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Meiotic Double-Strand Break Formation in C. elegans

By

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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular and Cell Biology in the Graduate Division of the University of California, Berkeley

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Abstract

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Abby Dernburg, Chair

For most eukaryotes, recombination between homologous chromosomes during meiosis is an essential aspect of sexual reproduction. Meiotic recombination is initiated by programmed double-strand breaks (DSBs) in the DNA, which have the potential to induce mutations if not efficiently repaired. The focus of the work presented here is to better understand the mechanisms that govern the initiation of recombination and regulate the formation of DSBs. The nematode Caenorhabditis elegans was used as a model system for these studies. Here I describe the identification of a novel gene, dsb-1, that is required for DSB formation in C. elegans. Through analysis of DSB-1 I illuminate two important regulatory pathways that control the initiation of meiotic recombination and regulate DSB number.

The first regulatory pathway presented in this work is the crossover assurance checkpoint, which promotes crossover recombination events on all chromosome pairs to ensure successful meiosis. Under the crossover assurance checkpoint, DSB formation is prolonged when one or more homologous chromosome pair fails to form a crossover precursor. This increase in meiotic recombination initiation events gives cells additional opportunities to produce the crossovers necessary for proper chromosome segregation.

I also present evidence for a separate regulatory pathway that functions to limit the number of DSBs. I describe a negative feedback loop that is mediated by DNA-damage response kinases ATM and ATR and acts though DSB-1 to down-regulate DSB formation. This regulatory pathway permits the formation of a limited number of meiotic DSBs, while preventing excess DSB levels. Furthermore, my results demonstrate the resilience of meiotic cells in tolerating excess levels of meiotic DSBs without negative consequences for genomic integrity or crossover regulation.

The work presented here provides important insights into the regulation of meiotic recombination initiation. Although the regulatory pathways described here were identified in C. elegans, recent studies suggest that similar regulatory pathways are likely to be conserved in other organisms. Therefore this work is important not only for understanding DSB regulation in C. elegans, but may also shed light on the regulation of meiotic DSBs in other eukaryotic organisms.
I dedicate this dissertation to my mother, who has supported and encouraged my interest in science throughout my life, and to my husband, who has stood by me throughout the ups and downs, stress, and long hours of graduate school.
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Introduction

Overview of meiosis

Sexual reproduction is important for many eukaryotic organisms. During sexual reproduction, organisms produce gamete cells through a specialized cell division process called meiosis. Gametes, usually from two different individuals, fuse to produce a zygote, the first cell of the new generation. Gametes contain a chromosome complement with half of the chromosome copy number as the parent cell, so that upon fertilization the parental copy number is restored for the next generation (Kohl and Sekelsky, 2013; Snustad and Simmons, 2011). The reduction in chromosome copy number that occurs during the production of gamete cells is the hallmark of the meiosis.

During meiosis, chromosomes undergo one round of DNA replication, followed by two rounds of chromosome segregation. In most organisms, homologous chromosomes segregate away from each other during the first round of cell division, followed by the segregation of sister chromatids during the second round of cell division (Snustad and Simmons, 2011). The segregation of homologous chromosomes during the first meiotic division results in a reduction of chromosome copy by half.

The strategy employed during meiosis to achieve the segregation of homologous chromosome to opposite cells relies on the physical association of homologous chromosomes, such that they line up alongside each other on the metaphase I plate and bi-orient on the meiotic spindle, allowing their subsequent segregation to oppose poles during the meiosis I division (reviewed in Petronczki et al., 2003). If association of homologous chromosomes fails, they will segregate randomly during the first meiotic division, leading to aneuploidy in the gametes and progeny. Therefore the events that occur leading up to the first meiotic division are pertinent for the ongoing survival of subsequent generations.

Meiotic prophase I

Several key events that set the stage for the segregation of homologous chromosomes during the first meiotic division occur during meiotic prophase I, which is by far the longest phase of the meiotic cell division program. At the onset of prophase I, chromosomes have already undergone DNA replication. Throughout the progression of prophase I, chromosomes condense and undergo several meiosis-specific events that will ultimately permit their proper segregation. These events are pairing and synapsis between homologous chromosomes and ultimately crossover recombination (Petronczki et al., 2003). Pairing occurs when a chromosome associates with its homologous partner (e.g. maternal chromosome 1 pairs with paternal chromosome 1). This association between homologous chromosomes is then reinforced by the assembly of the proteinaceous synaptonemal complex along the length of the chromosome pair (Petronczki et al., 2003). The synaptonemal complex is thought to serve as a scaffold for the completion of crossover recombination (Colaiácovo et al., 2003; MacQueen et al., 2002), which is the reciprocal exchange of DNA between homologous chromosomes (Petronczki et al., 2003). Crossover recombination is
essential for the proper segregation of homologous chromosomes in most sexually reproducing organisms. The formation of crossovers between homologous chromosomes, in collaboration with sister chromatid cohesion, physically links homologous chromosomes together, and is critical for them to remain associated up until the first meiotic division (Petronczki et al., 2003). At late stages of prophase I the points of genetic exchange, or crossovers, between chromosomes can be cytologically observed as chiasmata (Petronczki et al., 2003).

Based on cytological observations of chromosome morphology, meiotic prophase I has been subdivided into various stages. Leptotene, derived from Greek words meaning “thin threads”, is the first stage of prophase I where chromosomes condense into visible strands within the nucleus (Snustad and Simmons, 2011). During zygotene, meaning “paired threads”, homologous chromosomes pair and the synaptonemal complex assembles (Petronczki et al., 2003; Snustad and Simmons, 2011). The next stage is called pachytene, meaning “thick threads”; this is the stage where crossover recombination occurs (Petronczki et al., 2003; Snustad and Simmons, 2011). Diplotene, meaning “two threads”, is where the synaptonemal complex disassembles (Petronczki et al., 2003; Snustad and Simmons, 2011). The final stage is diakinesis, meaning “moving through”, where chromosome condense even further making the chiasmata clearly visible (Snustad and Simmons, 2011). After diakinesis nuclei progress into metaphase I, followed by the first meiotic division. The first meiotic division is followed by the second meiotic division, with no intervening S-phase (Snustad and Simmons, 2011).

**Meiotic recombination**

Genetic recombination, or homologous recombination, is the exchange of genetic information between chromosomes (Petronczki et al., 2003; Snustad and Simmons, 2011). Genetic recombination shuffles allele combinations between chromosomes. This serves to increase the range of genetic possibilities available to the next generation, and also offers an opportunity to remove deleterious alleles from chromosomes. However, in addition to promoting genetic diversity, recombination is essential for proper chromosome segregation during meiosis (Kohl and Sekelsky, 2013; Petronczki et al., 2003).

There are two classes of outcomes from the homologous recombination pathway: crossovers and non-crossovers (Kohl and Sekelsky, 2013). Crossovers are the reciprocal exchange of DNA between two chromosomes, resulting in a swap of genetic information between the two chromosomes. Non-crossovers occur when a small portion of one chromosome is replaced with genetic information from the homologous region of another chromosome. Only crossovers form physical linkages between chromosomes and are productive in promoting the proper segregation of homologous chromosomes during meiosis (Kohl and Sekelsky, 2013).

During the mitotic cell cycle, homologous recombination between sister chromatids is an important DNA repair pathway used to repair DNA damage resulting from double-strand breaks (DSBs) (Kohl and Sekelsky, 2013).
Homologous recombination during meiosis is also initiated by DSBs (reviewed in Keeney, 2001). However, the DSBs that initiate meiotic recombination during meiosis are produced intentionally by the cell. The enzyme that catalyzes DSB formation, Spo11, is homologous to a class of topoisomerases from Archaea (Keeney, 2001). These programmed DSBs then get repaired through the homologous recombination pathway, similar to that which is used during the mitotic cell cycle (Kohl and Sekelsky, 2013). However, one important way in which homologous recombination during meiosis differs from the pathway used in mitosis is that the homologous chromosome is used as a template for DNA repair. This is essential for promoting the proper segregation of homologous chromosomes during the first meiotic division.

Upon Spo11-induced catalysis of the DSB, Spo11 remains covalently attached to the 5’ end of the broken DNA strand (Keeney, 2001). The DSB is then processed by various nucleases to remove Spo11 from the DNA and produce a 3’ single-strand DNA tail (Keeney, 2001). The enzymes involved in this resection step are the MRN/MRX complex (consisting of Mre11, Rad50 and Nbs1/Xrs2) and Com1/Sae2 (Krogh and Symington, 2004). After resection, the ssDNA tail invades the homologous chromosome to repair the DSB. This strand-invasion step is catalyzed by the RecA homologs Rad51 and the meiosis-specific Dmc1 (Krogh and Symington, 2004; Neale and Keeney, 2006). If both ends of the DSB engage with the homologous chromosome a joint molecule or double Holliday junction is formed. These double Holliday junction intermediates are precursors of crossovers (Kohl and Sekelsky, 2013). In contrast, most non-crossovers are not thought to go through a double Holliday junction intermediate, but rather are formed from an earlier intermediate in the pathway (Allers and Lichten, 2001; Kohl and Sekelsky, 2013).

There are two distinct pathways that produce crossovers during meiosis. One pathway is dependent on the Msh4/5 proteins, while the other is dependent on the Mus81 protein (Kohl and Sekelsky, 2013). Many organisms utilize both pathways. One interesting difference between the two pathways is that only the Msh4/5 pathway is thought to produce crossovers that display interference (Kohl and Sekelsky, 2013). Crossover interference is the phenomenon where multiple crossovers occurring between the same pair of homologous chromosomes are spaced further apart then expected based on random chance (Muller, 1916). Crossover interference is observed in many organisms (Berchowitz and Copenhaver, 2010; Broman et al., 2002; Hodgkin et al., 1979; Meneely et al., 2002; Perkins, 1962). However, the precise molecular mechanisms that cause crossover interference remain unknown.

Although the meiotic recombination pathway is mechanistically very similar to the homologous recombination pathway used during the mitotic cell cycle to repair DSBs, there are some important differences. During the mitotic cell cycle, the preferred outcome of homologous recombination is usually a non-crossover, because crossovers between non-homologous chromosomes can result in genome rearrangements. However, during meiosis, crossover formation is crucial. Additionally, during the mitotic cell cycle, homologous recombination often uses the sister chromatid, which is in close proximity, as a repair template.
However, during meiosis, a substantial portion of recombination events occurs between homologous chromosomes (Kohl and Sekelsky, 2013). Various meiosis-specific factors, including protein components of the meiotic chromosome axes, have been implicated in favoring genetic exchange between homologous proteins (Martinez-Perez and Villeneuve, 2005; Niu et al., 2005; Terentyev et al., 2010; Wan et al., 2004).

**Meiotic double-strand break formation**

The discovery that meiotic DSBs are catalyzed by a topoisomerase-like mechanism was based on studies demonstrating that DSBs have a protein covalently attached to the 5' strand at each side of the DSB (Keeney and Kleckner, 1995; Liu et al., 1995; de Massy et al., 1995). These observations then led to the discovery that Spo11, which had already been identified as an important factor in meiosis, was the enzyme that catalyzed meiotic DSBs in *S. cerevisiae* (Bergerat et al., 1997; Keeney et al., 1997). Orthologs of budding yeast Spo11 were soon after identified and shown to be required for DSBs in other eukaryotic organisms (Dernburg et al., 1998; Grelon et al., 2001; McKim and Hayashi-Hagihara, 1998). Based on our current knowledge it appears that Spo11 is universally required to initiate meiotic DSB formation in all eukaryotes that undergo meiotic recombination (Keeney, 2001).

Although Spo11 is essential for the formation of DSBs, it does not function alone. In various organisms – including fungi, plants, and animals – additional proteins required for meiotic DSB formation have been identified (Keeney, 2008). In budding yeast, 9 proteins in addition to Spo11 are required for DSB formation (Keeney, 2008). Unlike Spo11, these additional proteins involved in DSB formation are poorly conserved. For example, of the five meiosis-specific DSB proteins found in *S. cerevisiae* (Rec102, Rec104, Mer2, Rec114, and Mei4), only two (Rec114 and Mei4) have known orthologs in diverse eukaryotic phyla and have been shown to be involved in DSB formation in mammals; however, even these DSB proteins are absent in several species, including *C. elegans*, *D. melanogaster*, and *N. crassa* (Kumar et al., 2010). Additional DSB proteins have also been identified in several organisms, none of which have widespread homology among eukaryotes (Cervantes et al., 2000; Libby et al., 2003; Liu et al., 2002; De Muyt et al., 2007, 2009). As meiotic DSB formation is essential to sexual reproduction in most organisms, the lack of conservation among DSB proteins is intriguing, and raises the question of whether different eukaryotic organisms rely on the same fundamental principles for DSB formation and regulation.

Spo11 is homologous to the catalytic subunit of TopoVI from archaea (Bergerat et al., 1997). TopoVI is a heterotetramer consisting of A (catalytic) and B (ATPase) subunits. Based on structural studies of TopoVI, the N-terminal region of the A subunit is predicted to form a protein-protein interaction surface for interacting with the B subunit (Corbett and Berger, 2003). This N-terminal region is conserved in Spo11, suggesting the N-terminus of Spo11 may also interact with a protein partner (Corbett and Berger, 2003). In budding yeast, Spo11 has been shown to interact with Ski8, a protein involved in RNA
metabolism in vegetative cells (Anderson and Parker, 1998; Araki et al., 2001; van Hoof et al., 2000; Masison et al., 1995), that is also required for meiotic DSB formation in *S. cerevisiae* (Arora et al., 2004). The *S. pombe* homolog of Ski8, Rec14, is also required for meiotic DSBs (Davis and Smith, 2001; Evans et al., 1997; Fox and Smith, 1998; Molnar et al., 2003), however Ski8’s role in meiotic DSB formation is not conserved in *Arabidopsis* (Jolivet et al., 2006). Furthermore, many species, including *C. elegans*, do not appear to have a homolog of Ski8. Therefore the interacting partner of Spo11 in other organisms, if there is one, remains unknown.

The MRX complex (Mre11-Rad50-Xrs2) has a primary role in the resection of DSBs. In some organisms, including budding yeast and *C. elegans*, Mre11 and Rad50 are also required for meiotic DSB formation (Ajimura et al., 1993; Chin and Villeneuve, 2001; Hayashi et al., 2007; Ivanov et al., 1992). However, this role of Mre11 and Rad50 in promoting meiotic DSB formation is not conserved in all organisms (Keeney, 2008).

The formation of DSBs is thought to be a highly regulated process since DSBs are not only essential for promoting proper chromosome segregation, but also introduce a potential hazard to the genome. Important aspects of DSB formation that are regulated include the timing and the number of DSBs. At the low end, a minimum number of DSBs – one per pair of homologous chromosomes – is required to produce the crossovers necessary for promoting the proper segregation of homologous chromosomes. However, having too many DSBs could lead to genomic instability if they are not repaired properly. The mechanisms in place to regulate DSB number are largely unknown. Recent studies have implemented the DNA-damage repair kinases ATM and ATR in the negative regulation of DSB number in various species including mice, budding yeast, and *Drosophila* (Carballo et al., 2013; Joyce et al., 2011; Lange et al., 2011; Zhang et al., 2011). In budding yeast, Mec1 and Tel1 (ATM and ATR) were shown to downregulate DSB activity through phosphorylation of Rec114 (Carballo et al., 2013).

The formation of meiotic DSBs occurs within a specific window of time during early meiotic prophase (Cervantes et al., 2000; Keeney, 2008; Padmore et al., 1991). In *S. cerevisiae*, DSB formation is closely coupled to DNA replication such that DSBs occur only after DNA synthesis is complete (Borde et al., 2000; Murakami et al., 2003). This timing of DSB initiation is controlled by phosphorylation of Mer2 by cell cycle-regulated kinases, CDK and DDK, involved in DNA replication (Henderson et al., 2006; Sasanuma et al., 2008; Wan et al., 2008). The timing of when to turn off DSB formation is also extremely important, because it must be shut off in a timely manner to permit the repair of DSBs prior to the meiotic divisions. Mechanisms that shut down DSB formation are not understood.

