf2*) or the synapsis checkpoint (*meDf2/+*) is activated. B) Effect of loss of LEM-2 on checkpoints.

Figure 3

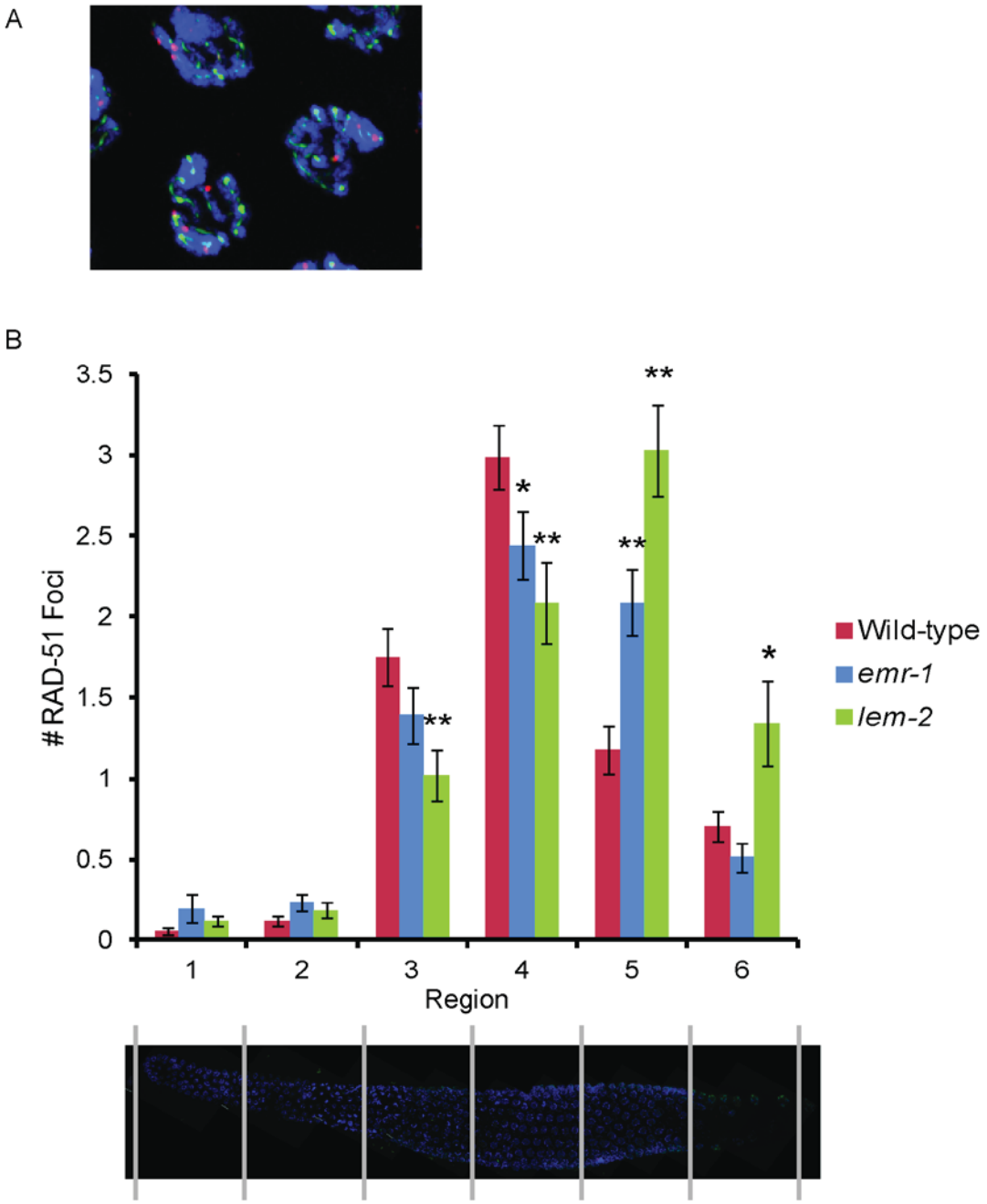
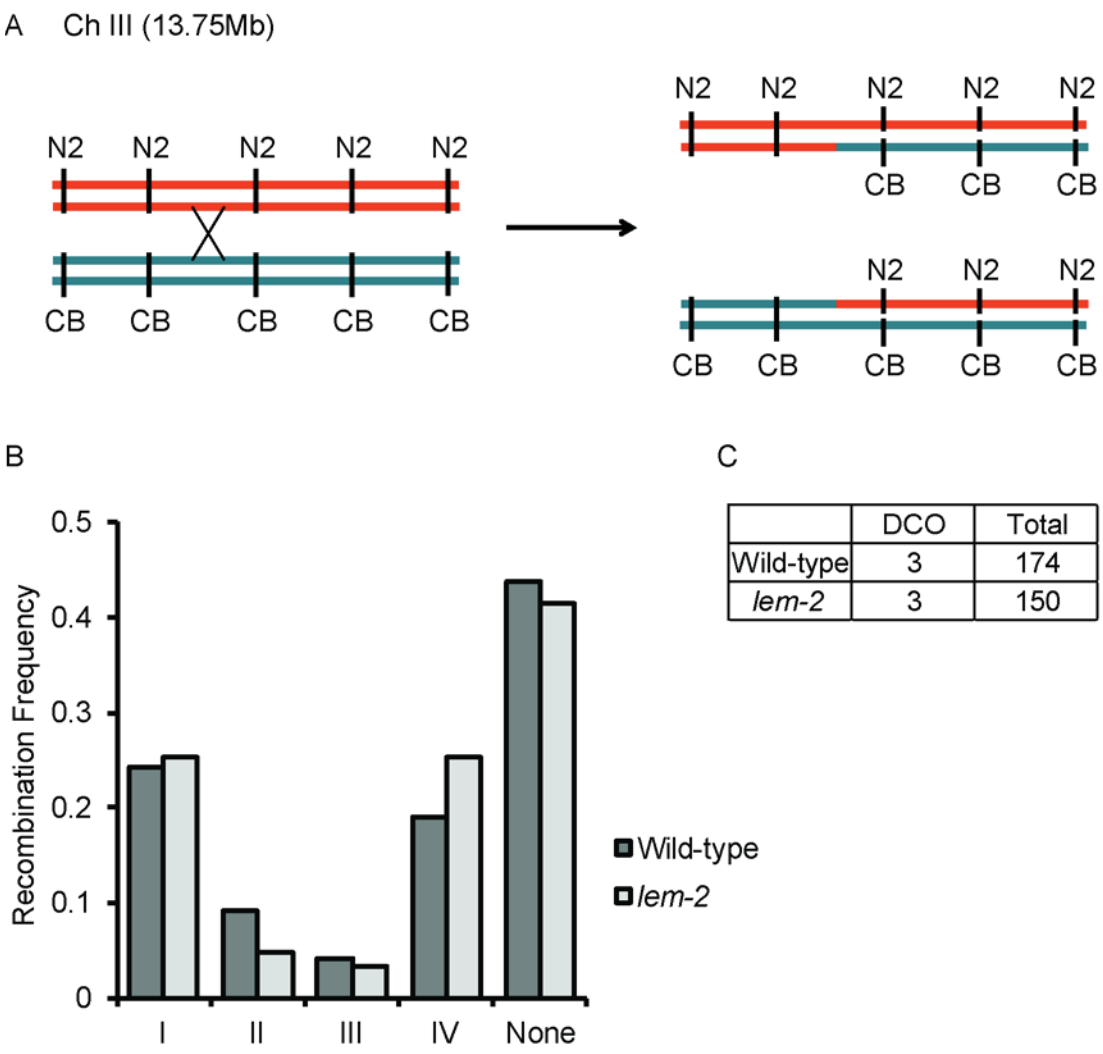


Figure 3. LEM mutants exhibit a delay in repair of meiotic DSBs. A) Staining of fixed hermaphrodite nuclei for SC component HTP-3 (green), DSB repair protein RAD-51 (red), and DAPI (blue). Wild-type mid-pachytene shown. B) Division of germline from the distal tip to the end of pachytene into six regions and quantification of average foci number per nucleus in each given region. * = p<.05 and ** = p<.01

Figure 4



	DCO	Total
Wild-type	3	174
lem-2	3	150

Figure 4. Recombination landscape in LEM mutant animals. A) Five snip-SNPs that differ between the Bristol (N2) and Hawaiian (CB) strain were chosen from previously described sites, dividing LGIII into four roughly equal regions by physical map. Homolog pair shown in the heterozygous animal above incur a CO in interval II. B) Frequency of COs in chosen intervals in the wild-type and the *lem-2* mutant. C) The number of double crossover chromosomes genotyped compared to the total number of chromosomes typed.

Figure 5

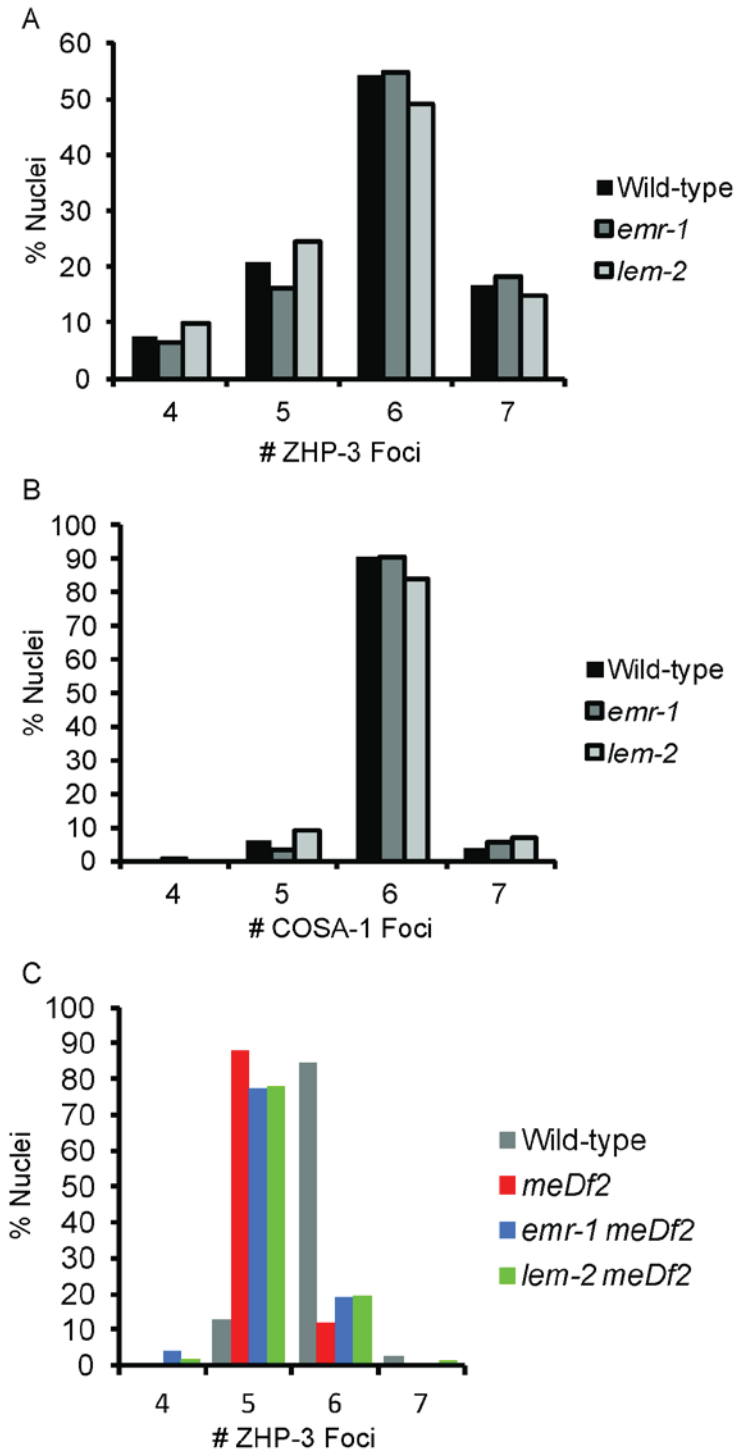


Figure 5. LEM mutants have a normal level of crossovers. A) The percentage of nuclei in late pachytene that have 4, 5, 6, or 7 COs marked with ZHP-3. B) The percentage of nuclei with 4, 5, 6, or 7 COs COSA-1 foci. C) ZHP-3 foci in the *meDf2* background, where X chromosomes are unsynapsed.

Figure 6

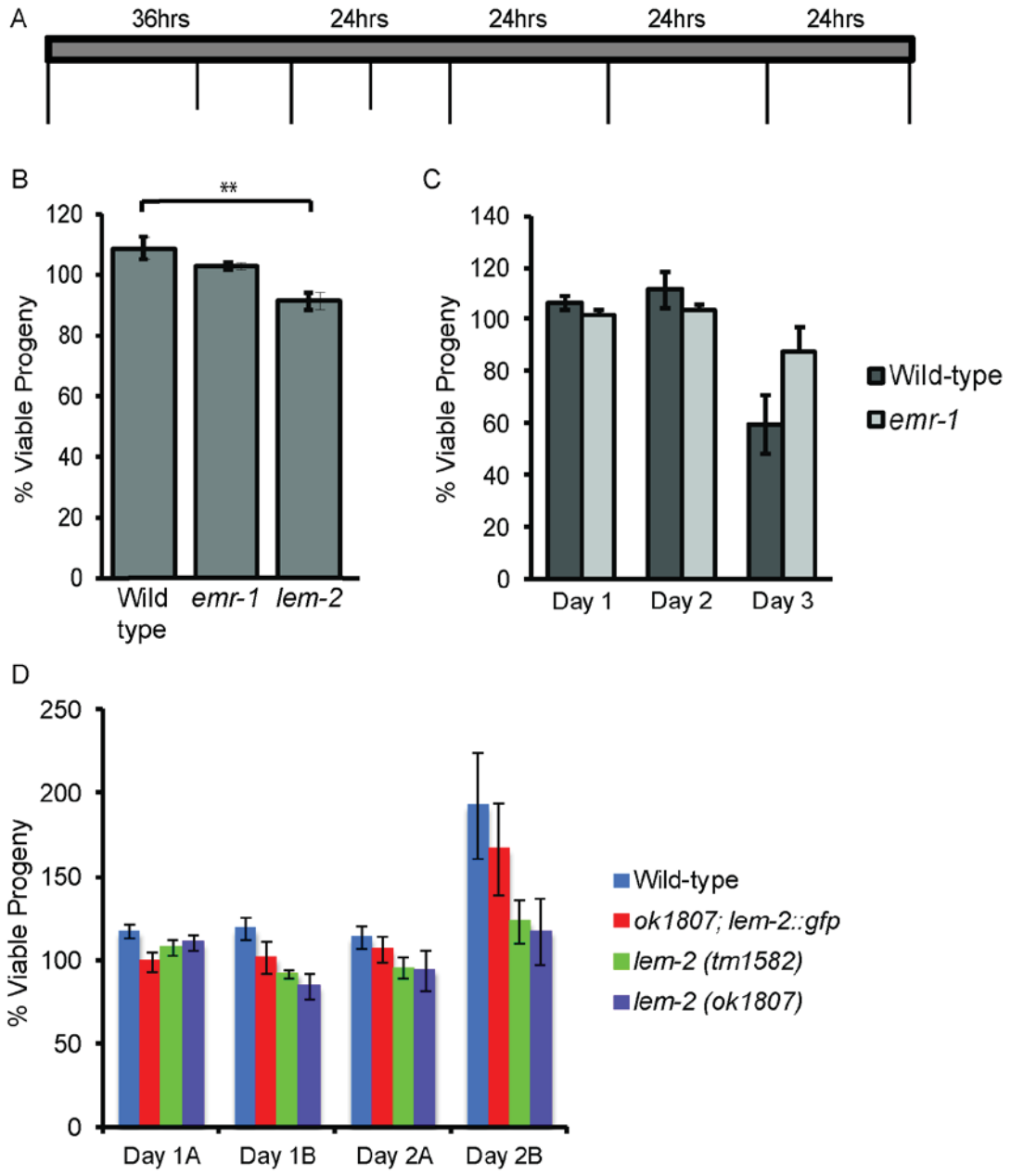
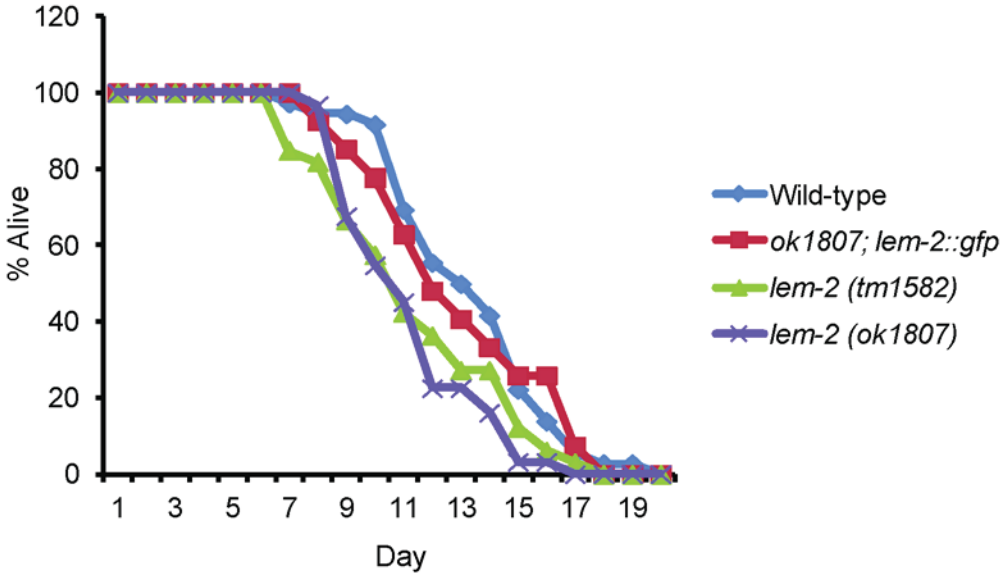


Figure 6. LEM mutants have differential effects on progeny viability. A) L4 hermaphrodites were plated individually and allowed to progress into gravid adults (12hrs), then scored either every 24 hours, or every 12 hours for the first two days, followed by every 24 hours. B) Lifetime % progeny survival. C) Progeny viability by day of adulthood in *emr-1* worms. D) Progeny viability over time in *lem-2* mutant worms.

Supplemental Figure 1



Supplementa Figure 1. Lifespan of *lem-2* mutants. Percent of worms alive on days post-hatch.

Chapter 2: Characterization of LEM-3 in meiosis

In order to monitor proper completion of meiotic events, checkpoints exist to allow the germline to assess progress and repair or delete defective cells. There are two checkpoints in *C. elegans*: the synapsis checkpoint and the DNA damage checkpoint (Meier and Gartner 2006). Active pairing centers, which transiently associate with the nuclear envelope during synapsis, are required for signaling to the synapsis checkpoint (Bhalla and Dernburg 2005). However, how the checkpoint is activated is unclear. Moreover, whether factors at the nuclear envelope are required for checkpoint activation is unknown.

The third LEM protein in C*. elegans* is the relatively uncharacterized LEM-3. LEM-3 is a smaller protein than either EMR or LEM-2 and does not contain a transmembrane domain. It was speculated to be part of a scaffold stretching into the interior of the nucleus in addition to serving a function at the inner nuclear membrane (Lee, Gruenbaum et al. 2000). Preliminary RNAi experiments done in the lab had suggested that *lem-3* had a role in meiotic checkpoint function. In mutants that activate the checkpoints, knockdown of *lem-3* reduced the levels of apoptosis. I sought to confirm this in a deletion mutant I uncovered and determine *lem-3's* precise role in checkpoint function. The results pointed toward a possible checkpoint role.

Results

I initially looked at *lem-3* by RNAi knockdown in the *syp-1 (me17)* mutant worm. *syp-1* mutants do not load the central component of the synaptonemal complex and therefore all homologs do not synapse. Since as a result DSBs also cannot be repaired off the homolog and processed into COs, *syp-1* worms experience greatly elevated levels of apoptosis due to activation of both the DNA damage and synapsis checkpoints. Loss of checkpoint function partially or fully restores apoptotic levels to wild-type. Knockdown of *lem-3* by RNAi reduced apoptosis significantly in *syp-1* worms (Figure 1A).

Initial experiments with a deletion mutant of *lem-3 (tm3468)* did not recapitulate the RNAi results (data not shown). However, as the deletion produced a truncated protein, the mutant may have been partially functional. Thus, I performed a deletion library screen and uncovered a deletion mutant that removed the start codon of *lem-3 (blt1)*. The null mutant recovered showed a similar but less pronounced reduction in apoptosis to the RNAi results (Figure 1B). The reduction also seemed to be specific to the synapsis checkpoint. In the *spo-11; syp-1* background, the DNA damage checkpoint is inactivated, and remaining elevated apoptosis is due to signaling through the synapsis checkpoint. Deletion of *lem-3* further rescues apoptosis in this background, suggesting that *lem-3* is important for signaling through the synapsis checkpoint (Figure 1B). However, loss of lem-3 did not reduce apoptosis to wildtype levels, complicating our ability to interpret this experiment.

Further, the loss of *lem-3* causes a slight defect in embryonic viability over the lifetime of the worm without decreasing the total number of progeny produced (Figure 2). This could be the result of defective nuclei inappropriately progressing through the meiotic checkpoints in the absence of *lem-3.* However, given the margin of error for viability and the range of fecundity seen in progeny number counts, the viability defect may not be severe enough to significantly alter the total progeny counts in hermaphrodite mothers. This leaves open the possibility that the embryonic viability defect is due to mitotic errors during development.

Conclusions

The experiments described in this chapter suggest that *lem-3* plays a role in the synapsis checkpoint. However, the defect in apoptotic signaling seemed mild – the checkpoint is not fully abolished when gene function is lost. Further, a study was published in late 2011, using another deletion mutant of *lem-3 (op444)* that described the function of *lem-3* in DNA Damage response as a putative endonuclease (Dittrich, Kratz et al.). It showed that *lem-3* is important for response to errors in mitosis, which would support the idea that the defect I saw in embryonic viability could be the consequence of loss of fidelity during embryogenesis. Due to this we were unable to reconcile the different lines of inquiry and find a strategy to pursue the function of *lem-3* in meiosis, independently of mitosis, in an impactful way, and so we did not pursue this project further.

Materials and Methods

Statistical Analysis

A Student's paired t-test was used for all significance analysis unless otherwise noted in the text or figures.

Strains Used

The same wild-type strains were used as in Chapter 1. The *lem-3* mutation initially used was *tm3468*. A deletion library screen generated another allele (*blt1*).

Apoptosis experiments were performed as in Chapter 1.

Deletion Screen

Access to the deletion library was generously provided by the Strome Lab. The screening protocol was adapted from the Koelle Lab, using the poison primer method and two rounds of amplification using nested primers (Edgley, D'Souza et al. 2002). The deletion library contained pooled lysates from worms subjected to random mutagenesis. The first round of amplification of each pooled lysate served to amplify small quantities of DNA from each worm genotype represented in the population of each well. The outward-facing poison primers for a given target gene helped to dilute the dominating proportion of wild-type amplicons for the gene, enriching the presence of amplicons from any deletion mutants lacking the poison primer complement. Secondary amplification with a primer pair inside the outside primers produced amplicons to the abundance that could visualized on a gel.

First round of amplification used outside primers and poison primers. Outside primers: 5'-acctcagtcgtgttacaaacg-3' (F), 5'-cgattgaacgagattgacgc-3' (R). Poison primers: 5'-cgtggcgctaatgttaatgc-3' (F), 5'-aagtttcactgcggagatgc-3' (R).

Second round of amplification used inside primers and was analyzed by gel electrophoresis. For template, trace amounts of the PCR product from the first round was added to the premix for the second round by hedgehog. Inside primers: 5'-aaacccaaccttgtgttcaag-3' (F), 5'-aaaaaatccgagacgctgcc-3' (R).

Any positives were confirmed by reamplification, and then the pooled DNA template was tracked to the appropriate 96-well microtiter plate. The two round PCR was repeated on the microtiter plate to identify the well with the desired mutant, which was then cut out of the frozen culture plate, thawed, and grown under standard conditions. A subset of the progeny were then individually cultured in wells and allowed to lay eggs before being genotyped for the correct deletion. The deletion mutant for *lem-3* discovered through this method was established as the line *blt1*.

Figure 1

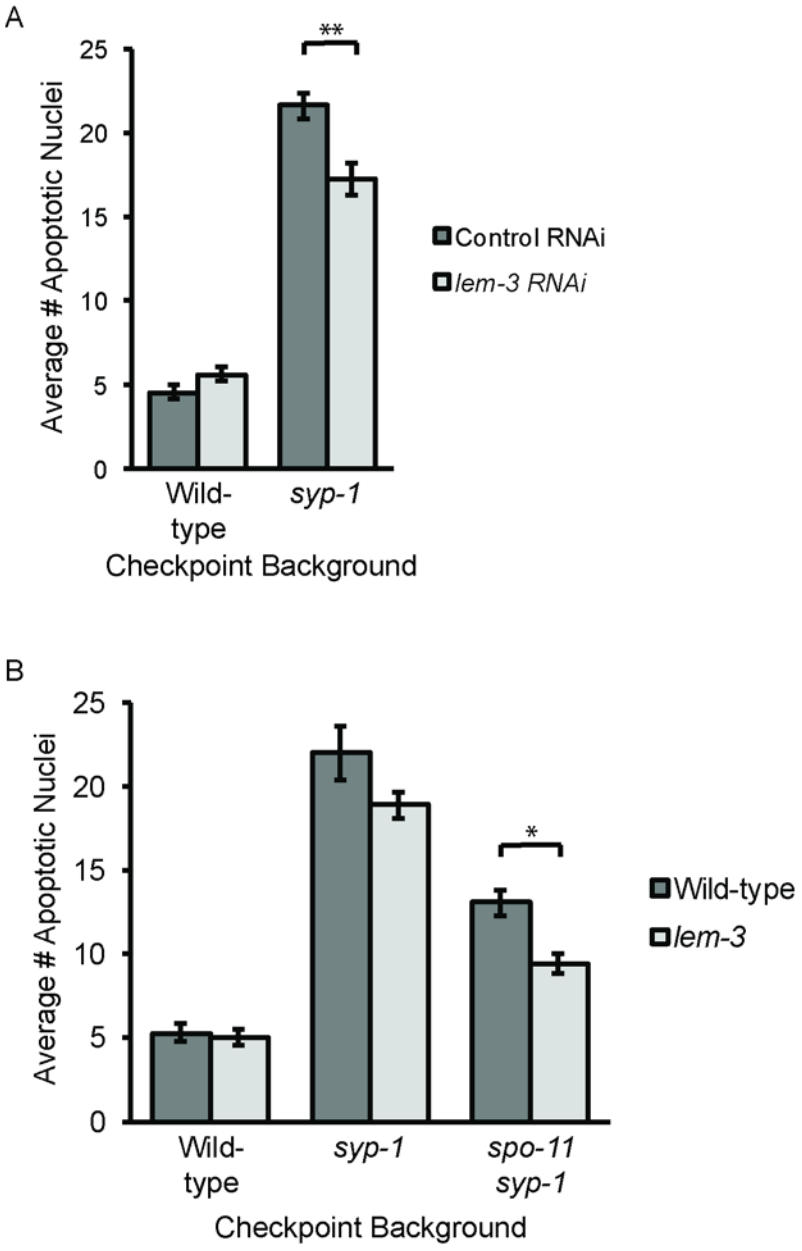


Figure 1. *lem-3* plays a role in the synapsis checkpoint. A) Knockdown of *lem-3* by RNAi results in a reduction of apoptosis in the *syp-1* mutant. B) Deletion mutant of *lem-3* has a milder reduction of synapsis checkpoint-induced apoptosis.

Figure 2

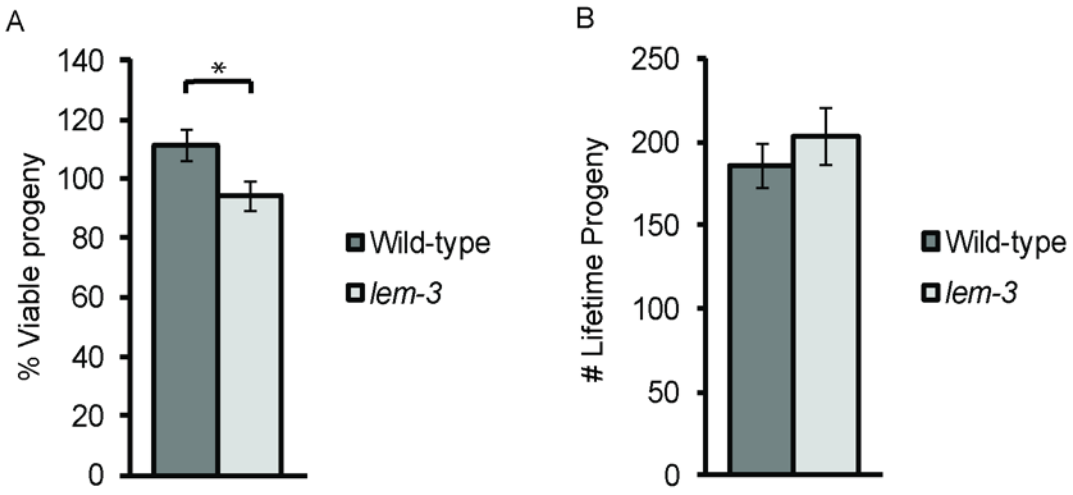


Figure 2. *lem-3* deletion causes a reduction in viability of offspring without lowering the lifetime number of progeny. A) Reproductive viability is lower in lem-3 mutants as compared to N2 Bristol. B) Living progeny produced across the reproductive lifespan.