**Crossover regulation**

As discussed above, crossover recombination promotes the accurate segregation of homologous chromosome pairs during meiosis (Page and Hawley, 2003). It is important that each chromosome pair attains at least one
crossover. Although many DSBs are formed during meiosis in most organisms, each chromosome pair typically receives very few crossovers (reviewed in Martinez-Perez and Colaiácovo, 2009), suggesting that too many crossovers may also have negative consequences. It is therefore important that each chromosome pair attains the optimal number of crossovers to promote its proper segregation. This is achieved through a mechanism known as crossover homeostasis (Martini et al., 2006). Crossover homeostasis describes a phenomenon where cells produce a similar number of crossovers, despite varying levels of meiotic DSBs, by altering the crossover to non-crossover ratio (Martini et al., 2006). Crossover homeostasis was first described in budding yeast, but has also been reported in C. elegans (Hillers and Villeneuve, 2003; Rosu et al., 2011; Yokoo et al., 2012). In C. elegans the typical outcome of one crossover per chromosome pair unaffected whether DSB levels are greatly diminished or greatly increased (Rosu et al., 2011; Yokoo et al., 2012). Crossover homeostasis is likely to be an important aspect for crossover regulation in many organisms.

In addition to the number of crossovers, the genomic distribution of crossovers is also important for proper chromosome segregation. This is exemplified by the observation that many cases of human aneuploidy show evidence of normal crossover numbers, but have aberrant placement of crossover events along the chromosome (Lamb et al., 2005). Crossovers do not occur randomly throughout the genome, but rather have a tendency to occur in a subset of genomic locations called crossover hotspots (reviewed in Lichten and Goldman, 1995). Several lines of evidence suggest that the initial distribution of DSBs made along the chromosome largely determines the distribution of crossovers (Buhler et al., 2007; Kauppi et al., 2004; Mets and Meyer, 2009; Pan et al., 2011; Petes, 2001; Smagulova et al., 2011). The placement of meiotic DSBs is largely influenced by chromosome structure an organization, including chromatin accessibility and histone modifications (for review see Brachet et al., 2012).

Another interesting feature that impacts the distribution of crossover events on a single chromosome pair is crossover interference. Crossover interference is a phenomenon where the occurrence of one crossover decreases the likelihood of having another crossover nearby. Crossover interference was first described a century ago in Drosophila (Muller, 1916), but has since been observed in many organisms (Berchowitz and Copenhaver, 2010; Broman et al., 2002; Hodgkin et al., 1979; Meneely et al., 2002; Perkins, 1962). The precise mechanism governing crossover interference remains unknown. Evidence from C. elegans suggests that meiotic chromosome structure, and specifically the synaptonemal complex, is important for mediating crossover interference (Hayashi et al., 2010; Hillers and Villeneuve, 2003; Libuda et al., 2013; Mets and Meyer, 2009; Nabeshima et al., 2004; Tsai et al., 2008). However, the role of the synaptonemal complex in mediating crossover interference in other organisms has been debated (Bishop and Zickler, 2004).
Meiosis in *Caenorhabditis elegans*

In addition to being an excellent model system for genetic manipulation and cytology, *C. elegans* has emerged as an important model system for studying meiosis. Within the germline of *C. elegans*, nuclei progress synchronously through the meiotic program in such a way that their location within the gonad is coupled to the phase of meiosis that they are undergoing (Phillips et al., 2009). In addition, chromosomes can be easily distinguished from one another during meiotic prophase without the need for special manipulation of nuclei (Dernburg et al., 1998; Mets and Meyer, 2009; Nabeshima et al., 2011). These features make *C. elegans* particularly amenable to cytological observation of meiotic processes.

As in other eukaryotes, SPO-11 catalyzes the formation of meiotic DSBs (Libby et al., 2003). Similar to *S. cerevisiae*, MRE-11 and RAD-50 are also required for DSBs formation in *C. elegans* (Chin and Villeneuve, 2001; Hayashi et al., 2007), in addition to their roles in the downstream processing of DSBs (Keeney, 2001). Meiotic axial element protein HTP-3 was also shown to be required for DSB formation in *C. elegans* (Goodyer et al., 2008). The importance of the meiotic chromosome axis in promoting DSBs has been demonstrated in other organisms as well (Daniel et al., 2011; Keeney, 2008; Shin et al., 2010). Additionally, the meiotic kinase CHK-2, which is important for regulating many key events during early meiotic prophase, is required for meiotic DSBs in *C. elegans* (MacQueen and Villeneuve, 2001). In addition to these proteins, there are several other factors that are known to promote meiotic DSB formation in *C. elegans*, although their affects may be indirect. These include the chromatin-associated proteins HIM-5, HIM-17, and XND-1, which promote normal levels of meiotic DSBs, but whose functions during meiosis are not well understood (Meneely et al., 2012; Reddy and Villeneuve, 2004; Wagner et al., 2010). However, apart from SPO-11, no protein that specifically functions in promoting meiotic DSB formation has previously been reported.

*C. elegans* meiosis has some unique features that set it apart from other organisms. In most organisms studied, including fungi, mammals, and plants, stable alignment and synapsis of homologous chromosomes during meiosis is dependent on the initiation of meiotic recombination (Baudat et al., 2000; Grelon et al., 2001; Loidl et al., 1994; Romanienko and Camerini-Otero, 2000; Storlazzi et al., 2003; Tessé et al., 2003). Exceptions to this include *Drosophila* and *C. elegans* (Dernburg et al., 1998; McKim et al., 1998). In *C. elegans*, homolog pairing and synapsis occur independently of SPO-11 and recombination initiation (Dernburg et al., 1998). This decoupling of meiotic recombination from homolog pairing and synapsis facilitates the investigation of defects specifically related to meiotic recombination.

Another interesting feature of meiosis in *C. elegans* is that under normal conditions each homologous chromosome pair usually attains exactly one crossover per meiosis, meaning that crossover interference is complete (Brenner, 1974; Hillers and Villeneuve, 2003; Hodgkin et al., 1979; Martinez-Perez and Colaiacovo, 2009; Wood, 1988). This is different from other organisms where each chromosome pair often receives multiple crossovers per meiosis.
(van Veen and Hawley, 2003). Disruption of meiotic chromosome structure, by either disrupting condensins or the synaptonemal complex, can cause the occurrence of more than one crossover per homolog pair, suggesting that chromosome structure plays an important role in mediating crossover interference (Hayashi et al., 2010; Hillers and Villeneuve, 2003; Libuda et al., 2013; Mets and Meyer, 2009; Nabeshima et al., 2004; Tsai et al., 2008). In addition, disruption of the anti-recombinase RTEL-1, which is thought to shuttle recombination intermediates into the non-crossover pathway, can also lead to an increase in crossovers (Yould et al., 2010). Interestingly, the excess crossovers observed in an rtel-1 mutant appear to be independent of the MSH-4/5 pathway that is typically used during C. elegans meiosis (Yould et al., 2010; Zalevsky et al., 1999), and may go through an alternate crossover pathway, such as the MUS81 pathway.

Another interesting feature of C. elegans chromosomes is that they are holocentric, meaning they do not have a single, specified location for kinetochore assembly. Rather, the kinetochore assembles along the entire length of chromosomes during mitosis. During meiosis, the chromosome reorganizes around the point where crossover recombination occurred, or the chiasma, and the kinetochore assembles in cup-like structures that encompass either side of the bivalent (reviewed in Schvarzstein et al., 2010). It has been speculated that the occurrence of a single crossover per chromosome pair per meiosis may be related to the holocentric nature of C. elegans chromosomes. However, not enough comparative data from other holocentric organisms is available to support this speculation.

Overview of presented work

Meiosis is an essential cellular process for sexually reproducing organisms. As described above, successful meiosis relies on several events, including the formation of crossovers between homologous chromosome pairs. Initiation of meiotic recombination is achieved through the induction of programmed DSBs, intentional DNA damage, demonstrating the importance of meiotic recombination. Although critical for the proper segregation of homologous chromosomes, meiotic DSB formation is thought to be tightly regulated to ensure genomic integrity for subsequent generations. Regulation of meiotic recombination initiation is an important aspect of meiosis that remains poorly understood. Studying the regulation of DSB formation in C. elegans has been the focus of my graduate research.

In the work presented in Chapter 1, I describe the identification of a novel factor, dsb-1, that is essential for the initiation of meiotic recombination in C. elegans. dsb-1 is required for meiotic DSB formation, and is therefore required for crossover formation and the proper segregation of homologous chromosome pairs during meiosis (Stamper et al., 2013). Apart from SPO-11, DSB-1 was the first factor identified that is specifically required for DSB formation in C. elegans. Although DSB-1 is essential for successful meiosis in C. elegans, it is not highly conserved. In fact, no homologs of DSB-1 can be readily identified outside of the Caenorhabditis genus. However, many Caenorhabditis species, including C.
*elegans*, contain a paralog of DSB-1. The paralog in *C. elegans*, which has been named DSB-2, is also involved in meiotic DSB formation (Rosu et al., 2013). DSB-1 and DSB-2 lack any known protein functional domains that might give us clues as to their biochemical function in promoting DSB formation. As of now we still do not understand how these proteins function to promote DSB formation. However, DSB-1 has been an invaluable tool for studying meiotic DSB regulation, and has enabled me to uncover important aspects of DSB regulation.

In Chapter 2, I describe how cytological analysis of DSB-1 localization under various mutant conditions allowed us to uncover a regulatory network coupling crossover assurance to DSB regulation. We have named this regulatory network the crossover assurance checkpoint (Stamper et al., 2013). Under the crossover assurance checkpoint model that we have developed, individual meiotic nuclei monitor the crossover status of each chromosome pair during early to mid meiotic prophase. If one or more chromosome pair lacks a crossover precursor by the time that DSB formation is normally shut off in early/mid pachytene, the nucleus responds by prolonging the formation of DSBs. This model provides a feedback mechanism by which crossover status is able to influence DSB levels based on the recombination requirements of the nucleus. Interestingly, a recent study provides evidence for a similar type of feedback mechanism coupling crossover status to DSB formation in *S. cerevisiae* (Thacker et al., 2014), suggesting that regulatory networks analogous to the *C. elegans* crossover assurance checkpoint may be conserved in other organisms.

In Chapter 3, I provide evidence for a negative feedback loop that acts through DSB-1 to limit the number of meiotic DSBs formed. In this feedback loop, DSB-1 becomes phosphorylated in response to DSBs, in a manner dependent on the DNA damage response kinases ATM and ATR. The phosphorylation of DSB-1 functions to downregulate DSB formation. Importantly, although DSB-1 is not conserved outside of *Caenorhabditis*, the ATM/ATR negative feedback loop limiting DSB number appears to be conserved in various organisms (Carballo et al., 2013; Joyce et al., 2011; Lange et al., 2011; Zhang et al., 2011).

By studying the negative feedback loop involving DSB-1 phosphorylation, I was able to develop a tool to increase the levels of endogenous DSBs without perturbing the meiotic recombination pathway, allowing me to access the consequence of having an excess of DSBs. I show remarkably, that meiotic nuclei are able to tolerate an estimated 2-3 fold increase in DSB levels with no consequences for genomic integrity, crossover distribution, or crossover number. This study reveals that *C. elegans* has robust mechanisms in place to ensure proper DNA repair and crossover homeostasis when faced with excess DSBs.

The work presented here describes two separate pathways that function to regulate DSB number in two very different ways. The crossover assurance checkpoint provides an opportunity to upregulate DSB levels by prolonging the timing of DSB formation in the event that one or more chromosome pairs lacks a crossover precursor. The negative feedback loop involving the phosphorylation of DSB-1 by ATM and ATR allows for the formation of a limited number of meiotic DSBs, while preventing the accumulation of excess DSB levels. Both of these regulatory pathways are important for meiotic cells to achieve an optimal number
of DSBs for successful completion of meiosis. Furthermore, recent studies suggest that both regulatory pathways identified in the presented work are likely to be conserved in other eukaryotic organisms.
Chapter 1: Identification of DSB-1, a novel factor required for meiotic double-strand break formation

Summary

In this chapter, I describe the identification of a novel protein factor, DSB-1, that is specifically required for meiotic DSB formation in C. elegans. I show that dsb-1 mutants do not disrupt the gene expression of spo-11, suggesting a distinct role for DSB-1 in promoting DSBs. I also show that DSB-1 localizes to chromosomes during early meiotic prophase, coincident with the timing of DSB formation.

Introduction

Meiotic recombination is initiated by programmed DNA double-strand breaks (DSBs), a subset of which is repaired to form crossovers between homologous chromosomes (for a review, see Keeney, 2001). Meiotic DSBs are catalyzed by the widely conserved, topoisomerase-related enzyme Spo11 (Bergerat et al., 1997; Keeney et al., 1997). Although Spo11 is essential for DSB formation, it does not function alone. In various organisms—including fungi, plants, and animals—additional proteins required for meiotic DSBs have been identified (for a review, see Keeney, 2008). Unlike Spo11, other known factors involved in DSB formation are poorly conserved. For example, of five meiosis-specific DSB proteins found in S. cerevisiae, only two (Rec114 and Mei4) have known orthologs in other phyla; and even these two proteins are absent in several species, including Caenorhabditis elegans, D. melanogaster, and Neurospora crassa (Kumar et al., 2010). Additional DSB proteins have also been identified in other organisms, but none are ubiquitous among eukaryotes (Cervantes et al., 2000; Libby et al., 2003; Liu et al., 2002; De Muyt et al., 2007, 2009). However, in C. elegans, apart from SPO-11, no protein that specifically functions in initiating recombination has previously been reported. Some aspects of C. elegans meiosis are unusual among model organisms, including the fact that synapsis between homologous chromosomes is independent of recombination (Dernburg et al., 1998). Thus, analysis of DSB regulation in C. elegans will likely reveal both conserved aspects of meiosis and how regulatory circuits are remodeled during evolution.

Here I describe the identification of a novel gene, dsb-1 (double-strand break factor 1), that is required for meiotic DSB formation in C. elegans. dsb-1 mutants lack meiotic DSBs, and show meiotic defects similar to spo-11 mutants. I also show that DSB-1 localizes to meiotic chromosomes coincident with the time of DSB formation, in a manner dependent on the CHK-2 kinase.
Results

Identification of dsb-1, a novel gene required for DSB formation

In *C. elegans*, mutations that impair meiotic chromosome segregation result in embryonic lethality and a high incidence of males (XO) among the surviving progeny (Hodgkin et al., 1979). The *dsb-1(we11)* mutant was isolated in a genetic screen for maternal-effect embryonic lethality, and was found to produce a high fraction of males among its few surviving self-progeny. A targeted deletion allele of the affected gene, *dsb-1(tm5034)*, was generated independently (see below), and results in defects identical to *dsb-1(we11)* based on all assays described here. Whereas self-fertilizing wild-type hermaphrodites produce nearly 100% viable progeny and 0.2% males (Figure 1.1A, Hodgkin et al., 1979), only 3% of progeny from self-fertilizing *dsb-1* mutant hermaphrodites survived to adulthood (n > 2000; 12 broods), (Figure 1.1A, Table 1.1). Among these survivors, 36-38% were male (Figure 1.1A, Table 1.1). The brood size (number of fertilized eggs) of self-fertilizing *dsb-1* hermaphrodites was also reduced relative to wild-type animals (Table 1.1).

Chromosome segregation errors in meiosis often reflect defects in crossover formation between homologs. The levels of embryonic lethality and male progeny observed in *dsb-1* mutants are quantitatively similar to several previously characterized mutants that fail to make any crossovers during meiotic prophase, such as *spo-11* (Figure 1.1A, Table 1.1), *msh-5*, and *cosa-1* (Dernburg et al., 1998; Kelly et al., 2000; Yokoo et al., 2012), suggesting that *dsb-1* mutants might also lack crossovers. Visualization of DAPI-stained oocytes at diakinesis provides a simple assay for crossover formation in *C. elegans*. In wild-type hermaphrodites, 6 DAPI-staining bodies are observed in each oocyte nucleus (average = 5.8, Figure 1.1B and 1.1C), corresponding to 6 pairs of homologous chromosomes, each held together by a chiasma (Villeneuve, 1994). In mutants that fail to make crossovers, oocytes typically display 12 DAPI-staining bodies. The number and morphology of DAPI-staining bodies observed in *dsb-1* mutant oocytes was similar to *spo-11* mutants (average = 11.6, Figure 1.1B and 1.1C), indicating an absence of chiasmata in *dsb-1* animals.

We investigated whether the disruption of crossover formation in *dsb-1* mutants might reflect a defect in homologous chromosome pairing or synapsis. Pairing was assessed using fluorescence in situ hybridization (Figure 1.1D). Early pachytene nuclei of both wild-type and *dsb-1* animals contained a single focus or closely apposed pair of foci, indicating that homologous chromosomes were paired (Figure 1.1D). Further, co-staining of the axial element protein HTP-3 and the synaptonemal complex central region protein SYP-1 indicated that chromosomes were fully synapsed by early pachytene in *dsb-1* animals (Figure 1.1E), as in wild-type animals. These results indicate that *dsb-1* mutants are proficient for homologous chromosome pairing and synapsis.

To assess whether *dsb-1* mutants initiate meiotic recombination, we used antibodies against the DNA strand-exchange protein RAD-51, which binds to single-stranded regions adjacent to resected DSBs (Pâques and Haber, 1999; Sung, 1994), as a cytological marker of recombination intermediates (Alpi et al.,
2003; Colaiácovo et al., 2003). Whereas wild-type oocytes in early pachytene showed abundant RAD-51 foci, dsb-1 gonads lacked RAD-51 staining (Figure 1.2A), indicating either failure to form DSBs or failure to load RAD-51. However, the lack of fragmented chromosomes at diakinesis seemed more consistent with an absence of DSBs.

To verify that dsb-1 mutants are defective in DSB formation, and to rule out the possibility of defects in the loading of RAD-51 or downstream steps of the recombination pathway, we tested whether exogenous DSBs could rescue the recombination defects observed in dsb-1 mutants. The same approach established a role for Spo11/SPO-11 in DSB formation (Dernburg et al., 1998; Thorne and Byers, 1993). Young adult dsb-1 mutant hermaphrodites were exposed to 10 Gy of gamma rays, a dose that has previously been shown to efficiently rescue crossovers in spo-11 mutants with minimal associated lethality (Hayashi et al., 2007). Wild-type and spo-11 controls were performed in parallel. At appropriate times after irradiation the animals were assessed for RAD-51 foci, chiasmata, and progeny viability. At 2 hours post irradiation, dsb-1 animals displayed abundant RAD-51 foci (Figure 1.2B), indicating that the mutants are proficient for resection and RAD-51 loading. At 18 hours post irradiation, both spo-11 and dsb-1 oocytes showed ~6 DAPI-staining bodies (Figure 1.2C and 1.2D). Additionally, the viability of embryos laid 20-30 hours post irradiation increased significantly for both spo-11 and dsb-1 animals, but decreased slightly for wild-type, compared to unirradiated controls (Figure 1.2E). The ability of exogenous DSBs to rescue the recombination defects of dsb-1 animals indicates that these mutants are specifically defective in meiotic DSB formation.

The defects observed in dsb-1 mutant hermaphrodites are virtually indistinguishable from spo-11(me44) mutants, except that mutations in dsb-1 were associated with reduced brood size (Table 1.1). Although dsb-1(we11) showed linkage to the middle of Chromosome IV, close to the spo-11 locus, complementation tests revealed that we11 is not an allele of spo-11. Quantitative RT-PCR also indicated that spo-11 mRNA levels were unaffected in dsb-1(we11) mutants (Figure 1.3).

dsb-1 encodes a member of a novel protein family

Whole-genome sequencing of backcrossed dsb-1(we11) animals identified several mutations in annotated coding sequences, including a nonsense mutation in the previously uncharacterized gene F08G5.1 (Figure 1.4A), which encodes a predicted protein of 385 amino acids and seemed a plausible candidate based on its meiosis-enriched expression pattern (Kim et al., 2001). We found that knockdown of F08G5.1 expression via transgene-mediated cosuppression (Dernburg et al., 2000) caused embryonic lethality and male progeny, as well as strong reduction of chiasmata, in the oocytes of treated animals (data not shown), supporting the hypothesis that the we11 mutation affects this gene. we11 introduces a premature stop (tca => taa) after leucine 96 (Figure 1.4A). A targeted deletion allele (tm5034) removes 290 bp from predicted exons 3 and 4 and the intervening intron (Figure 1.4A), resulting in a frameshift mutation that introduces a glutamine immediately followed by a stop codon after
leucine 96. The phenotype of dsb-1(tm5034) mutants is indistinguishable from dsb-1(we11) (Figure 1.1 and 1.2, Table 1.1). Both are predicted to lack functional protein based on the early stop codons, and this conclusion is supported by immunofluorescence and immunoblotting experiments (below). Based on the evidence described above that mutations disrupting F08G5.1 specifically interfere with meiotic double-strand break formation, we designated F08G5.1 as dsb-1, for double-strand break factor 1.

The DSB-1 protein has no apparent homologs outside of the genus Caenorhabditis, including other nematode genera. Interestingly, the genomes of C. elegans and several other Caenorhabditids each contain 2 predicted paralogs. Rosu et al. show that dsb-1 paralog F26H11.6/dsb-2 is also involved in meiotic DSB formation in C. elegans (Rosu et al., 2013). DSB-1, DSB-2, and their homologs cluster into two paralogous groups (Figure 1.4B). Even within Caenorhabditis, members of this protein family are not well conserved (Figure 1.5).

DSB-1 lacks identifiable domains that might give clues about its function in DSB formation. One notable feature is its high serine content: 60 of 385 amino acids (16%) are serine residues, compared to an average serine content of 8% encoded by all C. elegans ORFs (Echols et al., 2002). Protein structure prediction algorithms indicate that each end of DSB-1 may form alpha-helix secondary structures, but the central portion of the protein, which is especially serine-rich, is predicted to be largely unstructured. This central region is also the least conserved portion of the protein (Figure 1.5). Five serine residues within the central region are followed by glutamine, making them candidate phosphorylation targets for ATM or ATR DNA damage kinases. These clustered ATM/ATR consensus motifs are shared by other DSB-1 homologs, including DSB-2.

**DSB-1 localizes to chromosomes during early meiotic prophase**

To further probe the role of DSB-1 during meiosis, I generated tagged constructs of DSB-1 and integrated them into the C. elegans genome at a specific site on chromosome II (ttTi5605) using the single-insertion transposon-based mosSCI system (Frøkjær-Jensen et al., 2008). DSB-1 was tagged with either a 3xFLAG peptide or a GFP-strep tag on either the C- or N-terminus. For the majority of constructs made, dsb-1 expression was driven by its endogenous promoter and 3'UTR. Integrated transgene lines were outcrossed, then crossed to dsb-1(we11) to test for rescue of the mutant phenotype. Lines where dsb-1 expression was driven under its endogenous promoter largely rescued the dsb-1(we11) phenotype, displaying a 84% to 98% viability, compared to 100% viability for wild-type animals (Table 1.2). An N-terminally tagged DSB-1 was expressed under the htp-3 promoter: dsb-1(ie24)[phtp-3::GFP-strep-dsb-1]. However, this transgene was unable to substantially rescue the dsb-1(we11) mutant phenotype, and therefore was not used for further experiments.

The transgenic DSB-1 tagged lines were used to examine the localization of DSB-1 during meiosis. C- and N-terminally 3xFLAG tagged DSB-1, as well as C-terminally GFP-strep tagged DSB-1 lines all showed the same pattern of DSB-1 localization when stained with either anti-FLAG or anti-GFP antibodies,
respectively. DSB-1 localized to meiotic chromosomes beginning at the onset of meiotic prophase, upon entry into the “transition zone” (leptotene/zygotene), and persisted on chromosomes until the mid-pachytene region of the gonad (data not shown).

In addition to the tagged constructs, I also generated an antibody against the full-length protein expressed in *E. coli*. Immunofluorescence staining revealed the same pattern of DSB-1 localization as was observed with the tagged DSB-1 transgenes. DSB-1 was absent from somatic nuclei, and specifically localized to chromosomes during early meiotic prophase (Figure 1.6A and 1.6B), while *dsb-1* mutants showed only background staining (Figure 1.7). Accumulation of DSB-1 on chromosomes was first observed in nuclei marked by crescent-shaped DAPI-staining morphology, corresponding to the transition zone, and disappeared at mid-pachytene (Figure 1.6A). Chromosomal localization of DSB-1 preceded the appearance of RAD-51 foci, consistent with an early role for DSB-1 in meiotic recombination (Figure 1.6A). Thus, the localization of DSB-1 to chromosomes corresponds to the period during which DSBs are likely to be generated.

While most nuclei in the late pachytene region of the germline lacked DSB-1 staining, we consistently observed dispersed nuclei in this region that retained bright fluorescence (Figure 1.6A and 1.8). These nuclei also contained abundant RAD-51 foci and frequently displayed compact chromosome morphology resembling that seen in the transition zone, along with evidence of asynapsed chromosomes (Figure 1.8A and 1.8B). We tested whether these late DSB-1 positive nuclei might be apoptotic by examining *ced-4* mutants, which lack germline apoptosis (Bhalla and Dernburg, 2005; Gumienny et al., 1999), and found that they were still present in similar numbers (data not shown). The persistence of RAD-51 foci and asynapsed chromosomes suggest that these nuclei may be delayed in completing synapsis or other prerequisites for crossover formation, a conclusion reinforced by further analysis of DSB-1 regulation, described in Chapter 2.

DSB-1 was distributed as a network of foci and stretches of staining on meiotic chromosomes (Figure 1.6B). One chromosome consistently showed weaker DSB-1 staining. This seemed likely to be the X chromosome, which has many unique features in the germline, including distinct chromatin marks (Kelly et al., 2002; Schaner and Kelly, 2006) and genetic requirements for meiotic DSBs (Meneely et al., 2012; Wagner et al., 2010). Co-staining with antibodies against HIM-8, which specifically mark the X chromosome (Phillips et al., 2005), confirmed that the chromosome pair with weaker DSB-1 staining was the X (Figure 1.6C).

DSB-1 and RAD-51 both localized to chromosomes during early pachytene (Figure 1.6A). However, we found that RAD-51 did not colocalize with DSB-1 (Figure 1.6D). Similar findings have been reported for DSB proteins in mice and *Schizosaccharomyces pombe* (Kumar et al., 2010; Lorenz et al., 2006). This could indicate that DSB-1 does not act directly at DSB sites, or that it is removed from DSB sites prior to RAD-51 loading.

Meiotic chromosomes are believed to be organized as chromatin loops tethered at their bases to the proteinaceous chromosome axis (Moens et al., 2002).
Based on work from *S. cerevisiae*, it has been proposed that DSBs occur at sites within chromatin loops that are recruited to the chromosome axis (Blat et al., 2002; Panizza et al., 2011; Storlazzi et al., 2003). Most DSB-1 staining was associated with the periphery of DAPI-stained chromosomes rather than axes (Figure 1.6E), suggesting that DSB-1 is primarily associated with chromatin loops. This localization pattern is similar to what has been observed for several DSB proteins in *S. cerevisiae* (Arora et al., 2004; Henderson et al., 2006; Kee et al., 2004; Li et al., 2006; Maleki et al., 2007).

**Chromosome localization of DSB-1 is independent of SPO-11, the MRN complex, and a properly assembled meiotic chromosome axis**

I tested whether DSB-1 localization depends on other factors required for DSB formation. I crossed transgenic lines carrying the 3xFLAG-tagged DSB-1 (*ieSi23*) to *spo-11*, *mre-11*, *rad-50*, and *htp-3* mutants and examined the dependency of DSB-1-3xFLAG localization on the corresponding genes. DSB-1-3xFLAG localization appeared normal in *spo-11*, *mre-11*, and *rad-50* mutants. In *htp-3*(tm3655) mutants, DSB-1-3xFLAG localization was highly reduced, suggesting the DSB-1 localization is dependent on CHK-2 and HTP-3. These experiments were then repeated using the DSB-1 antibody to examine the localization of DSB-1 in various mutants by direct immunofluorescence. DSB-1 localized to meiotic chromosomes in various mutants by direct immunofluorescence. DSB-1 localized to meiotic chromosomes in the catalytically dead *spo-11*(me44) mutant (Hayashi et al., 2007), as well as in *spo-11*(ok79) mutants, which lack functional protein (Dernburg et al., 1998), indicating that DSB-1 localizes to chromosomes independently of DSBs and SPO-11 (Figure 1.9). DSB-1 localization was also independent of MRE-11 and RAD-50 (Figure 1.9, data not shown), which are required for DSB formation in *C. elegans* (Chin and Villeneuve, 2001; Hayashi et al., 2007). In *htp-3* mutants, which lack an essential axial element component that is important for DSB formation (Goodyer et al., 2008), DSB-1 was detected on meiotic chromosomes (Figure 1.9), but the staining appeared somewhat reduced compared to wild-type nuclei.

**Chromosome localization of DSB-1 is dependent on the meiotic kinase CHK-2**

The CHK-2 kinase is essential for several key events during early meiotic prophase in *C. elegans*, including DSB formation and homolog pairing (MacQueen and Villeneuve, 2001). I found that nuclear staining of DSB-1 was strongly reduced, albeit still detectable, in *chk-2*(me64) mutants (Figure 1.9). These immunofluorescence experiments were done using the DSB-1 antibody, as well as the *dsb-1*(ieSi23) 3xFLAG-tagged transgene. Although the intensity of DSB-1 staining was sharply reduced in *chk-2* mutants, it appears that the faint fluorescence observed upon prolonged exposure reflects DSB-1 localization, because the staining pattern resembles that seen in wild-type animals, and because chromosomal staining is not detected prior to meiotic entry. Western blot analysis revealed that DSB-1 protein is expressed in *chk-2* mutants, although the protein levels appear somewhat reduced compared to wild-type (data not shown). However, the reduction in DSB-1 protein levels in *chk-2*...
mutants does not appear to fully account for the sharply diminished chromosomal localization of DSB-1. These data indicate that DSB-1 localization to chromosomes is largely dependent on the CHK-2 kinase, and suggest that DSB-1 may act downstream of CHK-2 to promote DSBs.

Attempts to characterize the genomic distribution of DSB-1 localization

To better understand the relationship between the localization of DSB-1 and its role in promoting meiotic DSBs, I carried out chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-seq) experiments to characterize the genomic distribution of DSB-1 binding. ChIP-seq experiments were done on transgenic worms carrying either a 3xFLAG tagged or GFP-strep tagged DSB-1, or on wild-type N2 worms, using either anti-FLAG, anti-GFP, or anti-DSB-1 antibodies, respectively. Nuclear extracts were prepared from synchronized, young adult worms with the standard protocol developed for the modENCODE project using either formaldehyde or formaldehyde and EGS (ethylene glycol bis[succinimidylsuccinate]) as a crosslinking agent. Illumina high-throughput sequencing was used to sequence DNA that was pulled down from the ChIP experiments. After mapping reads to the genome and background subtracting, no reproducible or obvious pattern was observed from the data, indicating that the ChIP-seq experiments were not successful.

Probing the protein-protein interactions of DSB-1 to identify additional factors required for DSB formation

To better understand the role of DSB-1 in promoting DSBs I wanted to determine the protein factors that interact with DSB-1. I tested protein interactions between DSB-1 and other protein factors known to promote DSBs in *C. elegans* using yeast two-hybrid (Y2H) assays. Y2H was used to test pairwise interactions between DSB-1 and SPO-11, MRE-11, RAD-50, HTP-3, and DSB-2. Pairwise interactions were tested using either the full-length DSB-1, the N-terminal half (amino acids 2-192) or the C-terminal half (amino acids 193-385) of DSB-1. Truncated versions of SPO-11 (amino acids 2-162 or 2-286) were also used to test interactions with full-length or truncated DSB-1, or full-length DSB-2. No pairwise interaction was reproducibly detected using this assay. A Y2H screen was also preformed using DSB-1 as bait and cDNA library cloned into the prey vector (prepared by Aya Sato). However no positive interactions were detected. The inability to detect protein interactions using Y2H may be due to the fact that DSB-1 is phosphorylated during meiosis in worms and not in yeast (see Chapter 3).