SECTION II

Control of Germline Apoptosis:
Proapoptotic Factors EGL-1 and CED-13
and Their Regulation

Introduction

Meiotic checkpoint activation

In order for chromosomes to properly segregate during meiosis, homologous chromosomes must pair, synapse, and recombine. Since defects in these processes result in birth defects and infertility, checkpoints monitor meiotic events to ensure that they occur properly. In the *C. elegans* germline, two distinct checkpoints exist. The DNA damage checkpoint monitors the proper repair of double-strand breaks (DSBs) during meiotic recombination, and the synapsis checkpoint ensures chromosomes are synapsed. Activation of either of these checkpoints will induce apoptosis to remove defective meiotic nuclei, preventing aneuploidy and defective gametes (Bhalla and Dernburg 2008).The *C. elegans* model system allows us to use a number of strategies to dissect checkpoint requirements.

At the beginning of meiosis in worms, homologs typically pair and synapse with the aid of pairing centers (PCs) on chromosome ends. When synapsis is blocked in the presence of PCs, the synapsis checkpoint is activated (Bhalla and Dernburg 2005). For instance, when PC sequences are deleted from a single chromosome, such as in *meDf2* heterozygote mutants lacking the *X* chromosome PC from only one of the homologs, asynapsis results and the synapsis checkpoint is activated. When PCs are absent or nonfunctioning, however, the synapsis

checkpoint is silent (Bhalla and Dernburg 2005) Nonetheless, lack of synapsis of a pair of chromosomes still results in unresolved DSBs late in pachytene, which will activate the DNA damage checkpoint. *meDf2* homozygotes only activate the DNA damage checkpoint . Loss of PC associated proteins, such as HIM-8, hinders synapsis of their respective homologs and leads to unresolved DSBs (Phillips, et. al 2005).

Checkpoints can also be activated through global lack of synapsis in mutants that fail to load the SC. SYP-1 is a central component of the SC that loads between homologs; mutants lacking this protein have asynapsis of all homolog pairs in meiosis (MacQueen et al. 2002). These mutants are unable to repair meiotic DSBs in a timely manner and thus activate the meiotic DNA damage checkpoint. However, since these mutants possess functional PCs that are not synapsed, they will also activate the synapsis checkpoint (Bhalla and Dernburg 2005). Utilization of this variety of modes for checkpoint induction allows us to investigate the requirements for putative checkpoint components.

Apoptosis in the worm germline

In *C. elegans*, apoptosis occurs in two phases of the lifespan. In the first, an invariant population of somatic cells in developing eggs and larvae will undergo apoptosis before adult hermaphrodites reach their largely fixed complement of ~960 cells (Sulston and Horvitz 1977; Sulston, Schierenberg et al. 1983). The majority of the cells that die during development are intended for a neuronal fate (Nehme and Conradt 2008). These include the hermaphrodite specific neurons (HSNs), which die during embryogenesis in male embryos, and the male specific cephalic (CEM)

neurons, which die during hermaphrodite embryogenesis (Sulston 1983). Additionally, the neurosecretory motoneuron (NSM) sister cells die in both sexes (Sulston, Schierenberg et al. 1983).

In the second phase, a proportion of the nuclei in meiosis in the adult germline will undergo apoptosis (Gumienny, Lambie et al. 1999). It is estimated that roughly half of the nuclei that enter meiosis in the hermaphrodite germline are culled by apoptosis in healthy, wild-type worms (Gumienny, Lambie et al. 1999). This baseline level of “physiological apoptosis” increases drastically when the germline is subjected to genotoxic stressors such as irradiation, and when errors in meiosis activate checkpoints (Gartner, Milstein et al. 2000, Bhalla and Dernburg 2005).

The core mechanisms of programmed cell death have been well described in *C. elegans* literature. Cell death inhibitory protein CED-9, which is similar to a human proto-oncogene named bcl-2 (Hengartner and Horvitz 1994), work upstream of other cell death proteins CED-4 and CED-3, which activate the cell death cascade of corpse engulfment and DNA degradation (Hengartner, Ellis et al. 1992; Chinnaiyan, O'Rourke et al. 1997; Conradt and Horvitz 1998; Horvitz 1999). Most of the studies describing this machinery have focused on somatic apoptosis, given the highly restricted and predictable course of cell death. However, the core apoptotic machinery is conserved between germline and somatic apoptosis upon activation of CED-3 and CED-4 (Lettre and Hengartner, 2006). The upstream regulators of checkpoint activation are less clear, although EGL-1 is known to be important.

EGL-1 is a small proapoptotic protein with a conserved bcl-2 binding domain that is required for the highly regulated apoptotic program in the developing worm and in the adult germline in response to genotoxic stress. EGL-1 was initially

described through mutations that killed HSNs in hermaphrodites, leading to egg-laying defects (Ellis and Horvitz 1986). These mutations turned out to be gain-of-function mutations in a pro-apoptotic protein (Ellis and Horvitz 1986). *egl-1* is expressed in a sex-specific manner in both HSNs and CEM neurons, and is required to activate apoptosis appropriately in those cells (Nehme and Conradt 2008). It is also required for apoptosis in NSM sister cells (Thellmann, Hatzold et al. 2003). Further, while *egl-1* is not required for physiological apoptosis (Gumienny, Lambie et al. 1999), it has been shown to be required for apoptosis in response to genotoxic insults in the germline (Schumacher, Schertel et al. 2005).

CED-13 is a structurally similar protein to EGL-1 in *C. elegans*, and overexpression of this protein promotes apoptosis in the soma (Schumacher, Schertel et al. 2005). However, the impact of CED-13 on cell death is not as pronounced as that of EGL-1. While overexpression of *ced-13* induced inappropriate cell death through an egl-1 independent mechanism, loss of *ced-13* does not inhibit programmed cell death in development (Schumacher, Schertel et al. 2005).

The studies outlined above show that these proteins promote checkpoint-induced apoptosis in the germline, but their relative contributions are unclear. The following two chapters aim to clarify the downstream activators of the cell death response in response to meiotic errors. I discovered that both EGL-1 and CED-13 contribute to checkpoint activation. Each protein was responsible for varying checkpoints depending on the manner in which errors induced their activation: EGL-1 is required for both checkpoints when they are activated by lack of pairing centers on one pair of homologs, but only for the synapsis checkpoint when all homologs are asynapsed in the *syp-1* mutant. I also conducted experiments to find the genetic

mechanism of *egl-1* activation or repression by DNA-binding factors in germline nuclei and found regulatory elements that inhibit apoptosis in a *spo-11* dependent manner. These results, coupled with the apparent lack of DNA damage checkpoint role for *egl-1* in the *syp-1* mutant, suggest that there are mechanisms in the germline that work to suppress DNA damage checkpoint induced apoptosis in the presence of unresolved recombination intermediates.

Chapter 3: The Role of Meiotic Recombination in the synapsis checkpoint

The two distinct meiotic checkpoints in *C. elegans* have separable modes of regulation. Many factors in the regulatory pathway of the meiotic DNA damage checkpoint are known, such as the apoptotic regulator *cep-1* and the endonuclease *spo-11*, without which meiotic DSBs are not made (Dernburg, McDonald et al. 1998; Schumacher, Hanazawa et al. 2005). The synapsis checkpoint identified in 2005 required the worm ortholog of the yeast *PCH2*, which is necessary for the yeast pachytene checkpoint. However, many of the additional factors that are essential for apoptosis in response to these checkpoints are unclear.

Given the central importance of *egl-1* as a master regulator of cell death required for apoptosis in other contexts, we hypothesized that the gene may also be necessary for activation of the meiotic checkpoint responses. We also wished to clarify the potential germline function(s) of *ced-13*, which has previously been difficult to pinpoint. I found that *egl-1* is required for checkpoint induction in both checkpoints, but under specific conditions. When all chromosomes are unsynapsed and pairing centers are functional, *ced-13* appears to assume apoptotic function for the DNA damage checkpoint and *egl-1* is no longer required. I also uncovered a chromatin region that serves as a binding site for a transcriptional regulator of *egl-1*.

Results

Our readout for checkpoint activation was the same as in Chapter 1. By introducing the *ced-1::GFP(bcIs39)* transgene into my desired mutant backgrounds, I was able to score the resulting number of apoptotic nuclei. To answer whether either EGL-1 or CED-13 were necessary for meiotic checkpoints, I used the *meDf2* mutants to activate one checkpoint at a time (Figure 1A). Loss of *egl-1* in both the *meDf2* homozygote and heterozygote mutant backgrounds reduced apoptosis to wild-type levels (Figure 1B). This wild-type level of background physiological apoptosis is independent of *egl-1* and *ced-13* and is consistent with what has previously been reported. Mutation of *ced-13* in both checkpoint backgrounds did not significantly affect germline apoptosis (Figure 1B). We concluded that *egl-1*, but not *ced-13*, is required for both checkpoints when each is activated individually through loss of synapsis on a single homolog pair.

Next, I determined the role of *egl-1* in promoting germline apoptosis in a mutant that activates both checkpoints: the deletion mutant of *syp-1* (see Figure 2A), a mutant that does not load the SC and displays asynapsis on all homolog pairs. Additionally, because some of the components specific to a particular checkpoint are known, we were able to selectively inhibit one at a time. In contrast to our studies with *meDf2*, loss of *egl-1* in *syp-1* mutants revealed a role specific to the synapsis checkpoint (Figure 2B). Deletion of *egl-1* in the *syp-1* background reduced apoptosis to intermediate levels compared to *syp-1* alone, corresponding to loss of one checkpoint but not both. I prevented activation of the DNA damage checkpoint by mutating *spo-11* in the *egl-1 syp-1* mutant background. Loss of *egl-1* in the resulting

triple mutants (*spo-11;egl-1 syp-1*) further reduced levels of apoptosis to be comparable with wild-type, demonstrating that *egl-1* is required for the synapsis checkpoint. To verify this, I also inactivated the synapsis checkpoint by mutating *egl-1* in *pch-2;syp-1* mutants. There was no further reduction of apoptosis; persistent intermediate levels of apoptosis in these triple mutants (*pch-2;egl-1 syp-1*) established that *egl-1* is not required for the DNA damage checkpoint even when the synapsis checkpoint is abrogated. These data show that when both the synapsis checkpoint and the DNA damage checkpoint are activated, *egl-1* promotes germline apoptosis specifically in response to the synapsis checkpoint.

Because of the specification for *egl-1* in the *syp-1* background and the unknown functions of the similar *ced-13*, I next investigated the role of *ced-13* in promoting checkpoint-induced apoptosis in *syp-1* mutants. Loss of *ced-13* reduced the average number of apoptotic nuclei in *syp-1* mutants, indicating its requirement for checkpoint function (Figure 2C). In *spo-11;syp-1* mutants, loss of *ced-13* did not further reduce apoptosis. However, *pch-2;syp-1;ced-13* triple mutants had fewer average apoptotic nuclei than both *pch-2;syp-1* and *spo-11;syp-1;ced-13* mutants (Figure 2C). Thus, *ced-13* activates apoptosis in response to DNA damage in *syp-1* mutants, consistent with previous data illustrating a mild pro-apoptotic role for *ced-13* in response to genotoxic stress.

The reduction in germline apoptosis in *syp-1;ced-13* double and *pch-2;syp-1;ced-13* triple mutants was less severe than when *egl-1* was inactivated in the same mutant backgrounds (Figures 2B and C), leading us to wonder if *egl-1* might be contributing to germline apoptosis in the DNA damage response when *ced-13*

function is compromised. To test this possibility, I assayed germline apoptosis in *egl-1 syp-1;ced-13* triple mutants. Deletion of both *egl-1* and *ced-13* in the *syp-1* mutant further reduced apoptosis below the levels observed in *egl-1 syp-1* double mutants but did not rescue apoptosis to wild-type levels (Figure 2D). Therefore, germline apoptosis can be elevated even in the absence of two characterized pro-apoptotic factors, suggesting that either another pro-apoptotic factor promotes checkpoint-induced apoptosis or that physiological apoptosis can be upregulated in response to meiotic checkpoint activation. Indeed, scoring of apoptosis in the ced-13 mutant alone, in the absence of any checkpoint-inducing errors, sometimes yielded a level slightly elevated above physiological background levels (Figure 2D). This elevation hovered alternately on either side of a statistical significance cutoff of 95% confidence, and may have indicated a subtle defect in the *ced-13* mutant strain.

I also monitored germline apoptosis in *pch-2;egl-1 syp-1;ced-13* mutants (Figure 2D) and did not observe any further reduction in apoptosis from the levels observed in *pch-2;syp-1;ced-13* triple mutants (Figure 2C), indicating that there was no residual signaling through the synapsis checkpoint. These data allow us to conclude that the increase in germline apoptosis in *pch-2;syp-1;ced-13* triple mutants is not due to *egl-1* function compensating for the absence of *ced-13* during DNA damage checkpoint activation.

As regulation of *egl-1* is thought to be mostly at the level of transcription, I investigated how checkpoint activation affected transcription of *egl-1* by Fluorescent in situ hybridization (FISH) against *egl-1* mRNA. We obtained labeled FISH probes aginst the *egl-1* mRNA and observed elevated *egl-1* transcript levels in *syp-1*

mutants (Supplemental Figure 1). The elevation was observed in late pachytene (top right in figure). To obtain quantitative measurements, I used real-time reverse transcription PCR (RT-qPCR) with primers against both *egl-1* and *ced-13* in RNA isolated from whole worms. In both *meDf2* and *meDf2*/+ strains, *egl-1* mRNA was present at higher relative levels when compared to wild-type worms (Figure 3A), consistent with *egl-1*'s requirement for checkpoint-induced apoptosis in both of these mutant backgrounds (Figure 1B). In *syp-1* and *spo-11; syp-1* mutant worms, *egl-1* was also transcriptionally induced (Figure 3B). However, *egl-1* was not transcriptionally upregulated in *pch-2;syp-1* double mutants (Figure 3B), validating the genetic data placing *egl-1* in the synapsis checkpoint pathway in *syp-1* mutants (Figure 2B) downstream of the CO promoting PCH-2 protein. By contrast, *ced-13* mRNA was present at higher relative levels in *syp-1* mutants and *pch-2;syp-1* double mutants when compared to wildtype and *spo-11;syp-1* double mutants (Figure 3C), lending support to the finding that *ced-13* is required for the DNA damage checkpoint in *syp-1* mutants (Figure 2C). *ced-13* transcript is upregulated to a much higher extent than *egl-1* transcript in this study. Given that whole worms were lysed to obtain cDNA, it is likely that the greater involvement of *egl-1* in germline apoptosis and its importance in somatic apoptosis during embryogenesis results in a higher level of *egl-1* expression in wild-type worms as compared to *ced-13.*

During somatic apoptosis, regulatory elements at the *egl-1* locus contribute to its transcriptional regulation. We wondered if *egl-1* transcription was similarly regulated during germline apoptosis. In particular, we wondered what might be responsible for the restriction of *egl-1* to the synapsis checkpoint in *syp-1* mutants, since the DNA damage checkpoint is capable of *egl-1* activation in pairing center

mutants. I identified a sequence downstream of *egl-1* that is required to limit *egl-1's* contribution to germline apoptosis during checkpoint activation. The *egl-1(bc274)* allele removes a section of DNA ~1.6-3kb downstream of the *egl-1* stop codon (see Figure 4A). Deletion of this region elevated apoptosis in wild-type and *meDf2/+* mutant worms in a *spo-11*-dependent manner (Figures 4B and C), indicating that this region is specifically required to inhibit germline apoptosis in response to DNA damage incurred during meiotic recombination. In support of this interpretation, apoptosis was also enhanced in *syp-1* and *pch-2*;*syp-1* mutants but not in *spo-11;syp-1* mutants (Figure 4D), further suggesting that the increased apoptosis is activated through the DNA damage checkpoint and not the synapsis checkpoint. Quantitative RT-PCR in *egl-1(bc274)* mutants indicated that this regulatory region was required to inhibit *egl-1* transcription (Figure 4E). Loss of this region was also associated with a decrease in embryonic viability for the progeny of these worms (Supplemental Figure 2). The factors that bind to this region (discussed below) are required for *egl-1* induced somatic apoptosis in the worm, which may account for this drop.