In addition to Y2H experiments, immunoprecipitation coupled to mass spectrometry was also attempted in order to identify protein interactors of DSB-1. These experiments were carried out with nuclear extracts from synchronized adult worms carrying C-terminal 3xFLAG tagged or GFP-strep tagged DSB-1 transgenes. GFP-tagged DSB-1 was successfully immunoprecipitated using GFP-binding protein. Samples were submitted to the QB3 facility in Stanley Hall in for 2-D mass spec analysis. Although samples had been treated with benzonase to eliminate nucleic acids during the immunoprecipitation procedure,
mass spec analysis found that the sample was contaminated with large amounts of nucleic acid. Due to the poor quality of the mass spec data only a few proteins were identified including actin, myosin, and related proteins. Subsequent attempts to immunoprecipitate GFP-tagged DSB-1 failed. Immunoprecipitation 3xFLAG-tagged DSB-1 was also inefficient. Mass spectrometry of an excised band, running the expected sizes of DSB-1, from a silver stain gel of immunoprecipitation elutions found actin and myosin, and no trace of DSB-1 peptides. All attempts to identify protein factors that interact with DSB-1 have failed.

**Functional relationships between DSB-1 and DSB-2**

The DSB-1 paralog DSB-2 is also involved in meiotic DSB formation (Rosu et al., 2013). As reported by Rosu et al., the two proteins show very similar localization patterns (Figure 1.10A and 1.10B, (Rosu et al., 2013)). Both localize to nuclei from leptotene/zygotene through mid pachytene, although DSB-1 staining appears slightly earlier than DSB-2 staining (Figure 1.10A). They also disappear simultaneously from meiotic chromosomes, both in wild-type animals and various mutants that disrupt crossover formation (Figure 1.10A, data not shown). Additionally, both proteins show similar distributions along meiotic chromosomes (Figure 1.10B). Intriguingly, however, the two proteins do not extensively colocalize, but instead rarely coincide (Figure 1.10B).

To probe the functional interactions between DSB-1 and DSB-2, we localized each protein in the absence of the other. We found that DSB-1 localized to chromosomes in dsb-2(me96) mutants, although the fluorescence intensity was reduced relative to wild-type gonads (Figure 1.11A and 1.11B; see also (Rosu et al., 2013)). The DSB-1 positive region of the gonad was also somewhat shorter (Figure 1.11A), despite the reduction of crossovers in dsb-2 mutants (Rosu et al., 2013). This suggests that localization of DSB-1 to meiotic chromosomes does not require, but may be reinforced or stabilized by, DSB-2. By contrast, DSB-2 was not detected on meiotic chromosomes in dsb-1 mutants (Figure 1.11B). Immunoblotting of whole-worm lysates revealed that DSB-1 protein levels are moderately reduced in dsb-2 mutants, while DSB-2 protein levels are severely reduced in dsb-1 mutants (Figure 1.11C). This parallels our conclusions from *in situ* localization of these proteins, and suggests that the reduction of staining observed on chromosomes is a consequence of lower protein levels.

We also tested the effect of eliminating both DSB-1 and DSB-2 by constructing a double mutant strain. The phenotypes observed in dsb-1; dsb-2 mutant animals were indistinguishable from dsb-1 mutants (Figure 1.12A and 1.12B). This result is consistent with the idea that these proteins collaborate in some way to promote DSB formation, and argues against more complex epistasis scenarios.
Discussion

**DSB-1 and DSB-2 mediate initiation of meiotic recombination**

We have discovered a novel protein, DSB-1, required for meiotic DSB formation in *C. elegans*. Our data indicate that DSB-1 acts specifically to promote DSBs, and does not play a major role in DNA repair or other meiotic processes. DSB-1 localizes to chromosomes during meiotic prophase, concomitant with the period of DSB formation. It appears more abundant on the autosomes than the X chromosome. The significance of this finding is unclear, since DSB-1 is clearly required for DSBs on all chromosomes, but it may be related to observations that the X chromosome has distinct chromatin structure and differential genetic requirements for DSB formation (Kelly et al., 2002; Meneely et al., 2012; Schaner and Kelly, 2006; Wagner et al., 2010).

Both DSB-1 and its paralog DSB-2 are required for normal levels of meiotic DSBs. These proteins show a similar temporal and spatial pattern of localization to meiotic chromosomes. The localization of both proteins is also extended to a similar extent in mutants that disrupt crossover formation. In mutants where the localization of both DSB-1 and DSB-2 was assayed simultaneously, as well as in wild-type animals, the proteins localize to the same subset of meiotic nuclei, except that DSB-1 appears slightly earlier, suggesting that they are co-regulated. However, these proteins seem unlikely to act as a complex, since they show little if any colocalization.

Although DSB-1 and DSB-2 appear to play similar roles in meiotic DSB formation, the severity of their mutant phenotypes are not equivalent. As shown by Rosu et al., DSBs are reduced but not eliminated in young *dsb-2* mutant hermaphrodites (Rosu et al., 2013), while *dsb-1* mutants lack DSBs regardless of age. The less severe defects observed in young *dsb-2* mutants likely reflect the presence of substantial residual DSB-1 protein on meiotic chromosomes in *dsb-2* mutants, whereas DSB-2 is not detected on chromosomes in *dsb-1* mutants, and protein levels are severely reduced. DSB-1 appears to stabilize DSB-2, perhaps by promoting its association with chromosomes, and to a lesser extent is reciprocally stabilized/reinforced by DSB-2.

The CHK-2 kinase promotes the chromosomal association of DSB-1. CHK-2 is also required for DSB-2 localization on meiotic chromosomes (Rosu et al., 2013), although it is not clear whether CHK-2 promotes DSB-2 loading directly, or indirectly through its role in the loading of DSB-1. Our findings suggest a model in which DSB-1 and DSB-2 mutually promote each other’s expression, stability, and/or localization, with DSB-2 depending more strongly on DSB-1, to promote DSB formation (Figure 1.12C).

The number of sites of DSB-1 and DSB-2 localization per nucleus – too many to quantify in diffraction-limited images – appears to greatly exceed the number of DSBs, estimates of which have ranged from 12 to 75 per nucleus (Hayashi et al., 2010; Mets and Meyer, 2009; Saito et al., 2012). DSB-1 and DSB-2 may each bind to sites of potential DSBs, with only a subset of these sites undergoing DSB formation, perhaps where they happen to coincide. They could also be serving as scaffolds to recruit other factors required for DSB formation to...
meiotic chromosomes and/or to promote their functional interaction. This idea is currently difficult to test, since we have not yet been able to detect chromosomal association of SPO-11 in C. elegans, and no other proteins specifically required for DSBs have been identified. Alternatively, these proteins may influence DSB formation by modifying chromosome structure. We did not observe overt changes in chromosome morphology in dsb-1 mutants, but further analysis — e.g., mapping of histone modifications — may be necessary to uncover more subtle changes.

Rapid divergence among DSB-promoting proteins

Proteins with apparent homology to DSB-1 are restricted to the Caenorhabditis lineage. Even within Caenorhabditids, DSB-1, DSB-2 and their homologs are only weakly conserved. This reinforces abundant evidence from other organisms that apart from Spo11 itself and the Rad50-Mre11 complex, proteins that promote DSB formation diverge rapidly during evolution (Keeney, 2008; Kumar et al., 2010; Richard et al., 2005). This might seem surprising given that meiotic DSB formation is an essential aspect of sexual reproduction in most eukaryotes. However, potent evolutionary pressures are likely to act on meiosis.

The genome-wide distribution of DSBs appears to underlie the strongly biased distribution of crossovers observed in many species (Buhler et al., 2007; Kauppi et al., 2004; Petes, 2001), including C. elegans (Mets and Meyer, 2009; C. V. Kotwaliwale and AFD, unpublished). The nature of this biased distribution shows interesting variation among species (Borde and de Massy, 2013; Lichten and de Massy, 2011). Since crossover number and position have a direct impact on the fidelity of meiotic chromosome segregation, mechanisms governing DSB distribution have likely evolved in concert with changes in chromosome structure and the spindle apparatus to maintain reproductive fitness.

Several features of meiosis in C. elegans distinguish it from other organisms in which DSB-promoting factors have been identified. In particular, DSBs and early recombination steps contribute directly to homolog pairing and synapsis in many species, while in C. elegans homolog pairing and synapsis occur independently of DSBs. Additionally, C. elegans lacks Dmc1, Hop2, and Mnd1, which are thought to function together as an essential meiotic recombination module in most eukaryotes (Malik et al., 2008). C. elegans also lacks the DSB proteins Mei4 and Rec114, which are conserved between budding yeast and mice (Kumar et al., 2010). A correlation between the absence of DMC1/Hop2/Mnd1 and Mei4/Rec114 has been noted in several other lineages, and has been suggested to reflect a functional link between the formation of DSBs and their subsequent repair (Kumar et al., 2010). Interestingly, Rec114, like DSB-1/2, has several potential target sites for ATM/ATR phosphorylation (described in Chapter 3), and these are important for regulation of DSBs in budding yeast meiosis (Carballo et al., 2013). Thus, the DSB-1/2 family of proteins may play analogous roles to known mediators of DSB formation in other species, despite their lack of apparent sequence similarity.
Materials and Methods

**C. elegans Mutations and Strains.** All C. elegans strains were cultured under standard conditions at 20°C. The wild-type strain was N2 Bristol. The nonsense *dsb-1(we11)* allele was generated by EMS mutagenesis. The *dsb-1* deletion allele (*tm5034*) was generated by the Japanese National BioResource for the Nematode. Both *dsb-1* alleles were extensively outcrossed to wild-type (5-6x), and additionally outcrossed in a directed three-point cross to *dpy-20 unc-30* to eliminate most linked mutations. Additional mutants analyzed in this study were: *spo-11(me44, ok79), mre-11(ok179), rad-50(ok197), chk-2(me64), htp-3(tm3655), and ced-4(n1162).* Strains used in this study were:

- CA1104 *dsb-1(we11) IV/nT1[unc-30(n754) let-230](IV;V)*
- CA1105 *dsb-1(tm5034) IV/nT1[unc-30(n754) let-230](IV;V)*
- CA279 *spo-11(me44)/+IV*
- CA276 *spo-11(ok79)/+IV*
- CA1109 *mre-11(ok179) V/nT1[unc-30(n754) let-230](IV;V)*
- AV158 *rad-50(ok197) V/nT1[unc-30(n754) let-230 qls50](IV;V)*
- CA1110 *chk-2(me64) rol-9(sc148)/+V*
- CA821 *htp-3(tm3655) I/hT2[bli-4(e937) let-230 qls48](I;III)*
- CA1087 *ced-4(n1162) III*
- DP38 *unc-119(ed3) III*
- AV477 *dsb-2(me96) II*
- AV539 *rol-1(e91) dsb-2(me96)/+II*
- AV539 *rol-1(e91) dsb-2(me96)/+II*
- AV539 *rol-1(e91) dsb-2(me96)/+II*
- AV539 *rol-1(e91) dsb-2(me96)/+II*
- CA1111 *rol-1(e91) dsb-2(me96)/+II*
- CA1111 *rol-1(e91) dsb-2(me96)/+II*
- CB91 *rol-1(e91) II*

**Quantification of Viability and Male Progeny.** L4 hermaphrodites were picked onto individual plates and transferred to new plates every 12 hours, for a total of 6-8 12-hour laying periods, until newly-laid fertilized eggs were no longer observed. Eggs were counted immediately after each 12-hour laying period. Surviving hermaphrodite and male progeny were counted 3 days later.

**Immunofluorescence and Cytological Analysis.** Polyclonal antibodies against recombinant full-length DSB-1 protein were produced at Pocono Rabbit Farm & Laboratory. 6xHis-DSB-1 was purified from *E. coli* using Ni beads under denaturing conditions. The protein was resolved on an SDS-PAGE gel and the excised DSB-1 band was used to immunize guinea pigs. Rabbit anti-HTP-3 antibodies were raised against a synthetic peptide (PTEPASPVESPVKEQPQKAPK) by Strategic Diagnostics Inc., SDIX. Additional antibodies used in this study were: guinea pig anti-HTP-3 (MacQueen et al., 2005), rat anti-HIM-8 (Phillips et al., 2005), rabbit anti-RAD-51 (Harper et al., 2011), goat anti-SYP-1 (Harper et al., 2011), and rabbit anti-DSB-2 (Rosu et al., 2013), mouse anti-FLAG (Genescript, A00187), and mouse anti-AFP.

Immunofluorescence was performed as previously described (Phillips et al., 2009). Briefly, hermaphrodites 24-28 hours post L4 were dissected in egg buffer containing sodium azide and 0.1% Tween 20, fixed for 3 min in 1%
formaldehyde in the same buffer between a Histobond slide and coverslip, and frozen on dry ice. The coverslip was removed, and slides were transferred to methanol chilled to -20°C. After 1 min, slides were transferred to PBST (PBS containing 0.1% Tween 20), washed in two further changes of PBST, blocked with Roche blocking agent, and stained with primary antibodies in block for 2 hours at room temperature or overnight at 4°C. Slides were then washed with 3 changes of PBST and stained with secondary antibodies. Secondary antibodies labeled with Alexa 488, Cy3, or Cy5 were purchased from Invitrogen or Jackson ImmunoResearch. Following immunostaining, slides were washed, stained in 0.5 mg/ml DAPI, destained in PBST, and mounted in buffered glycerol-based mounting medium containing 4% n-propyl gallate as an antifading agent.

For quantification of DAPI-staining bodies in oocytes, animals were dissected, fixed, and DAPI-stained as described above, omitting the steps involving immunostaining.

FISH procedures have also been previously described in detail (Phillips et al., 2009). Probes used in this study included the 5S rDNA repeat (Dernburg et al., 1998) and a short repeat associated with the right end of the X chromosome (Phillips et al., 2005).

All images were acquired using a DeltaVision RT microscope (Applied Precision) equipped with a 100x 1.40 oil-immersion objective (Olympus) or (for whole gonad images) a 60x 1.40 oil-immersion objective (Olympus). Image deconvolution and projections were performed with the softWoRx software package (Applied Precision). Image scaling, false coloring, and composite image assembly were performed with Adobe Photoshop. All micrographs presented in the figures are maximum-intensity projections of 3D data stacks.

Irradiation Experiments. Young adult worms were irradiated with approximately 10 Gy (1000 rad) from a Cs-137 source. For each experiment, unirradiated controls were treated identically to irradiated animals, other than exposure to radiation. For quantification of DAPI-staining bodies at diakinesis, hermaphrodites were irradiated 4-5 hours post L4 and dissected 18 hours post irradiation. To assess progeny survival, animals were irradiated 4-5 hours post L4, eggs laid 20-30 hours post irradiation were quantified, and surviving progeny were quantified 3 days later. For RAD-51 immunofluorescence, animals were irradiated 24 hours post L4 and dissected 1 hour post irradiation.

Whole Genome Sequencing of we11. 1000 homozygous we11 animals were picked from an outcrossed, balanced strain. A genomic DNA library was prepared as described in the genomic DNA library protocol from Illumina. Libraries were sequenced using 76-bp single-end Illumina sequencing. MAQGene (Bigelow et al., 2009) was used to identify mutations present in the we11 mutant strain.