The region deleted in *egl-1(bc274)* removes the first exon of the gene *F23B12.1* (Figure 4A). To rule out the possibility that loss of this unknown gene caused defects in meiosis leading to the elevation of apoptosis and mild viability defect seen in *egl-1(bc274)*, I knocked down *F23B12.1* by feeding RNA interference (RNAi) in wild-type worms and did not observe any increase in germline apoptosis (Figure 5A) reminiscent of *egl-1(bc274)*.

I then used a candidate approach to test which transcription factors might be regulating *egl-1* through binding at this site. *egl-1(bc274)* includes a binding site for transcription factors that regulate *egl-1* in the soma, namely *ces-1*, *hlh-2 and hlh-3*. The HLH-2/HLH-3 heterodimer is required for the transcription of *egl-1* in the NSM sister cells during embryogenesis, leading to their death (Krause, Park et al. 1997). The cell death specification protein CES-1 antagonizes HLH-2 function to repress *egl-1* by binding at the same site (Thellmann, Hatzold et al. 2003). I inactivated both *ces-1* and *hlh-2* by feeding RNAi post-embryonically in wild-type worms and did not observe elevation of germline apoptosis in either case (Figure 5B). Efficacy of RNAi was confirmed by a strong incidence of larval arrest and abnormal progeny in the worms hatched and grown on the *hlh-2* RNAi lawn. A search for potential or predicted binding sites for other transcription factors in this region did not yield additional sites. From this we concluded that the regulatory function of *egl-1 (bc274)* in meiotic checkpoints does not proceed through factors previously characterized in relation to *egl-1*.

Conclusions

The experiments in this chapter establish that *egl-1* is required for meiotic checkpoint apoptosis, and that *ced-13* participates in one of these checkpoints in a proapoptotic role. They further uncover a *cis*-acting regulatory element for *egl-1* downstream of the gene. The phenotype resulting from deletion of this regulatory element may shed light on a puzzling question that has been unresolved in the field. In germlines of worms that are heterozygous for a PC deletion, such as the *meDf2/+*, only the synapsis checkpoint is activated, despite the persistence of DSBs that

cannot be repaired off the homolog due to lack of synapsis. How or why the meiotic DNA damage checkpoint is silenced in this background is unclear. The lack of *egl-1* activation in response to persistent DNA damage in the *syp-1* mutant could indicate a similar silencing, suggesting that there are mechanisms in the germline to suppress the DNA damage checkpoint response in response to unresolved double-strand breaks or recombination intermediates. Given that the *egl-1(bc274)* deletion elevates apoptosis in a DNA damage checkpoint dependent manner, in both the *meDf2/+* background and the *syp-1* background, it is possible that the checkpoint factor that represses DNA damage apoptosis binds within the region.

Unfortunately, we have not yet discovered the factor responsible for this regulation. The candidate genes that effect *egl-1* regulation in neuronal cells do not participate in this checkpoint, and a search for other predicted binding sites within the region did not lead us to other transcription factors as possibilities. Additional experiments will be required to generate other candidate genes. However, the studies as described in this chapter were submitted for publication in spring 2014, and experiments are currently underway to answer additional questions posed by reviewers, in preparation for resubmission in summer 2014.

Materials and Methods

Statistical Analysis

A Student's paired t-test was used for all significance analysis unless otherwise noted in the text or figures.

Strains Used

Apoptosis: as *egl-1* is on the same linkage group (V) as *syp-1* and the integrated transgene *bcIs39*, and very close to the morphology marker *dpy-11* initially used to help identify homozygote worms, the *dpy-11* mutation was included in mutant strains used for apoptosis. The gene has no known effect on apoptosis. The wild-type strains were *dpy-11(e224)bcIs39* and *mnDp66;bcIs39;meDf2*

egl-1(n1084n3082) is a compound mutation: a point mutation that disrupts regulation of egl-1 and a deletion.

bcIs39;ced-13 (*tm536)* is a null deletion of the gene on the X chromosome.

These strains were put into the strain backgrounds below to assay checkpoint involvement.

spo-11 (ok79);ced-1::gfp (bcIs39)/nt1(GFP)

spo-11 (ok79);dpy-11(e224)syp-1(me17)ced-1::gfp (bcIs39)/ nt1[qIs51]

dpy-11(e224)syp-1(me17)ced-1::gfp (bcIs39)/ nt1[qIs51]

pch-2 (tm1458); dpy-11(e224)syp-1(me17)ced-1::gfp (bcIs39)/ nt1[qIs51]

mnDp66; meDf2

RT-qPCR: wild-type was Bristol N2, and checkpoint strains were as below.

mnDp66; meDf2

spo-11 (ok79);syp-1(me17)/ nt1[unc-?(n754) let-?]

syp-1(me17)/ nt1[unc-?(n754) let-?]

pch-2 (tm1458); syp-1(me17)/ nt1[unc-?(n754) let-?]

To obtain heterozygotes for the *meDf2* deletion, *meDf2* homozygote males were crossed to non-*meDf2* hermaphrodites as before.

RT-qPCR

RNA was extracted from 100 worms of each genotype measured using TRIzol (Life Technologies) according to the manufacturer's directions. Homozygous progeny of heterozygote hermaphrodites were picked for balanced strains. RNA was converted to cDNA using the Superscript III First Strand Synthesis System (Life Technologies) according to instructions, with the provided HeLa RNA as a control. Quantitative RT-PCR was performed to determine relative mRNA levels using the following thermoprofile: 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 45 seconds for 45 cycles. Reactions were run using *Power* SYBR Green PCR Master Mix (Life Technologies) with a 15uL reaction volume, and fold enrichment was calculated using the ddCt method. The reference mRNA used to normalize the quantitave results was gamma-tubulin *tbg-1*.

Primers for qPCR were as follows.

egl-1: 5'-tactcctcgtctcaggactt-3' (F) and 5'-catcgaagtcatcgcacat-3 (R)';

ced-13: 5'-acggtgtttgagttgcaagc-3' (F) and 5'-gtcgtacaagcgtgatggat-3' (R);

tbg-1: 5'-cgtcatcagcctggtagaaca-3' (F) and 5'-tgatgactgtccacgttgga-3' (R).

Single-molecule FIuorescent In-Situ Hybridization

For smFISH, hermaphrodites were dissected ~24hours post-L4 into Egg Buffer with Tween and Formaldehyde as described in Chapter 1. Slides were

blocked with Hybridization buffer according to the Biosearch Stellaris probe protocol (1 g dextran sulfate, 1 mL 20X saline-sodium citrate (SSC), and 1 mL deionized formamide in 10mL nuclease-free water). Washes were performed with Wash Buffer (1 mL 20X saline-sodium citrate (SSC), and 1 mL deionized formamide in 10mL nuclease-free water), and probes were added in Hybridization Buffer to 1:100 dilution.

Stellaris FISH probes were designed against the mature *egl-1* mRNA, using the Biosearch Technologies online tool and ordered with a fluorescein tag. Two sets were ordered; the longer probe set (below) obtained the better signal.

Table 1. FISH Probes	attgctgctagcttggag
tcaactgaattgaaaaga	gagcatcgaagtcatcgc
caaaggtgagcatcagca	ggccgagtaggacatcat
aagatccgaagaggttga	gaggcttctgtcggaagc
aaaacgttggacattggt	aagtccagaagacgatgg
aaacggaagattgaacgt	tgatcacttaaaaagcga
acatgttcttttcgttgt	catggtacaaattggaga
gtcctgagacgaggagta	caccgggtattatgagaa
agaatcttcacacgagga	aatcacaaatgaagaaaa
gagtcgtcggcaaattga	gagacggagagatcgaaa
tgatctcagagtcatcaa	gcaataaaggactatggt
gatctcgtagccgatgct	acgactaatcggtgtgaa

RNAi feeding of worms for putative checkpoint components

RNAi feeding of *F23B12.1* was done using protocols previously described (Lamelza and Bhalla) on HT115 bacterial lawns with the appropriate vector.

Since knockdown of *hlh-2* is embryonic lethal, RNAi was performed post-embryonically for this study. Approximately ten gravid adult worms were picked to a blank agar plate and allowed to lay eggs. The eggs hatched and went into L1 larval arrest. Mothers were removed from the plate and the synchronized larvae were washed off with M9 and onto a plate spread with bacteria expressing the appropriate RNAi clone.

RNAi clones for knockdown of *ces-1, hlh-2,* and *F23B12.1* were isolated from the Ahringer RNAi library (Kamath and Ahringer 2003).

F23B12.1 (V-9B01)

ces-1: F43G9.11 (I-4C10)

hlh-2: M05B5.5 (I-9G10)

Figure 1

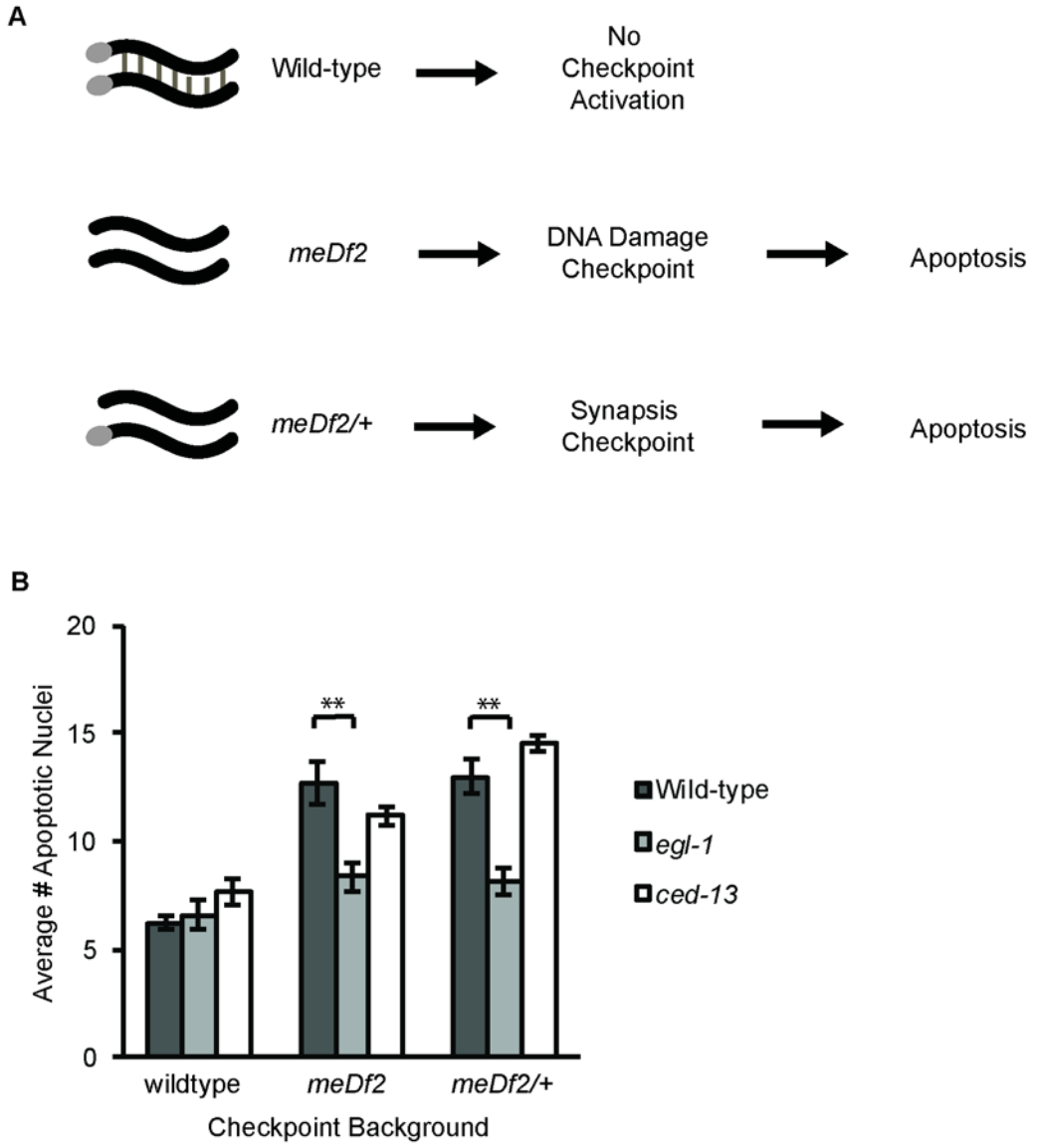


Figure 1. *egl-1* is required for the DNA damage and the synapsis checkpoint when each is activated individually. A) Checkpoint activation during meiotic prophase in strains homozygous or heterozygous for *meDf2*. B) Mutation of *egl-1* but not *ced-13* reduces germline apoptosis in both *meDf2* homozygous and heterozygous mutants. Except where indicated, *egl-1(n1084n3082)*and *ced-13(tm536)* were used. Error bars in all graphs represent 2X SEM. A ** indicates a p value of < 0.01.