Germline Cosuppression. A 2.1-kb region of genomic DNA including the dsb-1 coding sequence and promoter was amplified by PCR using the following primers: 5'-CCGCTTCCGAATACCGCC-3' and 5'-GGTGCCTGTGTAGAAGAAGC-3'. 100 ng/µl of dsb-1 PCR product was combined with 50 ng/µl of unc-119 rescuing plasmid pMM051 (Maduro and Pilgrim, 1995) and injected into unc-119 animals. Rescued non-Unc F1 animals
were picked to individual plates and assayed for embryonic lethality and male progeny. F2 animals were dissected, stained, and observed to quantify the number of DAPI-staining bodies in oocytes at diakinesis.

**Quantitative RT-PCR.** 12 young adult animals, 24 hours post L4, were used for each genotype. RNA was purified from animals and reverse transcribed into cDNA with the SuperScript kit from Invitrogen using poly-A primers. *spo-11* mRNA levels were compared by real-time PCR analysis with SYBR Green (Kapa Biosystems). *act-1* and *htp-3* mRNA levels were used as normalization controls. Primers used were as follows: *spo-11* (5'-TGAGCCCGGATCTGTAGAAT-3', 5'-TAGCTTGTTCCTTCGGTGGT-3'), *act-1* (5'-CCCCATCAACCATGAAGATC-3', 5'-TCTGTTGGAAGGTGAGGAG-3'), and *htp-3* (5'-CGAGTGATGACAGGGCTATATTC-3', 5'-TGCAAGATAACGCAGTTGG-3').

**MosSCI.** Single-copy genomic insertions of transgenes were generated using the transposon-based MosSCI protocol developed by the Jorgensen lab. The strain EG6249 that carries the *ttTi5605* mos site on chromosome II was used for injections to generate all strains. Mos insertion strains were then outcrossed to *unc-119(ed3)* animals.

**Yeast Two-Hybrid.** Yeast two-hybrid assays were done using the Invitrogen ProQuest Two-Hybrid System according to standard protocol. Gateway vectors pDEST32 and pDEST22 were used to clone the bait and prey hybrid fusion proteins. A cDNA library cloned by Aya Sato was used for the Y2H screen.

**Immunoblotting.** Lysate from 50 young adult hermaphrodites, picked at 24 hours post L4, was used for each lane. Gel electrophoresis was performed using 4-12% Novex NuPage gels (Invitrogen). Proteins were transferred to PVDF membrane. Guinea pig DSB-1 antibodies and rabbit DSB-2 antibodies (see above) were used for immunoblotting, followed by detection with HRP-conjugated secondary antibodies and ECL Western Blotting Substrate (Pierce).

**Preparing nuclei extract.** Worms were grown in liquid culture at 20 °C. Worms were synchronized by bleaching cultures with gravid adults to collect embryos, allowing embryos to hatch into liquid culture lacking food, and then feeding the culture with OP50 *E. coli*. Young adult worms were then harvested and flash froze in liquid nitrogen to make 'popcorn'. Worm popcorn was stored at -80 °C until used to make nuclear extracts. Frozen worms were broken up by grinding in the mixer mill for 2-3 minutes total. Worms were then thawed an immediately fixed in formaldehyde. Tissue was further broken up by treating with a Teflon-coated dounce homogenizer. Large pieces of worm tissue were removed by successive low-speed spins. Nuclei were then pelleted and resuspended in buffer appropriate for either ChIP-seq of IPs. Nuclei were lysed either by sonication in the biorupter (for ChIP-seq, to also break up chromatin) or by a tip sonication (for IPs). Samples used for IP were treated with benzonase to remove DNA.

**ChIP-seq.** ChIP-seq was done according the protocol developed by Andrea Dose for the modENCODE project. Standard formaldehyde crosslinking was used, as well as a dual crosslinking protocol with both formaldehyde and
EGS. Antibodies used were guinea pig anti-DSB-1, mouse anti-FLAG (Genescript, A00187), rabbit anti-FLAG (Sigma F7425), and GFP binding protein.

**IP/mass-spec.** DSB-1 immunoprecipitations were preformed against either DSB-1-3xFLAG using Sigma anti-FLAG magnetic beads or against GFP-DSB-1 using ChromoTek GFP-trap magnetic beads. For anti-FLAG IPs, DSB-1-3xFLAG was eluted from the beads using FLAG peptide. For anti-GFP IPs, GFP-DSB-1 was eluted from the beads using 0.2 M Glycine, pH 2.5. IPed samples were analyzed by Western blot and silver stain. Mass spectrometry was carried out in the QB3 Vincent J. Coates Proteomics/Mass Spectrometry Laboratory in Stanley Hall by Lori Kohlstaedt.
Table 1.1. Progeny viability, incidence of males, and brood size from *dsb-1* and *dsb-2* mutants. Quantification of the viable and male self-progeny from hermaphrodites of indicated genotypes, as well as average brood size (fertilized eggs), is shown. The numbers in parentheses indicate: fertilized eggs (for viability), adult progeny (for male counts), and broods (for brood size) counted. Each of the two sets of experiments was performed in parallel under identical conditions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Viability (n)</th>
<th>% Males (n)</th>
<th>Brood Size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>102 (2933)</td>
<td>0.20 (2991)</td>
<td>285 (10)</td>
</tr>
<tr>
<td><em>spo-11(me44)</em></td>
<td>2.5 (4063)</td>
<td>37 (103)</td>
<td>343 (11)</td>
</tr>
<tr>
<td><em>dsb-1(we11)</em></td>
<td>3.2 (2142)</td>
<td>38 (68)</td>
<td>184 (12)</td>
</tr>
<tr>
<td><em>dsb-1(tm5034)</em></td>
<td>3.2 (2134)</td>
<td>36 (69)</td>
<td>178 (12)</td>
</tr>
<tr>
<td><em>rol-1</em></td>
<td>98 (1741)</td>
<td>0.06 (1705)</td>
<td>290 (6)</td>
</tr>
<tr>
<td><em>rol-1 dsb-2(me96)</em></td>
<td>29 (789)</td>
<td>15 (229)</td>
<td>263 (3)</td>
</tr>
<tr>
<td><em>dsb-1(tm5034)</em></td>
<td>3.7 (782)</td>
<td>38 (29)</td>
<td>196 (4)</td>
</tr>
<tr>
<td><em>rol-1 dsb-2(me96); dsb-1(tm5034)</em></td>
<td>3.8 (1500)</td>
<td>35 (57)</td>
<td>214 (7)</td>
</tr>
<tr>
<td>DSB-1 construct</td>
<td>Promoter</td>
<td>3'UTR</td>
<td>Allele name</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>WT</td>
<td>dsb-1</td>
<td>dsb-1</td>
<td>ieSi22</td>
</tr>
<tr>
<td>N-3xFLAG</td>
<td>dsb-1</td>
<td>dsb-1</td>
<td>-</td>
</tr>
<tr>
<td>C-3xFLAG</td>
<td>dsb-1</td>
<td>dsb-1</td>
<td>ieSi23</td>
</tr>
<tr>
<td>C-GFP-strep</td>
<td>dsb-1</td>
<td>dsb-1</td>
<td>-</td>
</tr>
<tr>
<td>N-GFP-strep</td>
<td>htp-3</td>
<td>dsb-1</td>
<td>ieSi24</td>
</tr>
</tbody>
</table>

**Table 1.2. DSB-1 MosSCI transgenic constructs.** All DSB-1 constructs were integrated into the *C. elegans* genome in single copy on Chromosome II using MosSCI. The expression of transgenes was driven by the corresponding promoters and 3'UTRs. Allele names are listed for those that were named. Quantification of the viable and male self-progeny from hermaphrodites carrying the indicated genotype crossed into the *dsb-1(we11)* mutant background are shown.
Figure 1.1. dsb-1 mutants lack meiotic crossovers but are proficient for homologous chromosome pairing and synopsis. (A) Quantification of viable and male self-progeny for the indicated genotypes is shown. Homozygous dsb-1(we11 and tm5034) hermaphrodites produce many inviable and male self-progeny compared to wild-type (WT) animals, similar to spo-11 hermaphrodites. For each bar, the upper number indicates the percentage, and the lower number in parentheses indicates the total number of fertilized eggs (for viability) or adult progeny (for males) counted. (B) Histogram showing the number of DAPI-
staining bodies observed in oocytes at diakinesis for the indicated genotypes. 100 oocytes were counted for each genotype. (C) Each panel shows a representative DAPI-stained oocyte nucleus at diakinesis for the indicated genotype. WT oocytes display 6 DAPI-staining bodies (bivalents), while *dsb-1* oocytes display 12 DAPI-staining bodies (univalents). (D) Fluorescence *in situ* hybridization against the 5S rDNA genomic locus (5SrDNA) and a locus on the right end of the X chromosome (Xrt) indicated that pairing is not disrupted in *dsb-1* mutants. (E) Synapsis was assessed by immunofluorescence staining of the axial element protein HTP-3 and central region protein SYP-1, components of the synaptonemal complex. Full colocalization of the two markers indicates fully synapsed chromosomes. Scale bars, 5 µm.
Figure 1.2. *dsb-1* is required to initiate meiotic recombination. (A, B) Immunofluorescence staining of RAD-51 was used as a cytological marker of early recombination intermediates in early pachytene nuclei in (A) untreated and (B) irradiated animals. Scale bar, 5 µm. (A) WT nuclei display abundant RAD-51; however, *dsb-1* mutants lack RAD-51 foci. (B) *dsb-1* mutants were gamma-irradiated (10 Gy) as young adults, and then assessed 2 hours later for the presence of RAD-51 foci. RAD-51 foci were present on chromosomes in *dsb-1* mutants after irradiation. (C, D) WT, *spo-11(me44)*, and *dsb-1* mutants were gamma-irradiated (10 Gy) as young adults. After 18 hours, irradiated and control animals were fixed, and the number of DAPI-staining bodies in oocytes at diakinesis was quantified. (C) Each panel shows DAPI-stained chromosomes in a single, representative oocyte at diakinesis for the indicated genotype. Similar to *spo-11* and WT, irradiated *dsb-1* mutants displayed 6 DAPI-staining bodies. Scale bar, 5 µm. (D) Quantification of the number of DAPI-staining bodies observed for each genotype in untreated and irradiated animals. The top number for each bar indicates the average number of DAPI-staining bodies and the number in parentheses indicates the number of oocytes counted. (E) WT, *spo-11*, and *dsb-1* mutants were gamma-irradiated (10 Gy) at 4-5 hours post L4. Eggs laid 20-30 hours post irradiation were counted for progeny survival. The top
number for each bar indicates the percent survival; the numbers in parentheses indicates the total number of eggs counted. As for spo-11, irradiation of dsb-1 mutants partially rescued progeny survival.
Figure 1.3. Mutation of *dsb-1* does not affect *spo-11* expression. Real-time PCR was used to measure the levels of *spo-11* mRNA in *dsb-1* mutants and WT animals. RNA was purified from age-matched young adult hermaphrodites at 24 hours post-L4. *spo-11* mRNA levels were normalized either to (A) *act-1* or (B) *htp-3* mRNA levels, both of which gave similar results.
Figure 1.4. *dsb-1* is a novel gene that belongs to a poorly conserved gene family. (A) Structure of the *dsb-1* gene (*F08G5.1*) indicating the 2 mutant alleles analyzed in this study: *we11* and *tm5034*. The *we11* allele introduces a premature stop at codon 97, while the *tm5034* deletion allele causes a frameshift that introduces one amino acid followed by a stop codon after lysine 96. (B) Phylogenetic tree of DSB-1 homologs in *C. elegans*, *C. briggsae*, *C. remanei*, and *C. japonica*. Each species shown contains two paralogs belonging to DSB-1 protein family. These proteins appear to fall into 2 paralogous groups: the DSB-1 group and the DSB-2 group.
Figure 1.5. Amino acid alignment of DSB-1 homologs. Global alignment of DSB-1 homologs from *C. elegans*, *C. briggsae*, *C. remanei*, and *C. japonica*. Two
genes with homology to DSB-1 and DSB-2 were identified in the genome of each species included here. Alignment was performed using Geneious Pro (Geneious alignment, Blosum62, default settings).
Figure 1.6. DSB-1 localizes to chromosomes during early meiotic prophase. (A) Composite projection image of a gonad from a WT animal showing DAPI and immunofluorescence staining for DSB-1 and RAD-51. Scale bar, 30 µm. (B) Partial projection image of pachytene meiotic nuclei from WT showing DAPI and immunofluorescence staining of DSB-1. Scale bar, 5 µm. (C) Immunofluorescence staining of DSB-1 and HIM-8 in early pachytene nuclei in WT. HIM-8 was used to mark the X chromosome (arrows). DSB-1 staining was reduced on the X chromosome. (D) Partial projection image of early pachytene nuclei in WT showing immunofluorescence staining of RAD-51 and DSB-1. Little colocalization was observed between DSB-1 and RAD-51. (E) Partial projection image of early pachytene nuclei from WT showing immunofluorescence staining of DSB-1, HTP-3, and DAPI. Only a subset of DSB-1 colocalized with chromosome axes, marked by HTP-3. Scale bar, 5 µm.
Figure 1.7. Validation of DSB-1 antibody specificity. Immunofluorescence staining of DSB-1 in early pachytene nuclei in *dsb-1* mutants. Only faint nonspecific background staining is observed. Scale bar, 5 µm.
Figure 1.8. DSB-1 positive nuclei in the late pachytene region display RAD-51 foci and regions of asynapsed chromosomes. (A) Immunofluorescence staining of DSB-1 and RAD-51 in wild-type nuclei from the late pachytene region of the gonad. Nuclei positive for DSB-1 staining also show condensed, transition zone-like DAPI-staining morphology, and have abundant RAD-51 foci. (B) Immunofluorescence staining of DSB-1, HTP-3, and SYP-1 in wild-type nuclei from the late pachytene region of the gonad. Nuclei positive for DSB-1 staining contain asynapsed chromosome regions (HTP-3 positive axes not associated with SYP-1). DSB-1 positive nuclei are outlined with a dotted line.
Figure 1.9. DSB-1 localization is independent of *spo-11*, *mre-11*, and *htp-3*, but requires *chk-2*. Immunofluorescence staining of DSB-1 with DAPI in pachytene nuclei from *spo-11(ok79)*, *mre-11*, *htp-3*, and *chk-2* mutant hermaphrodites. Localization of DSB-1 was not reduced in *spo-11* or *mre-11*, but was slightly reduced in *htp-3* mutants. Localization of DSB-1 in *chk-2* mutants was greatly reduced compared to WT, but was not abolished, as shown in inset, in which the DSB-1 signal has been rescaled to highlight the faint chromosomal staining. Scale bar, 5 µm.
Figure 1.10. **DSB-1 and DSB-2 show similar patterns of localization, but do not colocalize.** (A) Composite projection image of a gonad from a WT animal showing DAPI and immunofluorescence staining for DSB-1 and DSB-2. Both proteins are detected on chromosomes by the transition zone (leptotene/zygotene), and persist until mid pachytene. Scale bar, 30 µm. (B) Projections of early pachytene nuclei showing DSB-1 and DSB-2 immunofluorescence and DAPI staining. Although DSB-1 and DSB-2 have similar staining patterns on meiotic chromosomes (Rosu et al., 2013), they do not colocalize extensively. Scale bar, 5 µm.
A: \( \text{dsb-2(me96)} \)

- DAPI
- DSB-1
- DSB-2

B: WT

- DAPI
- DSB-1
- DSB-2

- \( \text{dsb-2(me96)} \)
- DAPI
- DSB-1
- DSB-2

- \( \text{dsb-1(we11)} \)
- DAPI
- DSB-1
- DSB-2

- \( \text{dsb-1(tm5034)} \)
- DAPI
- DSB-1
- DSB-2

C: wild-type, \( \text{dsb-1(tm5034)} \), \( \text{dsb-2(me96)} \)