Figure 2

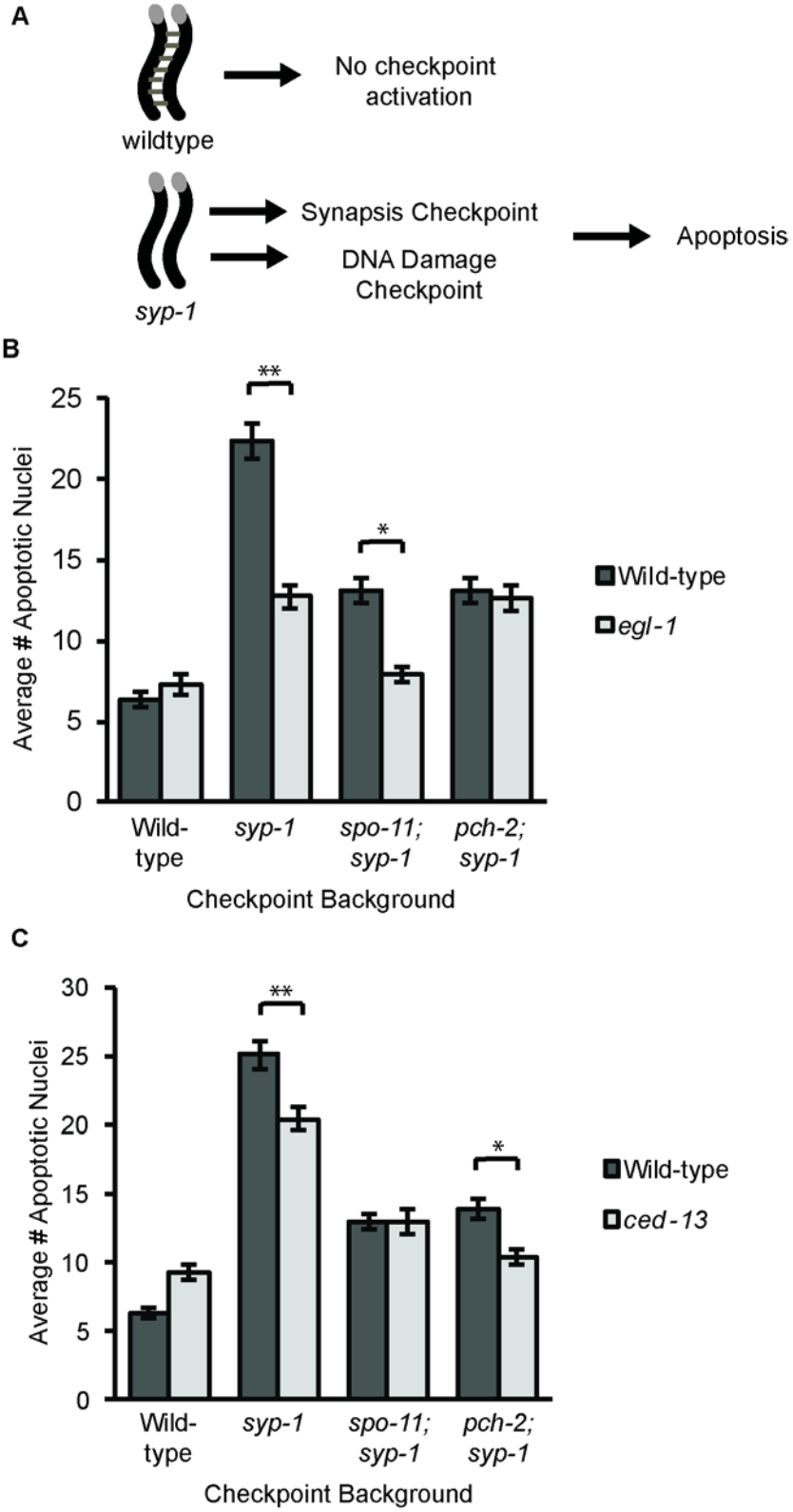

A
No checkpoint activation
wildtype
Synapsis Checkpoint
DNA Damage Checkpoint
Apoptosis
syp-1
B
Average # Apoptotic Nuclei
25
20
15
10
5
0
**
*
Wild-type
egl-1
Wild-type
syp-1
spo-11; syp-1
pch-2; syp-1
Checkpoint Background
C
Average # Apoptotic Nuclei
30
25
20
15
10
5
0
**
*
Wild-type
ced-13
Wild-type
syp-1
spo-11; syp-1
pch-2; syp-1
Checkpoint Background

Figure 2 (cont)

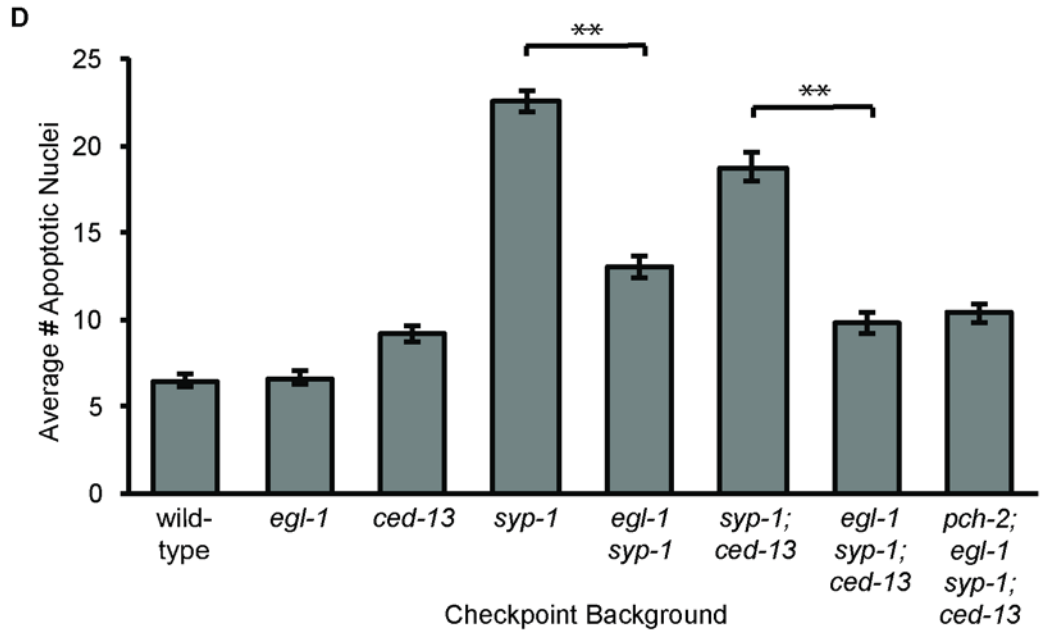


FIgure 2. egl-1 and ced-13 promote germline apoptosis in response to different checkpoints in syp-1 mutants. A. syp-1 mutants activate both checkpoints during meiotic prophase. B. Mutation of egl-1 reduces germline apoptosis in syp-1(me17) and spo-11(ok79);syp-1(me17) mutants but not in pch-2(tm1458);syp-1(me17) mutants. C. Mutation of ced-13 reduces germline apoptosis in syp-1(me17) and pch-2(tm1458);syp-1(me17) mutants but not in spo-11(ok79);syp-1(me17) mutants. D. Mutation of both egl-1 and ced-13 in syp-1(me17) and pch-2(tm1458);s-yp-1(me17) mutants does not reduce apoptosis to wildtype levels. * = $p < 0.05$ and ** = $p < 0.01$.

Figure 3

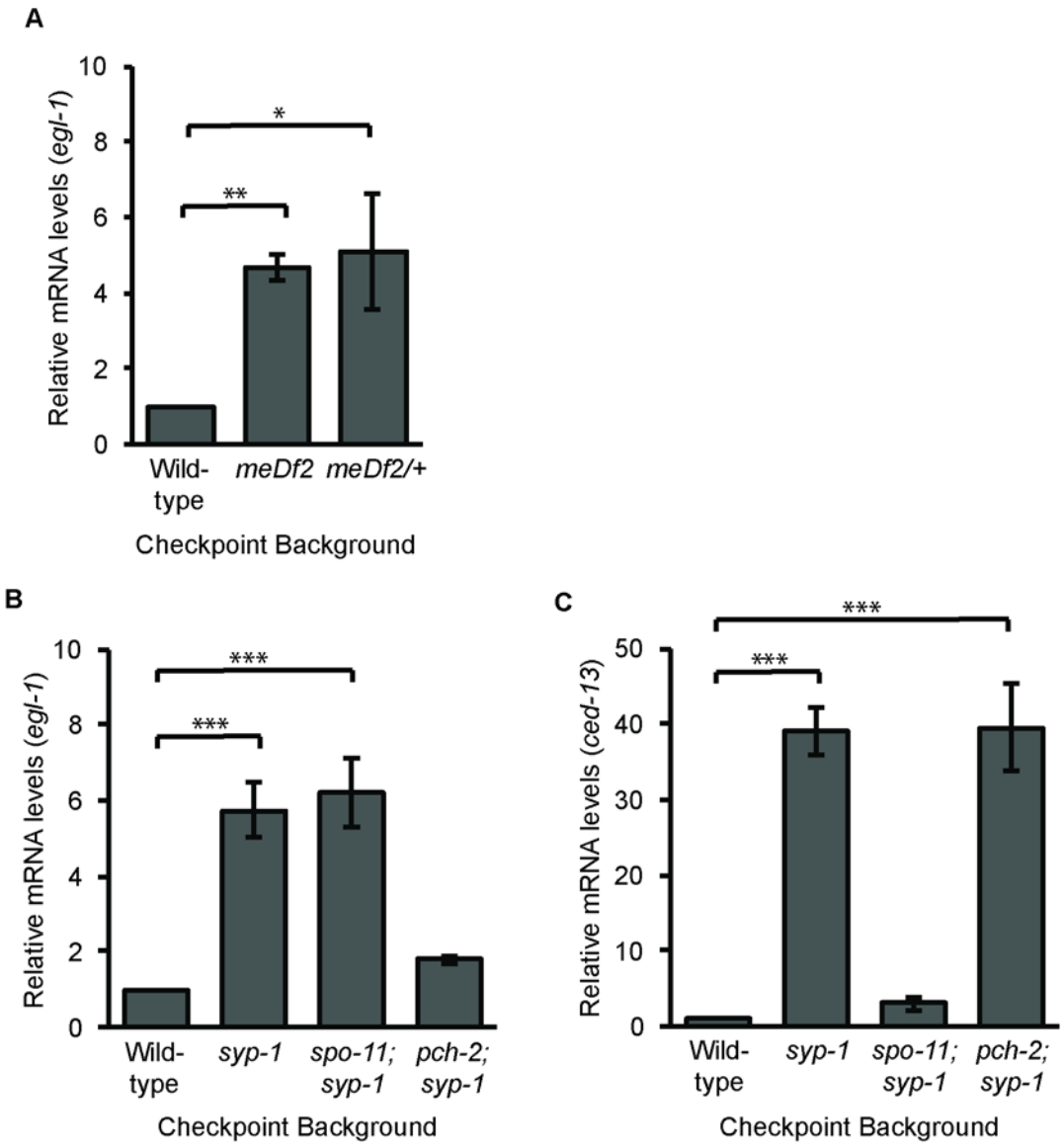


Figure 3. *egl-1* and *ced-13* are transcriptionally induced in response to different checkpoints in *syp-1* mutants. A) Relative transcript levels of *egl-1* mRNA are shown in wild-type, *meDf2* homozygous and *meDf2* heterozygous mutants. B) Relative transcript levels of *egl-1* mRNA in wild-type, *syp-1(me17), spo-11(ok79);-syp-1(me17)* and *pch-2(tm1458);syp-1(me17)* mutants. C) Relative transcript levels of *ced-13* mRNA in same mutants as part B. ** = p<.01 and *** = p < .001

Figure 4

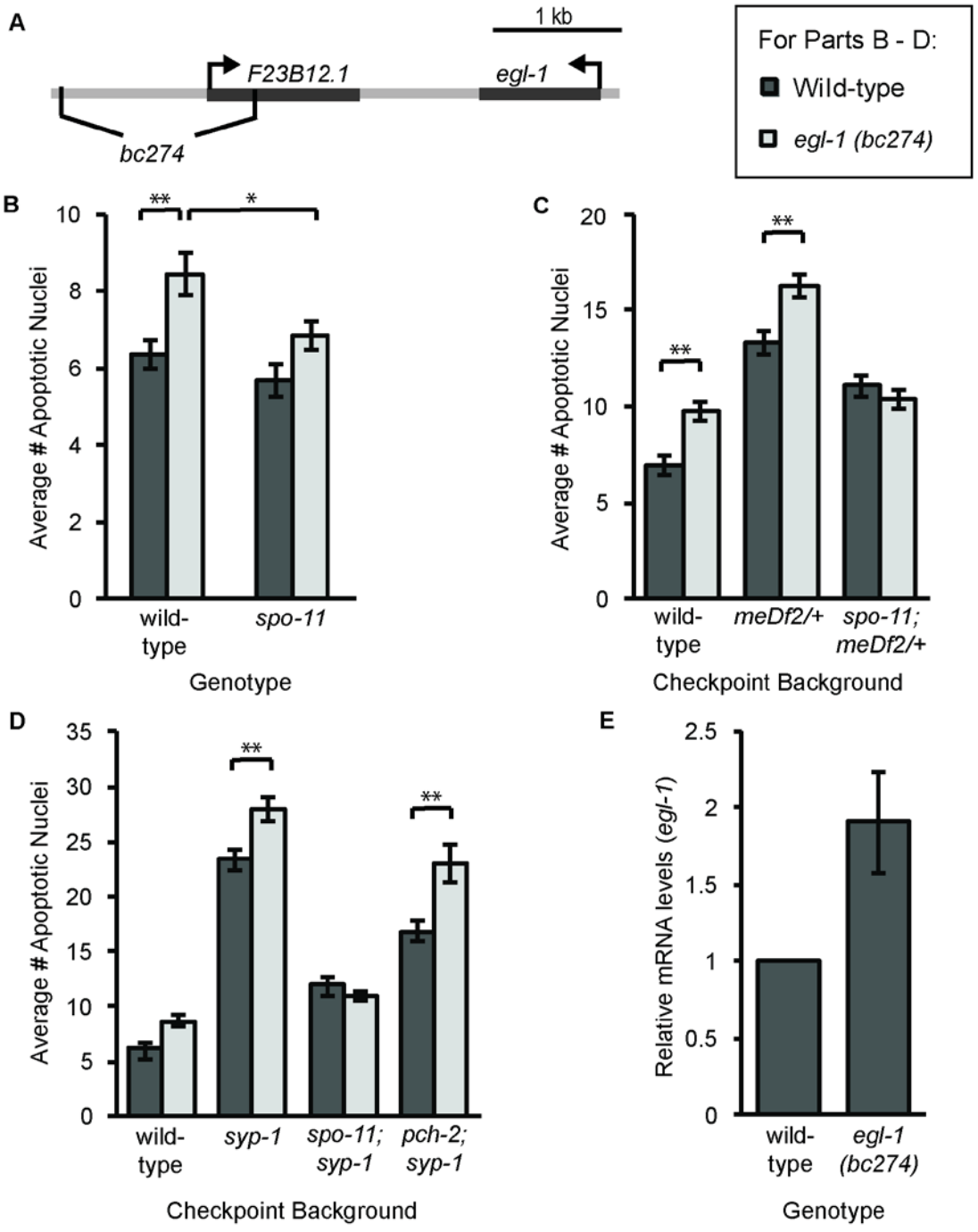


Figure 4. Identification of a negative regulatory element of *egl-1* in germline apoptosis. A) Schematic of the *egl-1* locus. B) *egl-1(bc274)* results in *spo-11* dependent elevation of apoptosis. (C and D) Apoptosis in *egl-1(bc274)* mutants when combined with mutants that activate meiotic checkpoints. E) Relative transcript levels of *egl-1* mRNA in wild-type and *egl-1(bc274)*. * = $p < 0.05$ and ** = $p < 0.01$.