- DSB-1
- DSB-2
- loading

D: CHK-2

- DSB-1
- DSB-2

- DSB formation
- crossovers

\* 65
\* 43
\* 32

40
Figure 1.11. DSB-1 promotes the protein localization and protein level of DSB-2. (A) Composite projection image of a gonad from a dsb-2(me96) animal showing DAPI and immunofluorescence staining for DSB-1 and DSB-2. Scale bar, 30 µm. (B) Immunofluorescence staining of DSB-1 and DSB-2 in early pachytene nuclei from WT, dsb-2(me96), dsb-1(we11), and dsb-1(tm5034) animals. Reduced DSB-1 staining is observed in dsb-2 mutants, while DSB-2 is undetectable on chromosomes in dsb-1 mutants. Scale bar, 5 µm. (C) Immunoblotting of DSB-1 and DSB-2 in WT, dsb-1(tm5034), and dsb-2(me96) animals. Lysate from 50 animals, 24 hours post L4, was used for each lane. DSB-1 protein levels are slightly reduced in dsb-2 mutants, while DSB-2 protein levels are severely reduced in dsb-1 mutants. The loading control (bottom panel) is a nonspecific band. Panels were taken from the same blot. * indicates a nonspecific background band.
Figure 1.12. *dsb-2; dsb-1* double mutants fail to make DSBs. (A) Quantification of viable and male self-progeny produced by *rol-1, rol-1 dsb-2(me96)*, *dsb-1(tm5034)*, and *rol-1 dsb-2(me96); dsb-1(tm5034)* hermaphrodites. Whole broods were counted for each genotype. *dsb-2; dsb-1* double mutants display levels of progeny viability and males similar to *dsb-1* mutants. For each bar, the upper number indicates the percentage, and the lower number in parentheses indicates the total number of fertilized eggs (for viability) or adult progeny (for males) counted. (B) A representative DAPI-stained oocyte nucleus at diakinesis for *rol-1 dsb-2(me96); dsb-1(tm5034)* showing 12 DAPI-staining bodies. Scale bar, 5 µm. (C) Diagram summarizing the dependencies of DSB-1 and DSB-2 on each other and CHK-2. CHK-2 promotes the chromosomal localization of DSB-1 and DSB-2. Because DSB-2 localization depends on DSB-1, it is unknown whether CHK-2 promotes the loading of DSB-2 directly or through its effect on DSB-1. DSB-1 and DSB-2 promote or stabilize the protein levels and chromosomal localization of each other, with the effect of DSB-1 on DSB-2 being much greater than the converse. DSB-1 and DSB-2 collaborate to promote DSBs and crossover formation.
Chapter 2: Identification of a crossover assurance checkpoint that modulates DSB activity

Summary

In this chapter, I describe evidence for a crossover assurance checkpoint that upregulates meiotic DSB activity when one or more homologous chromosome pairs fail to form a crossover precursor. Mutations that disrupt crossover formation result in prolonged DSB-1 association with chromosomes, suggesting that nuclei may remain in a DSB-permissive state. Extended DSB-1 localization is seen even in mutants with defects in early recombination steps, including spo-11, suggesting that the absence of crossover precursors triggers the extension. Strikingly, failure to form a crossover precursor on a single chromosome pair is sufficient to extend the localization of DSB-1 on all chromosomes in the same nucleus. Based on these observations we propose a model for crossover assurance that acts through DSB-1 to maintain a DSB-permissive state until all chromosome pairs acquire crossover precursors.

Introduction

Formation of crossovers between homologous chromosomes is essential for successful execution of the meiotic program in most sexually reproducing organisms. Disruption of crossover formation leads to chromosome nondisjunction and the formation of aneuploid gametes, and thereby greatly reduces fertility. Therefore, it seems likely that cells have mechanisms in place to assure that each pair of chromosomes obtains the obligate crossover needed to promote its proper segregation. The meiotic recombination pathway that leads to the formation of crossovers is initiated by DSBs. A sufficient number of DSBs are needed to generate the crossovers necessary to proper chromosome segregation, however too many DSBs could be problematic. Here we describe a feedback mechanisms that senses the crossover status of each chromosome pair and responds to an absence of a crossover precursor by upregulating DSB activity.

Results

Disruption of crossover formation prolongs chromosome localization of DSB-1

In testing the genetic requirements for DSB-1 localization, we noticed that the zone of DSB-1 staining in the gonad was extended in mutants that disrupt crossover formation. Previous studies have reported a persistence of RAD-51 foci in numerous mutants that are proficient for DSBs but not for crossovers (Carlton et al., 2006; Colaiácovo et al., 2003; Mets and Meyer, 2009). In wild-type
animals, and also in most mutants with extended RAD-51 staining, DSB-1 staining disappeared concomitant with, or slightly before, the disappearance of RAD-51 foci (Figure 1.6A and 2.1). Two exceptions were rec-8 and rad-54 mutants, in which DSB-1 staining disappeared by late pachytene, but RAD-51 staining persisted into diplotene (Figure 2.2, data not shown). Since DSB-1 is required for DSBs and its localization correlates with the timing of DSB formation, its presence on chromosomes may be indicative of proficiency for DSB formation. Although the presence of DSB-1 on chromosomes may not be sufficient for prolonged DSB formation, we interpret extension of the region of DSB-1 staining as evidence of a prolonged DSB-permissive state (see Discussion).

We quantified the extension of DSB-1 localization by comparing the length of the zone of DSB-1-positive nuclei to the total length of the region spanned by the transition zone through late pachytene nuclei, just before oocyte nuclei begin to form a single row near the bend region of the gonad, which coincides with diplotene (Figure 2.2A). We designated this entire zone as the LZP region (leptotene-zygotene-pachytene), although it also includes a few diplotene nuclei. We found that this metric – the length ratio of the DSB-1-positive region to the LZP region – was consistent across age-matched animals of the same genotype. In wild-type adult hermaphrodites, DSB-1 positive nuclei comprised about 50% of the length the LZP region (Figure 2.2A and 2.2B). However, in most mutants that disrupt crossover formation on one or more chromosomes, this zone of DSB-1 staining was significantly extended (Figure 2.2A and 2.2B). We saw some variability in this extension, which tended to correlate with the nature of the mutation: Mutations affecting late steps in the crossover pathway, including msh-5(me23) (Kelly et al., 2000), cosa-1(me13) (Yokoo et al., 2012), and zhp-3(ajf61) (Bhalla et al., 2008), extended the DSB-1 zone to ~75%, of the LZP region (Figure 2.2A and 2.2B). Mutations that block earlier steps in homologous recombination, including com-1(t1626) (Penkner et al., 2007), rad-51(lg8701) (Alpi et al., 2003), and rad-54(tm1268) (Mets and Meyer, 2009), extended the DSB-1 zone even further, to ~90% of the LZP region. Mutations that block crossover formation by disrupting synapsis, including syp-1(me17) (MacQueen et al., 2002) and syp-2(ok307) (Colaiácovo et al., 2003), also showed an extension of DSB-1 staining to ~90% (Figure 2.2B).

Significantly, mutants that lack meiotic DSBs, including spo-11(ok79 or me44) (Dernburg et al., 1998; Hayashi et al., 2007), mre-11(ok179) (Chin and Villeneuve, 2001), and rad-50(ok197) (Hayashi et al., 2007), also showed significant extension of the zone of DSB-1 staining to 69-78% of the LZP region (Figure 2.2A and 2.2B). Together these findings indicate that the absence of crossovers or crossover precursors, rather than the presence or persistence of earlier recombination intermediates, triggers extension of the DSB-1 zone.

Also of note, in htp-1 and htp-3 mutants (Couteau and Zetka, 2005; Goodyer et al., 2008; Martinez-Perez and Villeneuve, 2005), in which the axial element is disrupted, the region of DSB-1 staining was shorter than in other crossover-deficient mutants (Figure 2.2B), despite the fact that no crossovers form in these animals and DSBs are either eliminated or reduced (Couteau and
This suggests that axis structure may play a role in detecting or signaling the absence of crossover precursors to prolong DSB-1 localization, consistent with proposed roles for the axis in other species (Bailis et al., 2000; Daniel et al., 2011; Wojtasz et al., 2012; Woltering et al., 2000).

We tested whether irradiation could suppress the extension of the DSB-1 zone seen in spo-11 mutants. Young adult hermaphrodites were irradiated, then fixed and stained 8 hours later. As controls, we included mutants (mre-11 and msh-5) in which crossover defects are not rescued by exogenous DSBs (Chin and Villeneuve, 2001; Kelly et al., 2000). Irradiation reduced the zone of DSB-1 staining in spo-11(me44) animals to 56%, compared to 70% for unirradiated controls (Figure 2.2C). In contrast, the length of the DSB-1 zone in wild-type, mre-11, and msh-5 hermaphrodites was unaffected by irradiation (Figure 2.2C). These data reinforce the idea that the absence of crossovers or crossover precursors induces prolonged DSB-1 association with chromosomes.

Many mutations that result in extension of the DSB-1 zone also cause elevated oocyte apoptosis, which can be triggered in response to persistent DNA damage or asynapsis (Bhalla and Dernburg, 2005; Colaiácovo et al., 2003; Gartner et al., 2000; MacQueen et al., 2002). We considered the possibility that apoptosis might mediate the observed extension of DSB-1 staining, since this process primarily culls nuclei from the late pachytene, DSB-1 negative region of the gonad (reviewed in Gartner et al., 2008). To test this idea, a representative subset of meiotic mutations, including spo-11(ok79), msh-5, syp-2, him-8, and zim-2 (see below) were combined with ced-4(n1162), which abrogates germline apoptosis (Gumienny et al., 1999). These double mutants displayed extended DSB-1 localization similar to that observed in the corresponding single mutants (Figure 2.3). We conclude that apoptosis does not account for the extension of DSB-1 staining observed in crossover-defective mutants, nor can it explain the quantitative differences observed among different mutants.

**Extension of DSB-1 localization reflects a genome-wide and nucleus-autonomous response**

To further characterize the extension of DSB-1 localization that occurs in response to defects in crossover formation, we examined mutant situations in which crossover formation was disrupted on only one chromosome. _him-8(tm611)_ and _zim-2(tm574)_ specifically disrupt homolog pairing and thus crossover formation on chromosomes X and V, respectively (Phillips and Dernburg, 2006; Phillips et al., 2005). _him-5(ok1896)_ does not impair pairing or synapsis, but abrogates DSBs on the X chromosome (Meneely et al., 2012). All three of these mutations extended the DSB-1 zone to 83-86% of the LZP region (Figure 2.2A and 2.2B). Furthermore, irradiation of _him-5_ animals, in which the crossover defect can be rescued by exogenous DSBs (Meneely et al., 2012), but not irradiation of _him-8_, suppressed the extension of DSB-1 localization (Figure 2.2C). These results indicate that the absence of a crossover precursor on a single chromosome pair is sufficient to prolong DSB-1 association with meiotic chromosomes.
Analysis of mutants with chromosome-specific defects in interhomolog recombination also allowed us to test whether DSB-1 staining is specifically prolonged on crossover-deficient chromosomes. In *him*-5 and *him*-8 mutants, the autosomes, but not the X chromosomes, are proficient for crossover formation. X chromosomes can be specifically marked in these mutants using HIM-8 antibodies (in *him*-5 mutants) or by staining for synaptonemal complex components (in *him*-8 mutants). In both of these genotypes, we observed persistent DSB-1 staining on all chromosomes throughout the region of extended DSB-1 localization (Figure 2.4A, 2.4B, and 2.4C). As in wild-type nuclei, the X chromosome showed weaker DSB-1 staining than the autosomes (Figure 1.6C and 2.4A). These findings indicate that the extension of DSB-1 localization is a genome-wide response affecting all chromosomes within the nucleus.

To test whether the extension of DSB-1 localization is regulated by nuclear-intrinsic or extrinsic signals, we examined animals heterozygous for *meDf2*, a deficiency of the X chromosome pairing center (Villeneuve, 1994). In *meDf2/+* hermaphrodites, X chromosome pairing and synapsis is partially compromised, such that approximately half the nuclei achieve full pairing and synapsis by the end of the pachytene region (MacQueen et al., 2005). Nuclei with asynapsed X chromosomes can be recognized by their more condensed, transition zone-like chromosome morphology, or by co-staining for axial element and central region proteins of the synaptonemal complex (MacQueen et al., 2005). In the late pachytene region of these animals, we found that DSB-1 staining correlated with the status of individual nuclei: those with asynapsed chromosomes were positive for DSB-1 staining, while fully synapsed nuclei lacked DSB-1 staining (Figure 2.4D). These results indicate that the extension of DSB-1 localization is a response to a signal intrinsic to individual nuclei, and does not extend to neighboring nuclei within the same region of the gonad. However, as in all mutants examined, DSB-1 disappeared by the end of the pachytene region of the gonad, indicative of an extrinsic, spatially regulated “override” signal that triggers progression to late pachytene and loss of the presumptive DSB-permissive state, even when crossover precursors have not been attained on all chromosomes (see Discussion).

**Discussion**

**A crossover assurance checkpoint mechanism that regulates DSB formation**

DSBs normally occur within a discrete time window during early meiotic prophase. In *C. elegans* this corresponds to the transition zone and early pachytene, based on RAD-51 localization. As DSB-1 is necessary for DSB formation, and its appearance on meiotic chromosomes coincides with the timing of DSBs, we infer that the chromosomal localization of DSB-1 is indicative of a regulatory state permissive for DSB formation. We observed that when crossover formation is disrupted, this DSB-1-positive region is extended. Rosu *et al.* report a similar extension of DSB-2 in crossover-defective mutants (Rosu *et al.*, 2013).
Previous work has shown that RAD-51 foci persist longer and accumulate to greater numbers in various mutants that make breaks but not crossovers (Carlton et al., 2006; Colaiácovo et al., 2003; MacQueen et al., 2005; Mets and Meyer, 2009; Penkner et al., 2007). Extended or elevated RAD-51 staining could reflect an extension of the time that DSBs are made, a greater number of DSBs, or a slower turnover of the RAD-51-bound state. However, persistence of DSB-1 and DSB-2 on meiotic chromosomes in these mutants suggests that the period in which nuclei are competent for DSB formation is extended. In support of this idea, in many crossover-defective mutants defective, the number of RAD-51 foci per nucleus not only reaches a far higher level but also peaks later than in wild-type animals and continues to rise even after RAD-51 is normally cleared from meiotic chromosomes (MacQueen et al., 2005; Phillips et al., 2005; Saito et al., 2012), indicating that breaks continue to be generated after their formation would normally cease. Several mutations that impair crossover formation on a limited number of chromosome pairs result in altered crossover distributions on the crossover-competent chromosomes (Carlton et al., 2006; Herman et al., 1982), which, particularly in light of the current findings, seems likely to reflect changes in DSB activity. In addition, RAD-51 chromatin immunoprecipitation data from our laboratory (C. V. Kotwaliwale and AFD, unpublished) have indicated that the DSB distribution is altered in him-8 mutants, one of the genotypes that show extended DSB-1 staining.

Taken together, these findings strongly suggest that both the temporal and genomic distribution of DSBs is altered in many situations that perturb crossover formation. A similar phenomenon may also account for the “interchromosomal effect” first observed in Drosophila female meiosis (Schultz and Redfield, 1951). We note that this raises caveats about previously published estimates of DSB numbers in C. elegans that have been based on quantification of RAD-51 foci in genotypes that do not complete crossovers, such as rad-54 or syp-1 mutants (Hayashi et al., 2010; Mets and Meyer, 2009; Saito et al., 2012).

Extension of the DSB-1 zone occurs even when nuclei are unable to initiate meiotic recombination due to an absence of DSBs. This suggests that the extension is not due to the persistence of unresolved recombination intermediates, but is instead a response to the absence of a particular crossover-competent recombination intermediate, or crossover precursor. We found that disruption of crossover formation on a single pair of chromosomes is sufficient to prolong DSB-1 localization on all chromosomes. Based on this result we believe that chromosomes lacking a crossover precursor may send out a signal that sustains a DSB-permissive state within the affected nucleus. How the lack of a crossover precursor is detected and what signal may result remains unknown. However, it seems likely that this signal is mediated through the chromosome axis, as axial element components appear to be required for the prolongation of DSB formation in the absence of a crossover precursor (Rosu et al., 2013). Thus, a single chromosome pair lacking a crossover precursor elicits a genome-wide response that results in extension of DSB-1 localization, which may reflect a modulation of the timing, and perhaps the extent, of DSB formation. Such a
mechanism would help to ensure formation of an “obligate” crossover on every chromosome pair.

All mutants that we found to extend the localization of DSB-1 cause a disruption in crossover formation, although they have various primary molecular defects. It is possible that extension of DSB-1 localization occurs in response to distinct molecular triggers in different mutant situations. For example, spo-11 mutants may be responding to an absence of DSBs, rad-54 mutants to unrepaired DSBs, and syp-1 mutants to asynapsed chromosomes. A similar model in which different unfinished meiotic tasks can elicit delays in meiotic progression was proposed in a recent study (Woglar et al., 2013). However, we feel that a parsimonious interpretation of our data is that the absence of a crossover precursor on one or more chromosomes is sufficient to prolong DSB-1/2 localization. The varying degree of extension seen in different mutants could reflect the engagement of additional regulatory mechanisms, such as the synapsis checkpoint and/or DNA damage checkpoint, which might converge with a crossover assurance mechanism to modulate regulators of DSB-1.

We propose that an “obligate crossover” checkpoint mediates the extension of DSB-1 localization (Figure 2.5). Our data suggest that DSB formation is activated during early meiosis and normally persists long enough for most nuclei to attain crossover precursors on all chromosomes (Figure 2.5). If interhomolog recombination is impaired on one or more chromosome pairs, individual nuclei can prolong the DSB-permissive state in an attempt to generate a crossover on every chromosome. Our observation that a block to crossover formation on a single pair of chromosomes results in persistent DSB-1 throughout the affected nuclei is reminiscent of the spindle assembly checkpoint (SAC), in which failure of a single pair of sister kinetochores to biorient on the mitotic spindle triggers a cell-autonomous delay in anaphase onset that affects cohesion on all chromosomes (Lara-Gonzalez et al., 2012). Interestingly, a key mediator of the SAC, Mad2, is homologous to the meiotic axis proteins HTP-3 and HTP-1 (Aravind and Koonin, 1998; Couteau and Zetka, 2005), which appear to be important for the regulatory circuit that mediates prolonged DSB-1 localization in response to crossover defects.

An alternative model would be a negative feedback circuit in which the acquisition of all necessary crossover-intermediates triggers inactivation of DSB formation. According to this view, the presence of crossover precursors generates a signal to exit the DSB permissive state, rather than the absence of precursors extending this period. Such a model would require a ‘counting’ mechanism that enables exit from the DSB permissive state in response to a threshold number of crossover precursors. This seems less likely based on first principles, and also less consistent with our data.

Our observations also suggest that there is a minimum duration of proficiency for DSB formation that does not depend on how rapidly chromosome pairs attain crossover precursors. We would expect meiotic nuclei to achieve crossover precursors on every chromosome in a stochastic manner. If DSB-1 were removed from chromosomes upon reaching this state, we would likely see a patchwork of DSB-1 positive and negative nuclei in the early pachytene region,
but instead we observe homogenous staining in this region, and abrupt disappearance of DSB-1 within a narrow zone of the gonad. Additionally, in mutants that appear to be defective in triggering the obligate crossover checkpoint, such as htp-3 and htp-1, a zone of DSB-1-positive nuclei similar in length to that in wild-type animals is observed. Together these observations suggest that there is a preset temporal window for DSB formation that can be extended in individual nuclei but not shortened.

The nature of the recombination intermediate that satisfies the requirement for a crossover precursor on all chromosomes remains unknown. We distinguish “crossover precursors” from “interhomolog recombination intermediates” because components that are specifically required for crossovers, including MSH-5, ZHP-3, and COSA-1 (Bhalla et al., 2008; Kelly et al., 2000; Rosu et al., 2011; Yokoo et al., 2012), are all required for timely disappearance of DSB-1 from chromosomes. However, cytological markers for crossovers, including foci of ZHP-3 and COSA-1, do not appear until the late pachytene region of the gonad (Bhalla et al., 2008; Yokoo et al., 2012), after DSB-1 and DSB-2 disappear from meiotic chromosomes (Rosu et al., 2013). Thus, it seems likely that crossover precursors, rather than mature crossovers, are sufficient to allow exit from the DSB-permissive state.

Genetic and cytological evidence indicate that nuclei eventually cease to make DSBs, even when crossovers fail to be made on one or more chromosomes. As nuclei approach the bend region of the gonad at the end of pachytene, an “override” signal appears to shut off DSB formation (Figure 2.5). Unlike in mammals, where crossover failures result in extensive apoptosis (Cohen and Pollard, 2001), C. elegans hermaphrodites produce both sperm and oocytes in roughly normal numbers even when homolog pairing, synapsis, and/or recombination are severely impaired.

The relationship between the crossover assurance mechanism and meiotic progression

Numerous studies have documented a phenomenon known as the “extended transition zone” in mutants with defects in homolog pairing and/or synapsis (Colaiácovo et al., 2003; Couteau et al., 2004; MacQueen et al., 2002; Phillips et al., 2005). An extended transition zone has been defined as a longer region of the gonad containing nuclei with crescent-shaped DAPI-staining morphology, multiple patches of the nuclear envelope proteins SUN-1 and ZYG-12, and strong foci of the ZIM proteins (MacQueen et al., 2002; Penkner et al., 2009; Phillips and Dernburg, 2006). An extended transition zone appears to be a response to asynapsed chromosomes (Colaiácovo et al., 2003; MacQueen et al., 2002). Previous work from our lab showed that the extension of the transition zone in synapsis-defective animals such as him-8 hermaphrodites was suppressed by mutations in recombination factors, including spo-11 and msh-5, and we therefore proposed that it might reflect a response to unresolved recombination intermediates (Carlton et al., 2006). However, subsequent work has revealed that these double mutant situations actually resulted in precocious
fold-back synapsis of unpaired chromosomes, thereby silencing the asynapsed chromosome response (Stacia Rodenbusch and Abby Dernburg, unpublished).

Since mutations that abrogate pairing or synapsis also impair interhomolog recombination, it is not surprising that most genotypes with extended transition zones also show persistent DSB-1 localization. However, not all mutants that disrupt crossover formation extend the transition zone. spo-11 and him-5 mutants, for example, are deficient for DSB formation on one or more chromosomes and show extended DSB-1 staining, but do not show typical extended transition zones. Instead, these mutants appear to have extended regions of early pachytene nuclei. Based on these observations, we believe that the obligate crossover checkpoint mechanism is distinct from the response to asynapsed chromosomes. However, these two regulatory circuits serve similar purposes – to enable meiotic nuclei more time to complete synapsis or achieve crossovers on all chromosomes – and they may also involve common molecular components.
Materials and Methods

**C. elegans Mutations and Strains.** All *C. elegans* strains were cultured under standard conditions at 20°C. The wild-type strain was N2 Bristol. Mutants analyzed in this study were: *spo-11(me44, ok79), mre-11(ok179), rad-50(ok197), com-1(t1626), rad-51(lg8701), rad-54(tm1268), msh-5(me23), cosa-1(me13), zhp-3(jf61), htp-3(tm3655), htp-1(gk174), syp-1(me17), syp-2(ok307), him-8(tm611), zim-2(tm574), dsb-2(me96), and ced-4(n1162).* Strains used in this study were:

- CA279 *spo-11(me44)/mIs11 IV*
- CA276 *spo-11(ok79)/mIs11 IV*
- CA1109 *mre-11(ok179) V/nT1[unc?-? (n754) let-?] (IV;V)*
- AV158 *rad-50(ok197) V/nT1[unc?-? (n754) let-? qIs50] (IV;V)*
- GE4132 *unc-32(e189) com-1(t1626)/qC1 dpy-19(e1259) glp-1(q399) III*
- CA538 *rad-51(lg8701)/mIs11 IV*
- CA855 *rad-54(tm1268) I/hT2[bli-4(e937) let-? (q782) qIs48] (I;III)*
- AV115 *msh-5(me23) IV/nT1[unc?-? (n754) let-?] (IV;V)*
- AV424 *cosa-1(me13)/qC1[dpy-19(e1259) glp-1(q339) qIs26] III*
- CA685 *zhp-3(jf61) I/hT2[bli-4(e937) let-? (q782) qIs48] (I;III)*
- CA821 *htp-3(tm3655) I/hT2[bli-4(e937) let-? (q782) qIs48] (I;III)*
- AV393 *htp-1(gk174) IV/nT1[unc?-? (n754) let-?] (IV;V)*
- AV307 *syp-1(me17) V/nT1[unc?-? (n754) let-? qIs50] (IV;V)*
- AV276 *syp-2(ok307) V/nT1[unc?-? (n754) let-?] (IV;V)*
- CA257 *him-8(tm611) IV*
- CA258 *zim-2(tm574) IV*
- CA1087 *ced-4(n1162) III*
- CA1090 *ced-4(n1162) III; spo-11(ok79)/mIs11 IV*
- CA1092 *ced-4(n1162) III; msh-5(ne23) IV/nT1[unc?-? (n754) let-?] (IV;V)*
- CA1091 *ced-4(n1162) III; syp-2(ok307) V/nT1[unc?-? (n754) let-?] (IV;V)*
- CA1088 *ced-4(n1162) III; him-8(tm611) IV*
- CA1989 *ced-4(n1162) III; zim-2 (tm574) IV*

**Immunofluorescence and Cytological Analysis.** Immunofluorescence was performed as described in Chapter 1.

**Irradiation Experiments.** Young adult worms were irradiated with approximately 10 Gy (1000 rad) from a Cs-137 source. For each experiment, unirradiated controls were treated identically to irradiated animals, other than exposure to radiation. For quantification of DSB-1 localization, animals were irradiated 16 hours post L4 and dissected 8 hours post irradiation.
Figure 2.1. Extension of DSB-1 staining is correlated with the extension of RAD-51 staining in mutants that disrupt crossover formation. Composite projection image of a gonad from a him-8 hermaphrodite, showing DAPI and immunofluorescence staining for DSB-1 and RAD-51. The disappearance of DSB-1 coincides with the disappearance of RAD-51 foci.
Figure 2.2. The region of the germline with nuclear DSB-1 localization is extended in mutants with impaired crossover formation. (A) Composite
projection images of gonads from indicated genotypes showing immunofluorescence staining of DSB-1 and DAPI. Lines represent the start (left line) and end (right line) of the leptotene-zygotene-pachytene (LZP) region of the gonad, and the end of the zone of DSB-1 localization (middle line). (B) Quantification of the zone of DSB-1 localization, showing the percent, by length, of the LZP region positive for DSB-1 staining. Numbers in parentheses indicate the number of gonads quantified for each genotype. All genotypes showed significant differences from wild type, p < 0.003 except for htp-1, for which p < 0.05. Error bars indicate standard deviation. Genotypes are color-coded based on the category of meiotic defect that they cause (listed above the graph). (C) Quantification of the zone of DSB-1 localization in gamma-irradiated animals and unirradiated controls. Animals were irradiated (10 Gy) at 16 hours post L4, then dissected and fixed 8 hours later to measure the length of the zone of DSB-1 localization relative to the length of the LZP region. Numbers in parentheses indicate the number of gonad arms quantified. Error bars indicate standard deviation. **p = 0.0005, *p = 0.002.
Figure 2.3. Extension of the DSB-1 region in crossover-deficient mutants is not a consequence of apoptosis. Quantification of the zone of DSB-1 localization, showing the percent, by length, of the LZP region positive for DSB-1 staining. The genotypes indicated along the x-axis are present either as single mutants in the wild-type ced-4 background or as double mutants combined with ced-4(n1162). Mutation of ced-4 abrogates germline apoptosis, but does not markedly or consistently alter the extended zone of DSB-1 localization to chromosomes. Error bars indicate standard deviations.
Figure 2.4. Extension of DSB-1 localization is a nuclear-autonomous response. (A, B) Immunofluorescence staining of DSB-1 and HIM-8 in pachytene nuclei in (A) WT and (B) him-5 mutants. HIM-8 was used to mark the X chromosome (arrows). (B) Nuclei were imaged from the mid-late pachytene region of the gonad with extended DSB-1 localization. DSB-1 localization occurs throughout the nucleus and is not restricted to the X chromosome. (C) Immunofluorescence staining of DSB-1, HTP-3, and SYP-1, with DAPI in him-8 mutants. Regions of HTP-3 staining that do not colocalize with SYP-1 identify the asynapsed X chromosomes (arrows). Nuclei from the mid-late pachytene region of the gonad, where DSB-1 would normally have disappeared, are shown. DSB-1 is observed throughout the nuclei and is not restricted to the X chromosome. (D) Hermaphrodites heterozygous for a deficiency of the X chromosome pairing center (mnDp66/+; meDf2/+) were stained for HTP-3, SYP-1, and DSB-1. Fully synapsed nuclei in the mid-late pachytene region lack DSB-1 staining (broken circles), while adjacent nuclei with asynapsed X chromosomes retain DSB-1 staining as well as more condensed DAPI morphology. Scale bar, 5 µm.
Figure 2.5. Model: Nuclei remain in a DSB-permissive state until a crossover precursor has been attained on each chromosome pair. At the onset of meiotic prophase, DSB-1 and DSB-2 are targeted to chromosomes to mediate DSB formation. A standard duration of the resulting DSB-permissive state is usually sufficient to ensure establishment of crossover (CO) precursors on most chromosome pairs. However, chromosome pairs that fail to form a crossover precursor emit a signal that prolongs the DSB-permissive state within individual nuclei. Once all chromosome pairs within a nucleus attain at least one crossover precursor, DSB-1 and DSB-2 are removed from meiotic chromosomes and DSB formation is thereby inactivated. As nuclei approach the bend region of the gonad, an override signal acts to shut off DSB formation regardless of crossover status. Upon exit from the DSB-permissive state, nuclei progress to late pachytene and complete crossover formation.
Chapter 3: A negative feedback loop acts through DSB-1 to downregulate DSB formation

Summary

In this chapter, I describe evidence for a negative feedback loop that is mediated by DNA-damage response kinases ATM and ATR and acts though DSB-1 to downregulate DSB formation. In response to meiotic DSB formation, DSB-1 is phosphorylated in a manner dependent on ATM and ATR. DSB-1 is likely to be a direct target of ATM and ATR as mutation of the ATM/ATR consensus phosphorylation motifs in DSB-1, in the dsb-1SAQ mutant, abolishes its phosphorylation. dsb-1SAQ mutants display an increase in DSBs, suggesting that phosphorylation of DSB-1 acts to downregulate DSB levels and may be important in preventing the accumulation of excessive DSBs. Importantly, the increase in DSBs observed in the dsb-1SAQ mutant appears to have little phenotypic consequence for meiosis. Despite the increase in DSB levels, dsb-1SAQ mutants do not display decreases in genomic stability or differences in crossover regulation, indicating that C. elegans has robust mechanisms in place to tolerate variation in DSB number. The downregulation of DSB formation through DSB-1 phosphorylation may nonetheless play an important contributing role in promoting genomic stability and crossover homeostasis by providing one layer of protection against excessive DSB formation. Although DSB-1 is not conserved, this ATM/ATR dependent negative feedback loop regulating DSB number appears to be conserved across various eukaryotic phyla.

Introduction

Meiotic crossover recombination is essential for the proper segregation of homologous chromosomes during the first meiotic division. Meiotic recombination is initiated by the formation of programmed DSBs, which are catalyzed by the widely conserved topoisomerase-related protein SPO-11. While meiotic DSBs are essential for the successful completion of the meiotic program, they may also pose a threat to genomic stability if they are not regulated and repaired properly. One important aspect of DSB regulation is controlling the number of meiotic DSBs. A minimum number of DSBs is needed to produce the obligatory crossover for each pair of homologs. One way to accomplish this is by simply inducing high levels of DSBs throughout the genome, thereby increasing the probability that each chromosome pair receives at least one DSBs that will be repaired as a crossover. However, inducing too many DSBs may lead to genomic instability. Therefore nuclei must achieve a balance between having enough DSBs to promote crossover formation, without having too many that genomic stability is compromised. Here, I describe a negative feedback loop that senses meiotic DSBs, and responds by downregulating the DSB formation. This negative
feedback loop is mediated through the phosphorylation of DSB-1 by DNA damage response kinases ATM and ATR.