Figure 5

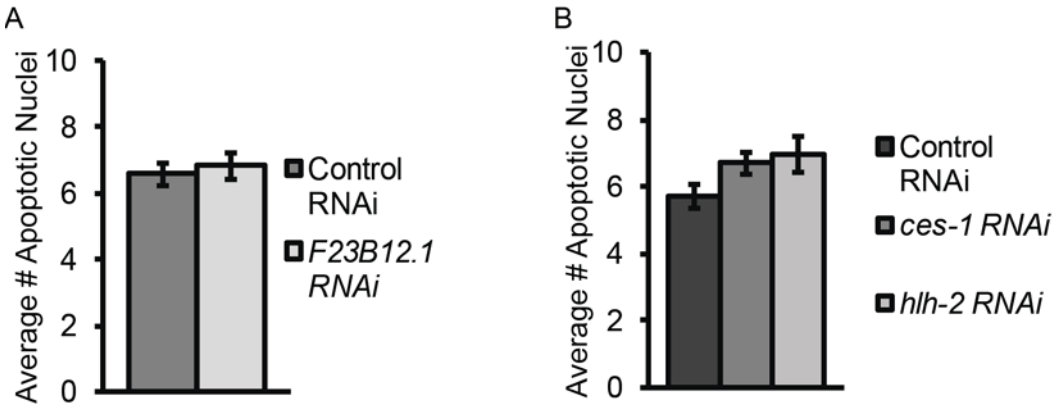
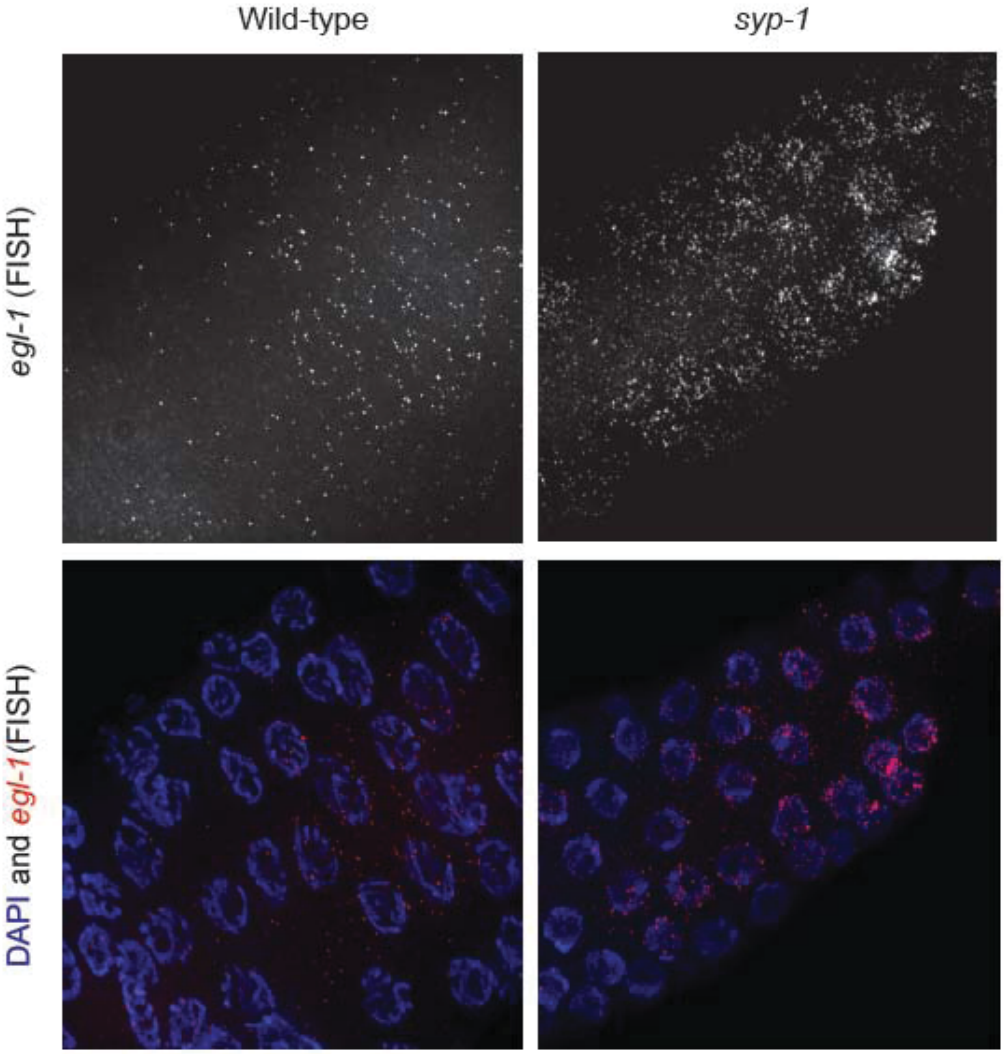


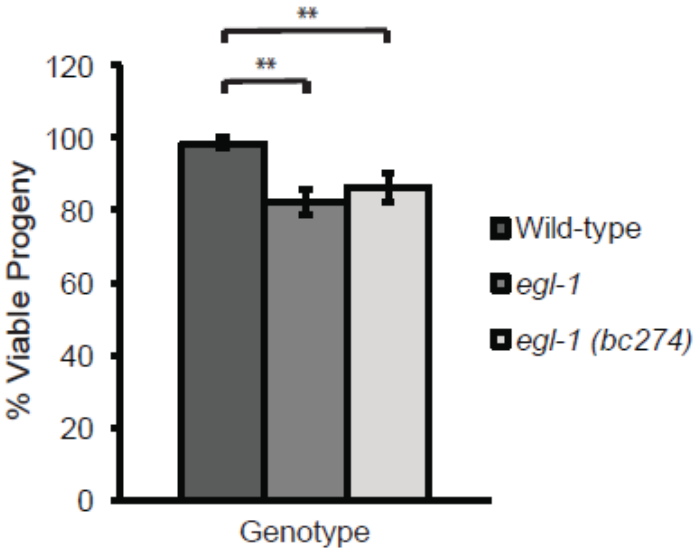
Figure 5. Transcription factors that regulate somatic *egl-1* do not regulate meiotic checkpoints. A) RNAi against the gene partially deleted by *bc274* does not alter apoptosis. B) Binding sites for CES-1 and HLH-2/3 were found in the deleted region of the *egl-1 (bc274)* allele. Apoptosis in

Supplemental Figure 1



Supplemental Figure 1. single-molecule FISH against *egl-1* mRNA . Probes designed against the mature *egl-1* transcript hybridize to the end of pachytene in hermaphrodite germlines.

Supplemental Figure 2



Supplemental Figure 2. *egl-1 (bc274)* and *egl-1 (n1084n3082)* both exhibit mild defects in progeny viability over the lifetime of the worm. Average of 12 worms shown. ** = p<.01

Chapter 4: Characterization of transcriptional regulation of *egl-1*

The work in the previous chapter demonstrated a role for *egl-1* in regulation of meiotic checkpoints. However, I wanted to further dissect the mechanism of regulation of *egl-1* itself, to pinpoint additional factors in the checkpoint activation pathway. One candidate we wanted to test was the transcription factor TRA-1, which represses *egl-1* in HSN cells in hermaphrodites and regulates egl-1 in the CEM neurons (Conradt and Horvitz 1999).

Additionally, we obtained several deletion mutants from our collaborator Barbara Conradt of regions immediately surrounding the *egl-1* gene, of which *egl-1 (bc274*) was described in Chapter 3 (Figure 1A). These regions, shown as grey boxes in the schematic, had been identified by homology to the *C. briggsae* species as being potentially important to regulation of *egl-1.* I tested these deletion mutants for meiotic or meiotic checkpoint defects related to the function of *egl-1.*

Results

The putative regulatory regions of *egl-1* are shown in the schematic in Figure 1A. The four remaining regions aside from *bc274* were tested for checkpoint involvement by crossing into the *syp-1* background. None of the regions appear to be important for checkpoint activation when all chromosomes are unsynapsed (Figure 1B-E). Of note, the region deleted in *egl-1(bc359*) likely contains a binding site for

TRA-1. Lack of a phenotype when this region is lost suggests that TRA-1 is not involved in regulating *egl-1* in meiosis. This was further validated in the *egl-1(n1084)* mutant, which specifically disrupts TRA-1 binding (Supplemental Figure 1).

I demonstrated in Chapter 3 that *egl-1* is required for meiotic checkpoints differentially depending on the manner of activation. Loss of pairing centers in the *meDf2* deletion mutants activates *egl-1* through both checkpoints, while asynapsis in the *syp-1* mutant only activates *egl-1* through the synapsis checkpoint. Because of this specification, I also tested the regulatory deletions in the *meDf2* and *meDf2/+* backgrounds. The results echoed those in the syp-1 background. Loss of these regulatory regions did not inactivate or upregulate either checkpoint in a statistically significant manner (Figure 2), indicating that none of these regions contribute to the regulation of apoptosis in response to meiotic checkpoints. Loss of any of these regions also does not cause gross progeny defects, as the viability of offspring is unaltered (supplemental Figure 2).

Recently, it was revealed that crossover promoting factor ZHP-3 and the DSB repair proteins MSH-4 and MSH-5 are required for the DNA damage checkpoint in *C. elegans* (Silva, Adamo et al.). These proteins play a role in later stages of DSB and CO processing, and their loss results in accumulation of recombination intermediates (Kelly, Dernburg et al. 2000; Jantsch, Pasierbek et al. 2004). Unexpectedly, single mutation of these proteins disabled the DNA damage checkpoint response; apoptosis was not upregulated in *msh-5* mutants despite the resulting unresolved intermediates (Silva, Adamo et al.). This suggested a requirement for these pro-CO proteins in the *egl-1* dependent checkpoint pathway, possibly through regulation of

egl-1 activation. We wanted to test the regulatory region deletions for requirement in this pathway. I tested our regulatory deletions for apoptotic activation in the *msh-5* mutant background. Of the four remaining deletions, only *egl-1(bc373*) had a phenotype in this background. *egl-1(bc373*) is a deletion of a region approximately 6kb upstream of the *egl-1* start codon (Figure 1). Loss of this region in the *msh-5* background elevates apoptosis (Figure 3), suggesting the possibility that this region is important for the apoptotic response of *egl-1* in stalled recombination intermediates.

Conclusion

This chapter contributes to our understanding of the mechanisms of *egl-1* regulation. *egl-1* is an important modulator of apoptosis in a number of contexts, and its regulation is likely to involve a number of factors and regulatory regions, including regions that have not been found to have significant homology to the *C. briggsae* locus. The data in this chapter also complements the previous chapter and further strengthens the case that there are mechanisms in place to specifically dampen the apoptotic response when DNA damage is present. To further explore which regions may be responsible for the recent discovery that procrossover proteins are required for checkpoint-induced apoptosis, experiments will be performed with the *egl-1(bc373*) in the *zhp-3(jf61)* mutant background.

Materials and Methods

Strains used

Wild-type worms for apoptosis assays were *dpy-11(e224) bcIs39* and *mnDp66;bcIs39;meDf2*

The regulatory deletion mutants were as below.

dpy-11(e224)egl-1(bc276)bcIs39

dpy-11(e224)egl-1(bc359)bcIs39

dpy-11(e224)egl-1(bc369)bcIs39

dpy-11(e224)egl-1(bc373)bcIs39

The TRA-1 binding mutant was *dpy-11(e224)egl-1(n1084)bcIs39,* a point mutation disrupting the binding site.

Crosses for meDf2/+ experiments were performed as described before. The regulatory mutants were then crossed into checkpoint activated strains below, using males generated from the non-dumpy checkpoint strains. *egl-1(n1084)* was also crossed into the *msh-5* mutant strain.

dpy-11(e224)syp-1(me17)ced-1::gfp (bcIs39)/ nt1[qIs51]
mnDp66;meDf2

msh-5(me23);bcIs39/ nt1[qIs51]

The wild-type strain for viability was Bristol N2

Apoptosis was scored as before

Embryonic Viability Assay

This was performed as done in Chapter 1 with each *egl-1* mutant. However, because we were not concerned about age-dependent defects in viability, the first timepoint was at 36 hours post-L4 seeding, and worms were moved every 24 hours therafter.

Figure 1

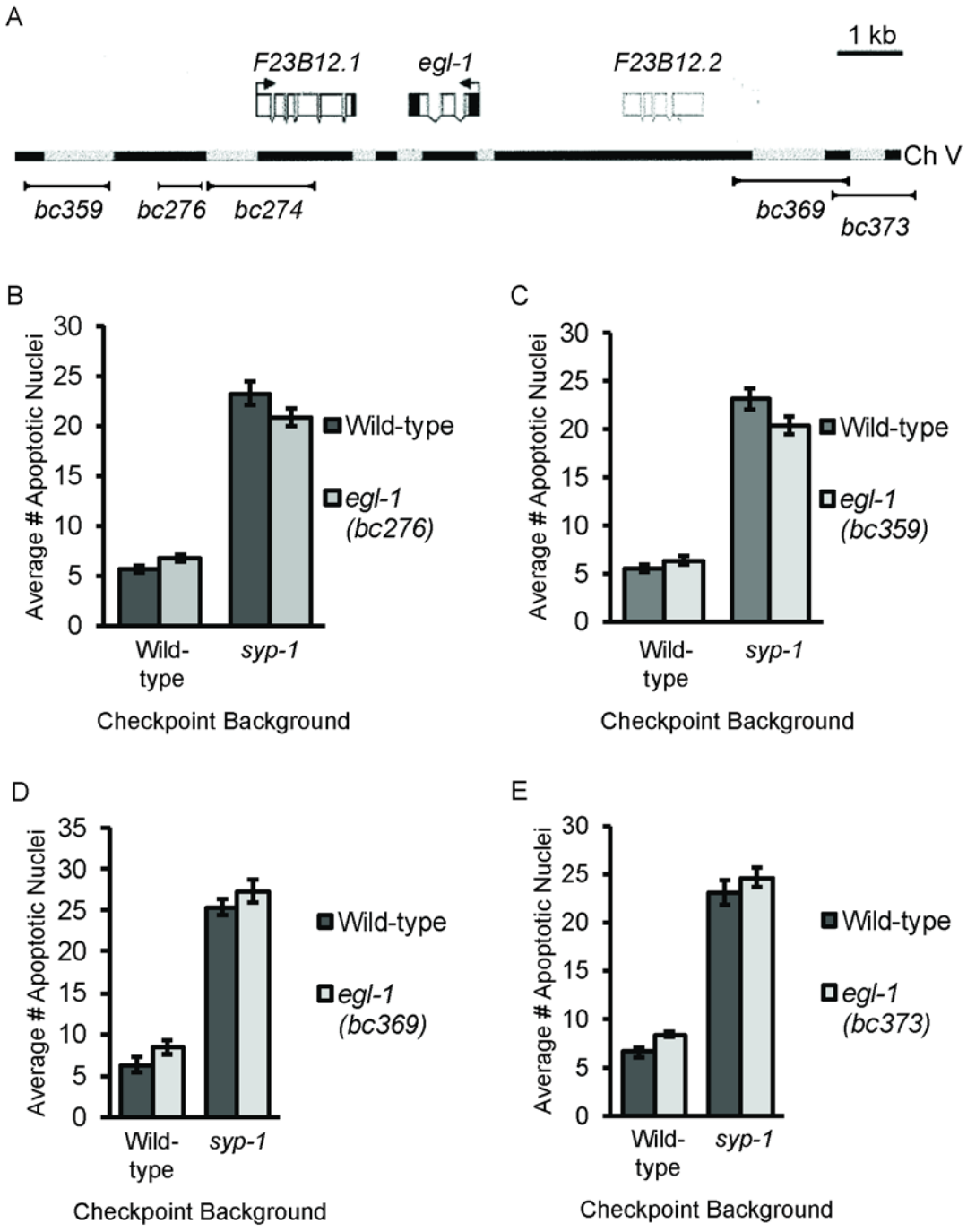


Figure 1. *egl-1* regulatory deletions do not regulate checkpoints when all chromosomes are usynapsed. A) Schematic of the deletions surrounding the *egl-1* locus. (B-E) Activation of the checkpoint in the *syp-1* mutant in four putative regulatory region deletions of *egl-1*.

Figure 2

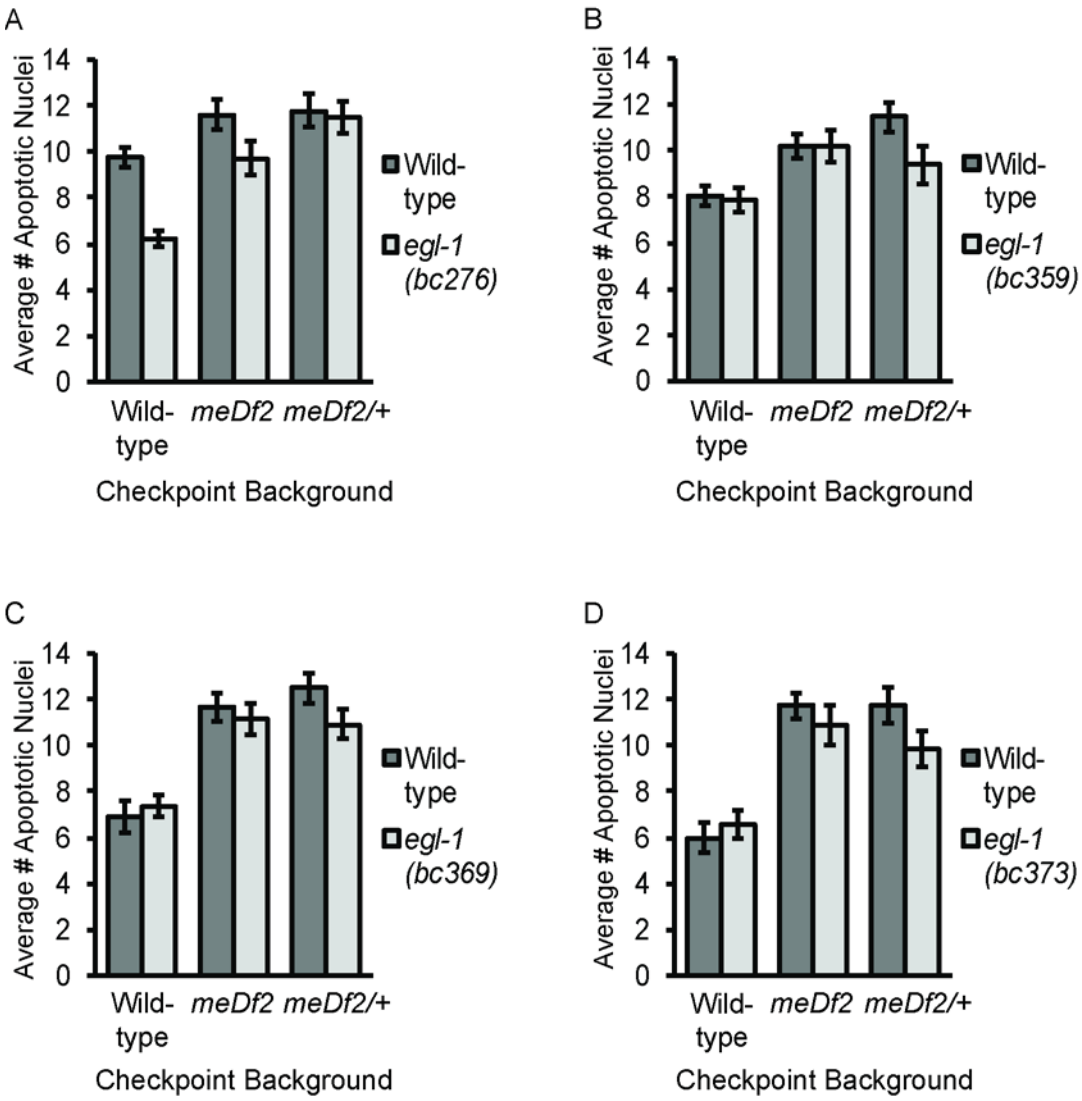


Figure 2. *egl-1* regulatory regions do not regulate the synapsis checkpoint when it is activated in *meDf2/+* worms. (A-D) Regulatory regions were mated into checkpoint activated backgrounds for apoptosis scoring. Drop in apoptosis is not significant in any checkpoint. Elevation of wild-type apoptosis in A is unusual, but the *egl-1 (bc276)* single mutant is within range for normal physiological apoptosis.

Figure 3

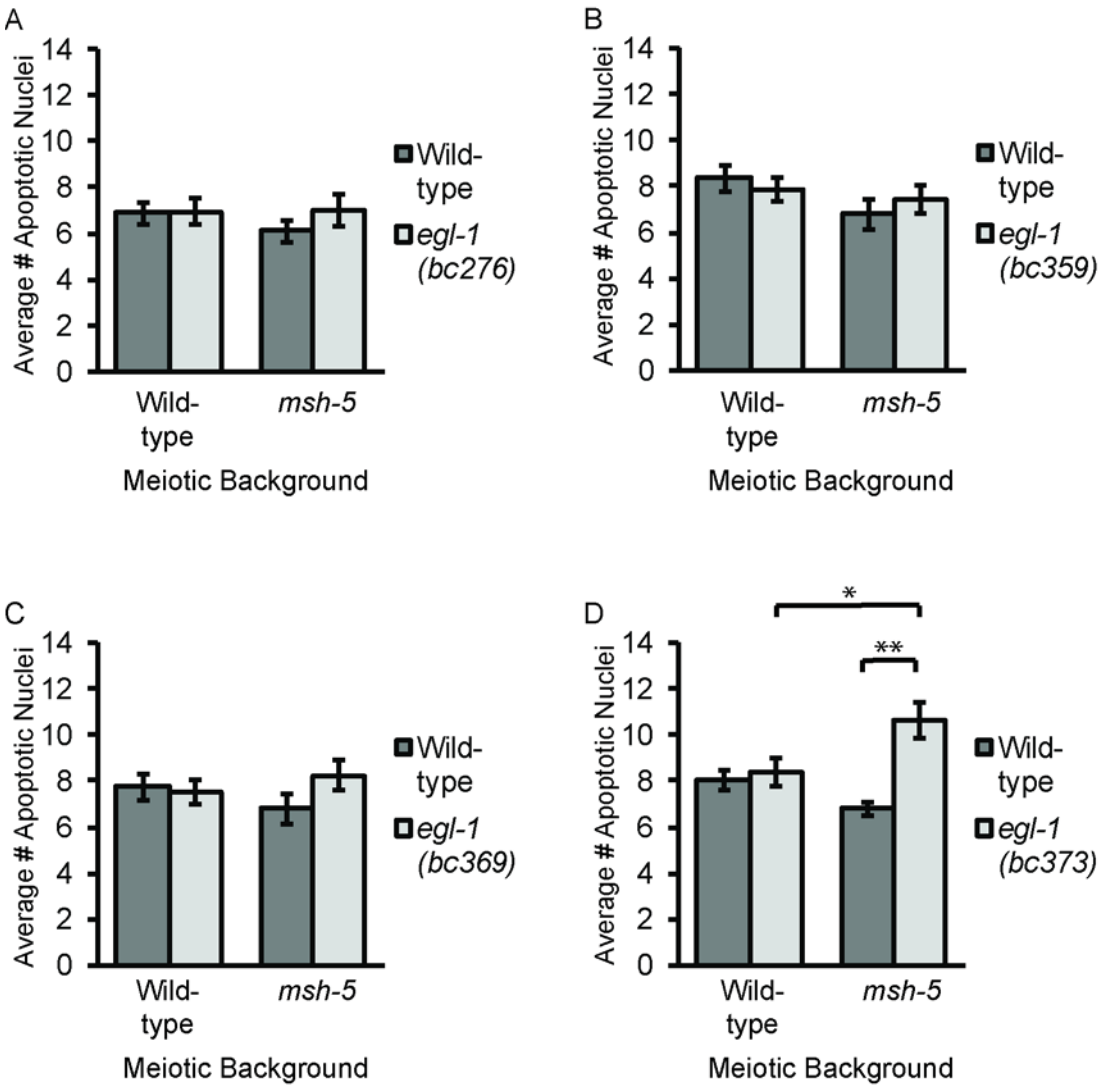
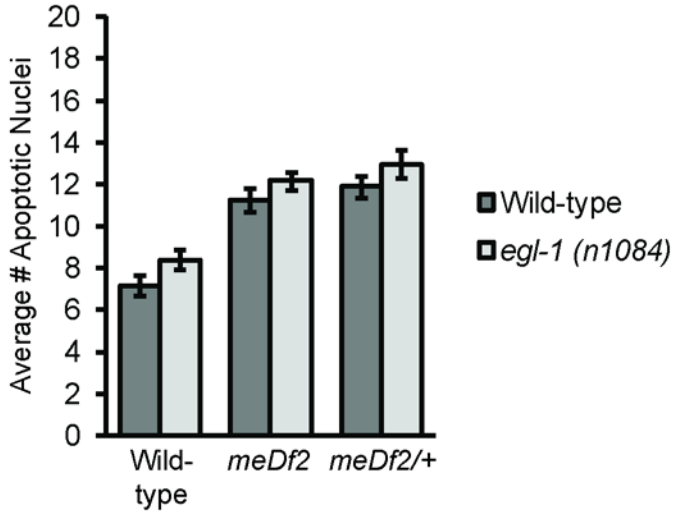


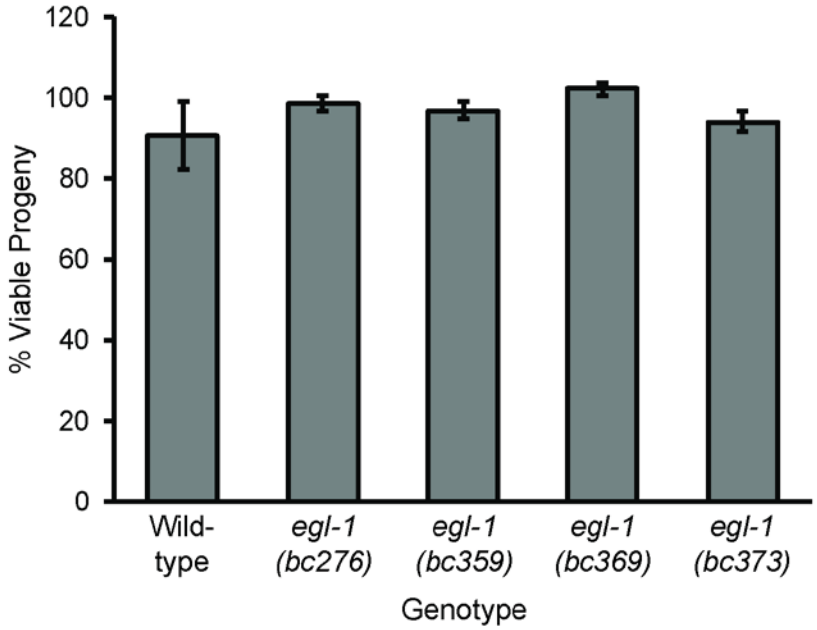
Figure 3. Regulatory deletion *egl-1 (bc373)* upregulates apoptosis in the msh-5 mutant. (A-D) Regulatory deletions were mated into the *msh-5(me23)* background and scored for apoptosis. * = p<.05 and **= p<.01

Supplemental Figure 1



Supplemental Figure 1. Disruption of TRA-1 binding does not perturb meiotic checkpoint signalling. The *egl-1(n1084)* allele destroys the TRA-1 binding site outside the *egl-1* locus. Apoptosis was scored after putting the mutation into checkpoint-induced strains

Supplemental Figure 2



Supplemental Figure 2. Lifetime progeny viability is unperturbed by deletion of putative regulatory regions other than *bc274*.

SECTION III

Coordination of Meiotic Prophase:

PCH-2 Regulation of Recombination

This section contains material previously published in

A quality control mechanism coordinates meiotic prophase events to promote crossover assurance.

Deshong AJ, Ye AL, Lamelza P, Bhalla N

PLoS Genetics, April 2014

Material is adapted here with permission of all authors

Chapter 5: PCH-2 Regulation of Recombination

Introduction

During meiotic prophase, double-strand breaks (DSBs) are made in DNA, and a subset of those breaks on each chromosome will be repaired to form crossovers (COs). In most systems, DSBs outnumber COs, and can be repaired in multiple ways (Baudat and de Massy 2007). In meiosis, DSB repair is biased towards inter-homolog repair and blocked towards sister repair (Bhalla and Dernburg 2008). In *C. elegans*, there is one obligate CO per homolog pair and it will be marked by CO promoting proteins late in pachytene (Yokoo, Zawadzki et al.).

PCH-2 is a conserved meiotic protein, an AAA-ATPase that has been implicated in checkpoint regulation and recombination. In *C. elegans*, it was identified as necessary for the synapsis checkpoint, and in *Drosophila,* it has been implicated in a recombination checkpoint (Joyce and McKim 2009). In budding yeast, loss of *pch2* was revealed to upregulate inter-sister DSB repair (Ho and Burgess ; Zanders, Sonntag Brown et al.). Similarly, in mice, where *pch-2* is called *Trip13*, knockdown causes defects in meiotic recombination (Li and Schimenti 2007, Roig 2010).

To dissect the role of *C. elegans pch-2* in regulating meiotic pairing, synapsis, and recombination, we used a variety of cytological and genetic techniques. I performed scoring of DSB repair by immunofluorescence and genetic analysis of CO rate and placement. These experiments contribute to work by two other members of

the lab – Alison Deshong and Piero Lamelza – pointing to *pch-2* as a regulator of multiple meiotic processes.

Results

To investigate the effect of *pch-2* mutation on recombination, I first looked at the ssDNA binding protein RAD-51. As described in Chapter 1, the germline was divided into six regions of equal length and the average number of foci was scored in each section as a readout of the introduction and repair of DSBs over the course of prophase. In the *pch-2* mutant, I saw fewer foci on average in the peak zones 4 and 5, corresponding to mid-pachytene (see Figure 1B). This could have pointed to a lower level of SPO-11 catalyzed breaks overall. However, since staining is done in fixed worms and RAD-51 presence on recombination intermediates is transient, this could also indicate that breaks are repaired faster in the *pch-2* mutant. To distinguish between these two possibilities, I looked again at RAD-51 staining in the germlines of *rad-54* mutant worms, with and without *pch-2*. RAD-54 is required to process recombination intermediates. Since mutants are unable to unload RAD-51, foci will accumulate (Mets and Meyer 2009). There was a similar level of RAD-51 staining between *rad-54* and *pch-2;rad-54* worms (Figure 1C), indicating that the lower level of staining in Figure 1B was not the product of a reduction in the number of DSBs made and might instead be due to faster repair.

In budding yeast, the effects of *pch-2* on meiosis are temperature-dependent (Joshi, Barot et al. 2009). Accordingly, I checked RAD-51 dynamics at both 15°C and 25°C. In both conditions, the peak average number of RAD-51 foci was similar between wild-type and *pch-2* (Figure 1D and E). However, at 15°C, RAD-51 foci

were cleared from chromosomes in zones 5 and 6 faster in *pch-2* than in wild-type, and at 25°C, there were more foci at the onset of meiosis in zone 3 in *pch-2* than in wild-type Figure 1D and E). Together, these data suggest that *pch-2* regulates meiotic recombination in a temperature-dependent manner. Loss of function leads to faster repair of recombination intermediates, with this phenotype being stronger at 20°C than at lower or higher temperatures.

These data complemented work by Alison Deshong in our lab analyzing synapsis in this background. By colocalizing central element protein SYP-1 and axial element protein HTP-3 by immunofluorescence, she showed that synapsis also occurs faster in *pch-2* mutant worms at 20°C. The *pch-2* synapsis phenotype was also more pronounced at 20°C than at other temperatures. She also confirmed that the accelerated synapsis and DSB repair were not due to earlier meiotic entry. The staining pattern of phosphorylated SUN-1, a nuclear envelope protein that is phosphorylated from meiotic entry to completion of synapsis (Rosu, Libuda et al. ; Woglar, Daryabeigi et al.), is normal in *pch-2* worms.

The subtle effect of *pch-2* on synapsis and recombination above led us to wonder specifically how loss of *pch-2* influenced homolog interactions, particularly in mutants that incur defects in meiosis. Alison found that loss of *pch-2* suppresses defects in pairing and synapsis. She also established that the localization of PCH-2 in the germline is consistent with a role in recombination. PCH-2 loads onto chromosomes just after synapsis and is present on the SC until mid-pachytene. This portion of the germline is associated with meiosis specific DNA repair mechanisms.

The observed pattern of localization, combined with *pch-2's* regulation of recombination, suggested the possibility that it might be required to promote inter-homolog DNA repair. To test this, I monitored RAD-51 loading in the *syp-1* background. In *syp-1* mutant worms, the lack of SC results in persistence of unresolved DSBs in late pachytene (Figure 2A, as compared to wild-type in Figure 1B). With loss of *pch-2* in this background I observed a significant drop in the number of DSBs persisting in zone 5, despite similar peak numbers to *syp-1* in zone 4 (Figure 2A). Since in the *syp-1* mutant, the homolog is unavailable to serve as a repair template, an early drop in RAD-51 foci implicated another pathway in DNA repair, most likely the use of the sister chromatid.

To directly test whether *pch-2* had an effect on the availability of the homolog vs the sister in DSB repair, Alison took advantage of a recently described transposon site engineered within the gene of a physiological marker that causes uncoordinated movement (Rosu, Libuda et al.). Heat shock of young adult hermaphrodites generates a specific DSB at the site of transposon excision, which can be repaired into a CO off the homolog or into a noncrossover. Because the timing of meiotic progression is known in C. elegans, this allowed Alison to look specifically for progeny produced from nuclei in various stages of meiotic prophase when heat shock occurred. In the 22-28 hour window, corresponding to nuclei that were in mid-pachytene at time of heat shock, she saw significantly fewer recombinant progeny from *pch-2* worms as compared to wild-type, reflecting an earlier disruption of access to the homolog as a repair partner.

We wondered if early inactivation of inter-homologous access in DSB repair would disturb the CO landscape. I performed snip-SNP analysis in *pch-2* mutant worms across chromosome III using the same alleles described in Chapter 1. I observed a slightly lower global level of recombination, manifested as a higher proportion of noncrossover chromosomes in *pch-2* (Figure 2C), as well as a statistically significant drop in the number of double crossover chromosomes. The lower levels of recombination frequency were more pronounced at chromosome centers than on the arms. A similar phenomenon was seen when I looked at SNP recombination on the X chromosome (Figure 2C).

We extended our analysis of recombination phenotypes in *pch*-2 mutants by testing the condition under which a single pair of chromosomes is unsynapsed (*meDf2* mutants). The presence of a single unpaired chromosome has been shown to extend the region in which nuclei undergo pairing and synapsis (transition zone), and also results in elevated numbers of unresolved DSBs in late pachytene and misregulated crossover control (Carlton, Farruggio et al. 2006). Interestingly, while Alison found that *meDf2* worms have an extended zone of PCH-2 staining, mutation of *pch-2* in this background does not allow for faster repair of recombination intermediates (Figure 2B), suggesting that there may be another mechanism to promote inter-homolog repair in this background.

I also examined SNP recombination in these two backgrounds. The recombination landscapes in *meDf2* and *pch-2; meDf2* are not significantly different from one another (Figure 2D).

Conclusions

The analysis of RAD-51 and SNP alleles allowed me to demonstrate that *pch-2* is required for regulation of meiotic recombination. Its promotion of inter-homolog DSB repair is reinforced by Alison's work on the importance of *pch-2* in regulating pairing and synapsis. Further, like the budding yeast *pch2* mutant, the phenotypes of *pch-2* mutants are temperature-dependent. This work was published in the spring of 2014 in PLoS Genetics (Deshong, Ye et al.), along with other data showing that *pch-2* mutants worsen meiotic and viaibility defects in mutants that are deficient in germline apoptosis. Thus, the model proposed in our paper is that *pch-2* acts as a meiotic "brake" to restrain the progression of meiotic events to ensure that they proceed properly and to promote crossover assurance on each chromosome.

Materials and Methods

Statistical Analysis

A Student's paired t-test was used for all significance analysis unless otherwise noted in the text or figures. For SNP recombination analysis, Fisher's exact test was used to calculate confidence.

Strains Used

Wild type for RAD-51 staining and SNP mapping was Bristol N2.

pch-2 (tm1458) was was mated into the strains below.

mnDp66;meDf2

syp-1(me17)/nT1[qIs51]

rad-54&tag-157(ok615)

Immunofluorescence for RAD-51

Dissection, fixation, and staining conditions were described in Chapter 1. For temperature-shifted experiments, worms were transferred by agar chunk to clean plates and placed at either 15°C or 25°C. The day before dissection, L4 animals were picked to plates and incubated overnight at 15°C or 25°C and dissection proceeded as at 20°C.

Snip-SNP genotyping

Mating strategies, growth conditions, and lysis/PCR protocols were described in Chapter 1. The snip-SNPs typed on chromosome III were the same as previously described. The X chromosome SNPs are as follows:

Table 1. snip-SNP alleles and primers

Allele	Primer		Primer	
pkP6139	01 for_X	aagagtgaacctttccgtgag	01 rev_X	tgatgcaatttatacacacgcc
pkP6120	13 for_X	tcgtggcaccataaaagtg	13 rev_X	gattcagatcaaacagaggtgg
pkP6157	36 for_X	ggggtataatgaaccaacctg	36 rev_X	tgtaggaaccgtttgtttcttc
pkP6161	61 for_X	atcgaccccaacaatgcac	61 rev_X	tccgtcatccaaatctccg
pkP6170	80 for_X	cgctgtcacaatctctaaaatg	80 rev_X	aaaccctccccactttgttgtc

Table 2. Restriction enzymes for snip-SNP alleles

Allele	Restriction Enzyme	N2 fragment sizes	CB4856 fragment sizes
pkP6139	MseI	[401, 31]	[279, 122, 31]
pkP6120	DraI	[243]	[128, 115]
pkP6157	ApoI	[261, 48]	[150, 111, 48]
pkP6161	AseI	[542]	[287, 255]
pkP6170	ApoI	[249, 118, 56]	[197, 118, 56, 52]

Figure 1

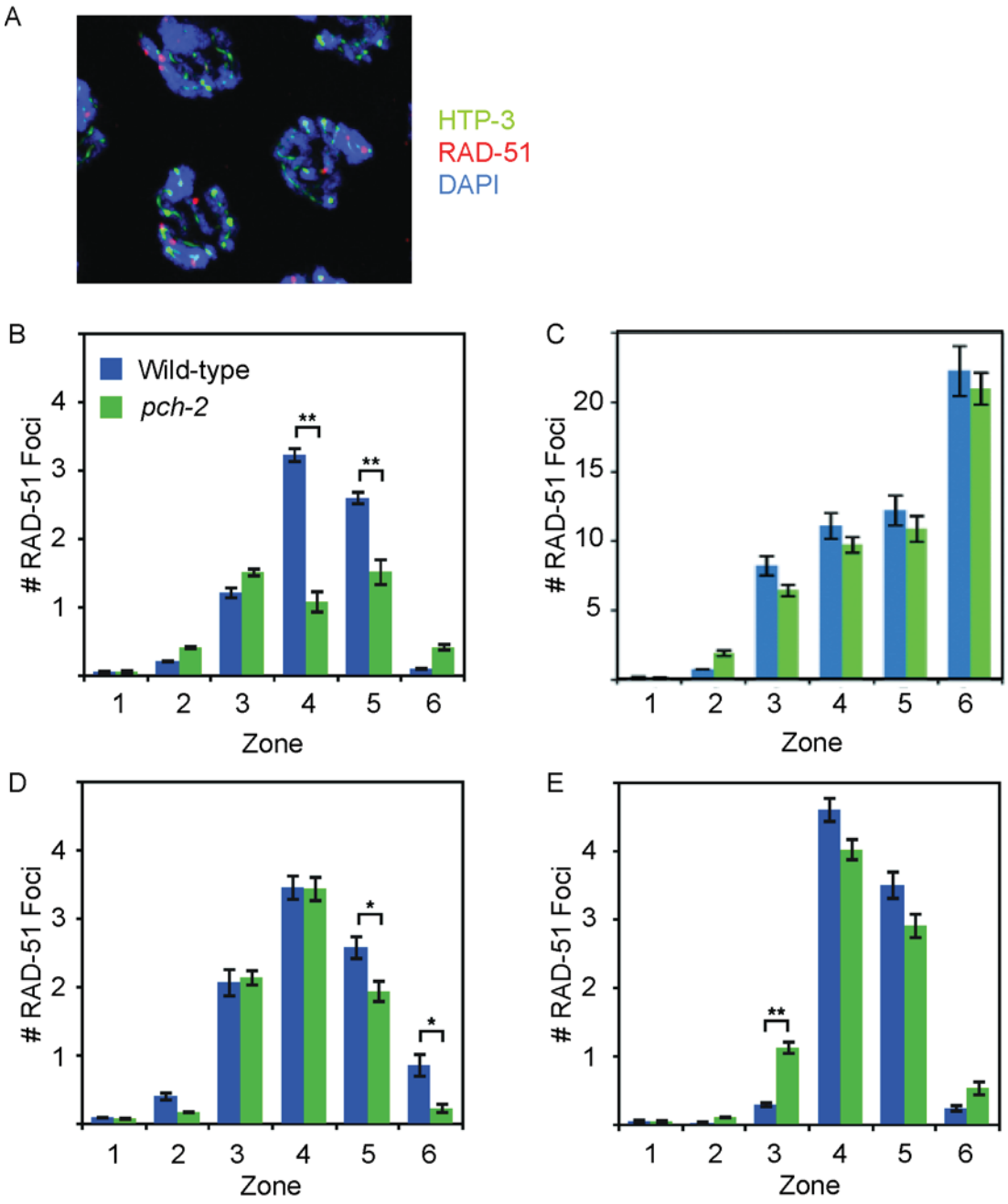
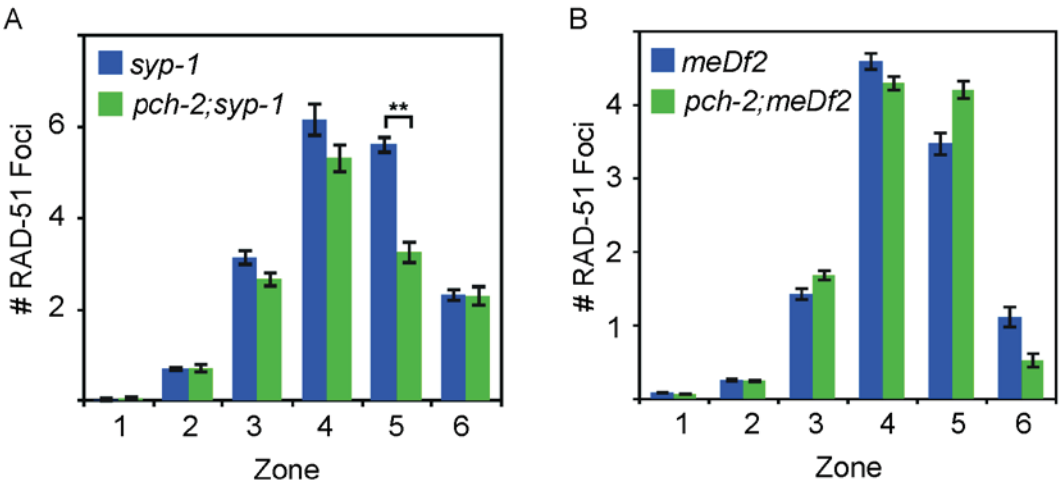


Figure 1. *pch-2* regulates meiotic DSB repair in a temperature-dependent manner. A) Example image of pachytene nuclei with DSBs stained in red. HTP-3 is a marker for the SC. B) Effect of loss of *pch-2* on RAD-51 at standard temperature (20°C). C) RAD-51 staining in the *rad-54* mutant that fails to repair DSBs. D) RAD-51 at 15°C. E) Rad-51 at 25°C. *=p<.05 and **=p<.01

Figure 2



C

X Chromosome

Interval	I	II	III	IV	2 CO	% NCO	n
Wild-type	13.1	9.8	9.2	23.1	10	48	173
pch-2	12	8.7	6.6	15.8	2*	57	183

Chromosome III

Interval	I	II	III	IV	2 CO	% NCO	n
Wild-type	26.3	7.6	3.5	21.1	8	46	171
pch-2	26.1	2.1	1.6	16	0*	54	188

D

Chromosome III

Interval	I	II	III	IV	2 CO	% NCO	n
meDf2	28	6.59	4.4	13.2	4	50	182
pch-2;meDf2	35.43	5.14	3.4	9.7	2	47	175

Figure 2. *pch-2* promotes access to inter-homolog DSB repair in *syp-1* but not *meDf2* mutants, resulting in altered recombination patterns. A) RAD-51 staining in the *syp-1* mutant background upon loss of *pch-2*. B) RAD-51 staining when *pch-2* is lost in the *meDf2* background. C) Recombination rates across two chromosomes assayed by snip-SNP analysis, with number of double-CO and % noncrossover chromosomes. D) Recombination across LGIII in the *meDf2* background. *=p<.05 and **=p<.01

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