Results

**DSB-1 is phosphorylated in a manner dependent on meiotic DSBs.**

Immunoblotting experiments DSB-1 revealed that a fraction of the DSB-1 protein is phosphorylated. By analysis of the DSB-1-3xFLAG construct, I observed that a fraction of the protein consistently ran as a slower migrating band by gel electrophoresis (Figure 3.1A). This slower migrating band was also observed by immunoblotting endogenous DSB-1 in wild-type animals, although the band was less pronounced (Figure 3.1B). Phosphatase treatment of immunoprecipitated DSB-1-3xFLAG protein collapsed the DSB-1-3xFLAG signal into a single, faster migrating band, demonstrating that the slower migrating band represents a phosphorylated form of the protein (Figure 3.1C). These results indicate that a fraction of DSB-1 is phosphorylated under normal conditions. I wanted to investigate this further to understand if DSB-1 phosphorylation could be involved in regulating DSB formation.

By analysis of DSB-1 phosphorylation in various mutant backgrounds (Table 3.1) I noticed that the band corresponding to the phosphorylated form of DSB-1 was absent in mutant conditions where no DSBs are made, such as in *spo-11, htp-3, mre-11, rad-50,* and *chk-2* mutants (Figures 3.2A and 3.2B and Table 3.1). I tested whether the absence of phosphorylated DSB-1 could be due to the lack of DSBs by ectopically inducing DSBs with gamma irradiation in these mutants and then assaying for DSB-1 phosphorylation. Ectopic induction of DSBs rescued the phosphorylation of DSB-1 in *spo-11, htp-3,* and *chk-2* mutants (Figures 3.2A and 3.2B). Furthermore, treating wild-type worms with increasing doses of gamma irradiation was able to increase the proportion of DSB-1 that was present in the phosphorylated form (Figure 3.2C). These results indicate that DSB-1 phosphorylation is dependent on the formation of DSBs. Additionally, the CHK-2 kinase is not required for DSB-1 phosphorylation. Since DSB-1 is required for DSB formation, and is phosphorylated in a DSB-dependent manner, it suggests the existence of a feedback loop that may be acting to regulate DSB activity.

Immunoblotting experiments show that only a fraction of DSB-1 is phosphorylated, even upon induction of high levels of DSBs (Figures 3.1 and 3.2C). In order to further examine the fraction of DSB-1 that is phosphorylated we attempted to generate a phospho-specific antibody against DSB-1. The phospho-specific antibody was not successful, however, as it failed to show any specific signal by immunofluorescence or immunoblotting experiments.

**DSB-1 phosphorylation is dependent on DNA damage response kinases ATM and ATR**

We next wanted to determine which kinase(s) could be responsible for the phosphorylation of DSB-1. DSB-1 has many potential phosphorylation sites. 16%
of DSB-1’s amino acids are serine residues, and include consensus motifs for several different kinases. Because DSB-1 phosphorylation is DSB dependent, we were interested to know whether the phosphorylation could be mediated through the DNA damage response kinases ATM and ATR, ATM-1 and ATL-1 in *C. elegans*, respectively. DSB-1 has five SQ motifs, which are consensus sites for ATM/ATR phosphorylation. I analyzed DSB-1 phosphorylation in *atm-1* and *atl-1* single mutants, as well as in *atm-1; atl-1* double mutants (Figure 3.3). In *atm-1* mutants, which have elevated levels of genomic instability, but are otherwise normal with mild meiotic defects (Jones et al., 2012), DSB-1 phosphorylation appears comparable to that observed in wild-type animals (Figure 3.3A). *atl-1* mutants display a much more severe phenotype than *atm-1* mutants and are severely affected during meiosis (Aoki et al., 2000; Garcia-Muse and Boulton, 2005). *atl-1* mutants display elevated levels of RAD-51 foci throughout the gonad (data not shown), indicating elevated levels of DNA damage and/or DSBs, which are likely to be due in large part to defects in DNA repair that have been carried over from the mitotic region of the gonad. The excessive amounts of RAD-51 foci are independent of SPO-11, as *spo-11; atl-1* double mutants also display high levels of RAD-51 foci throughout the gonad (data not shown). DSB-1 phosphorylation is elevated in *atl-1* mutants, as well as *spo-11; atl-1* double mutants, compared to wild-type animals (Figure 3.3A and Table 3.1), which is likely due to the elevated level of DNA damage and/or DSBs observed in these animals. However, when both ATM and ATR functions are abolished in an *atm-1; atl-1* double mutant, DSB-1 phosphorylation is absent, even upon exposing worms to high doses of gamma irradiation (Figure 3.3B). These results demonstrate that DSB-1 phosphorylation is dependent on ATM and ATR, and that either kinase can promote the phosphorylation of DSB-1 in the absence of the other.

In order to address the question of whether DSB-1 could be a direct target of ATM and ATR, I made a nonphosphorylatable DSB-1 mutant, mutating the 5 SQ motifs to AQ motifs. The *dsb-1*5AQ(*ieSi25*) transgene was integrated into the *C. elegans* genome using MosSCI, and crossed into the *dsb-1*(tm5034) mutant background. In *dsb-1*5AQ(*ieSi25*); *dsb-1*(tm5034) animals (hereafter referred to as *dsb-1*5AQ), DSB-1 was not phosphorylated, even upon exposing the animals to gamma irradiation (Figure 3.3C). These results indicate that DSB-1 is likely a direct target of ATM and ATR.

**DSB-1 phosphorylation is dependent on MRE-11 and RAD-50**

I next wanted to determine what factors may be upstream of ATM-1 and ATL-1 in responding to meiotic DSBs. Studies have shown that the MRN/ MRX complex is required for activation of ATM in mice and budding yeast, and that this requirement for MRN/MRX is independent of its nuclease activity (Deshpande et al., 2014; Lee and Paull, 2004; Lee et al., 2013). Furthermore, MRE-11 has been shown to be required for the recruitment of ATL-1 to DSB sites in *C. elegans* (Garcia-Muse and Boulton, 2005). I tested whether DSB-1 phosphorylation was dependent on MRE-11 and RAD-50 by irradiation *mre-11* and *rad-50* animals. Note that DSB-1 is not phosphorylated in unirradiated *mre-
11 and rad-50 mutants because no DSBs are made under these conditions (Chin and Villeneuve, 2001; Hayashi et al., 2007). Induction of DSB-1 phosphorylation was not observed upon irradiation in mre-11 animals, and was largely absent from rad-50 animals (Figure 3.2A). Occasionally, a faint, slower-migrating smear was observed in irradiated rad-50 animals, indicating that DSB-1 may be getting phosphorylated at a very low level. To test whether DSB-1 phosphorylation was dependent on the resection of DSBS, I analyzed DSB-1 in a com-1 mutant, which fails to process meiotic DSBS into ssDNA tails that can bind to RAD-51 and invade the homologous chromosome (Penkner et al., 2007). DSB-1 phosphorylation was not affected in the com-1 mutant (data not shown), indicating that DSB resection is not required for DSB-1 phosphorylation. Overall, these results indicate that DSB-1 phosphorylation is largely dependent on MRE-11 and RAD-50 in C. elegans, consistent with an upstream role of MRE-11 and RAD-50 in the activation of ATM-1 and ATL-1 in response to DSBS.

**DSB-1 phosphorylation acts to downregulate DSB formation**

I next wanted to investigate the function of DSB-1 phosphorylation on the regulation of meiotic DSB formation by analyzing the dsb-1<sup>5AQ</sup> mutant. The timing of RAD-51 foci in dsb-1<sup>5AQ</sup> animals is the same as in wild-type animals; RAD-51 foci first appeared towards the end of the transition zone and disappeared by mid pachytene. These observations suggest that the timing of DSB formation and repair in dsb-1<sup>5AQ</sup> animals is similar to wild-type. Strikingly, dsb-1<sup>5AQ</sup> meiotic nuclei showed elevated levels of RAD-51 foci, an average of 11.5 foci per nucleus, compared to wild-type, which have an average of 5.5 RAD-51 foci per nucleus (Figure 3.4A), suggesting the dsb-1<sup>5AQ</sup> may have increased levels of DSBs. However, increased levels of RAD-51 foci could also signify a delay in processing DSBS and a slower turnover of recombination intermediates marked by RAD-51.

To further examine whether dsb-1<sup>5AQ</sup> animals have an increased number of DSBs, we tested whether dsb-1<sup>5AQ</sup> could rescue the crossover defects observed in dsb-2 and him-17 animals, both of which suffer from low levels of DSBS (Reddy and Villeneuve, 2004; Rosu et al., 2013). Whereas self-fertilizing wild-type hermaphrodites produce nearly 100% viable progeny and low levels of male progeny (<1%), only 26% of dsb-2(tm6047) progeny survived until adulthood, 11.2% of which were male (Figure 3.4B). These defects are due to the low levels of meiotic DSBS formed in dsb-2 mutants, and get worse with increasing age of the mother (Figure 3.4C; (Rosu et al., 2013)). Crossing the dsb-1<sup>5AQ</sup> allele into the dsb-2 mutant background was able to largely rescue the meiotic defects of dsb-2 animals. dsb-1<sup>5AQ</sup> dsb-2(tm6047); dsb-1(tm5034) animals produced 80% viable progeny, only 2% of which were male (Figure 3.4B and 3.4C). Furthermore, dsb-1<sup>5AQ</sup> was also able to partially rescue the meiotic defects observed in him-17 mutants, which also show very low levels of DSBS (Reddy and Villeneuve, 2004). Whereas self-fertilizing him-17 hermaphrodites produced only 6.9% viable progeny and 33.3% male progeny, dsb-1<sup>5AQ</sup>; dsb-1(tm5034); him-17 animals produced 63% viable progeny and 11% males (Figure 3.4D). These results provide further evidence dsb-1<sup>5AQ</sup> have increased...
levels of DSBs, and suggest that phosphorylation of DSB-1 is important for
downregulating the levels of DSBs.

I next made phosphomimetic DSB-1 mutants to see whether mimicking
constitutively phosphorylated DSB-1 would cause a decrease in the level of
DSBs. Both dsb-1^{5DQ}(ieSi26) and dsb-1^{5EQ}(ieSi27) mutants were generated by
MosSCI and crossed into the dsb-1(tm5034) mutant background. Contrary to my
expectations, neither dsb-1^{5DQ}(ieSi26); dsb-1(tm5034) or dsb-1^{5EQ}(ieSi27); dsb-
1(tm5034) strains (dsb-1^{5DQ} and dsb-1^{5EQ}, respectively) showed a decrease in
DSBs. The levels of RAD-51 foci observed in both of these strains were similar to
wild-type (data not shown). Furthermore dsb-1^{5DQ} and dsb-1^{5EQ} animals showed
nearly 100% embryo viability and low male progeny, similar to wild-type and dsb-
1^{WT} animals (Figure 3.5A and Table 3.2), indicating little to no defect in meiotic
crossover formation. I also tested these alleles for haplo-insufficiency by crossing
dsb-1^{5DQ}(ieSi26); dsb-1(tm5034) or dsb-1^{5EQ}(ieSi27); dsb-1(tm5034) lines to dsb-
1(tm5034) and examining the heterozygote progeny, such that animals only had
one functional copy of DSB-1, either DSB-1^{5DQ} or DSB-1^{5EQ}. Self-progeny from
these animals also had near wild-type levels of embryo viability and male
progeny (Figure 3.5B and Table 3.2), indicating that these ‘phosphomimetic’
alleles are indistinguishable from the wild-type dsb-1 allele. One possible
explanation for this lack of phenotype is that these ‘phosphomimetic’
dsb-1 alleles may not accurately mimic the phosphorylated state of DSB-1. An
alternative explanation is that they do mimic the phosphorylated state of DSB-1,
but that there is a more complicated regulation pathway at play that is buffering
the DSB phenotype.

A modest increase in meiotic DSB levels does not affect genomic stability
or crossover homeostasis

The dsb-1^{5AQ} mutant causes an increase in endogenous DSB levels
without greatly perturbing the meiotic system (see Discussion). Although we
observe a >2 fold increase in the numbers of RAD-51 foci, it is uncertain exactly
how many more DSBs are getting made (see Discussion), however we can be
confident that there is at least a modest increase in the number of DSBs being
made. This presents a nice opportunity to interrogate the effects of having
increased levels of meiotic DSBs. I first wanted to ask whether increased DSB
levels would cause genomic instability. If increased levels of DSBs caused
genome rearrangements or detrimental mutations, you would expect to see a
decrease in embryo viability. I therefore assessed genomic instability by
quantifying embryo viability and the frequency of male progeny. dsb-1^{5AQ} animals
had nearly 100% viability and low percentage of males, similar to dsb-1^{WT}
animals (Figure 3.5A and Table 3.2). A low brood size can also be an indication
of genomic instability (Jones et al., 2012), however the brood size of dsb-1^{5AQ}
animals was also similar to wild-type (Table 3.2). These results suggest that the
increase in DSB levels observed in the dsb-1^{5AQ} mutant does not overtly affect
genomic stability. Additionally, I tested whether germline apoptosis could be
culling nuclei with more severe levels of DNA damage by assessing genomic
instability of dsb-1^{5AQ} in the ced-4 mutant background. ced-4; dsb-1^{5AQ}(ieSi25);
dsb-1(tm5034) animals showed similar levels of embryo viability and male progeny as ced-4 mutants (Figure 3.5C and Table 3.2), further demonstrating that the increase in DSBs levels observed in dsb-1^{SAQ} animals does not overtly affect genomic stability.

I next wanted to determine whether the increase in DSB levels observed in the dsb-1^{SAQ} mutant could cause an increase in the number of crossovers. In C. elegans, wild-type animals typically receive exactly one crossover per pair of homologous chromosomes per meiosis (Brenner, 1974; Hillers and Villeneuve, 2003; Hodgkin et al., 1979; Martinez-Perez and Colaiácovo, 2009; Wood, 1988). However, there are specific mutant conditions that can increase the number of meiotic crossovers per chromosome (Libuda et al., 2013; Mets and Meyer, 2009; Nabeshima et al., 2004; Tsai et al., 2008; Youds et al., 2010). Additionally, increasing the levels of DSBs through gamma irradiation can cause an increase in the number of crossovers (Mets and Meyer, 2009), although these DSBs are distinct from SPO-11 induced breaks in several important ways (see Discussion).

We therefore tested whether the >2 fold increase in DSB levels observed in the dsb-1^{SAQ} would be sufficient to cause an increase in the number of crossovers by using an array-based SNP mapping approach to examine crossover events throughout the genome. Crossovers occurring in hermaphrodite dsb-1^{SAQ} or dsb-1^{WT} animals heterozygous for Bristol and Hawaiian SNPs were examined. 102 SNPs throughout the genome, excluding chromosome II, were examined. (Chromosome II had been excluding from analysis because the dsb-1^{SAQ} and dsb-1^{WT} alleles had been integrated on chromosome II and had not been completely introgressed into the Hawaiian strain.) For dsb-1^{WT} animals, 33 F2 progeny were examined, representing 84 total crossovers, 15-20 crossovers per chromosome. For dsb-1^{SAQ} animals, 48 F2 progeny were examined, representing 110 total crossovers, 18-26 crossovers per chromosome. By analysis of crossover events, a double crossover was never observed for either dsb-1^{SAQ} or dsb-1^{WT} animals on any chromosome. These results indicate that the number of crossovers is not increased and crossover interference is not compromised in dsb-1^{SAQ} animals, even though there is an increase in DSB number.

I next wanted to determine whether the increase in DSBs observed in dsb-1^{SAQ} mutants could lead to a change in the genomic distribution of crossover events. In wild-type animals, crossover frequency is suppressed in the center portion of each chromosome, and is elevated on the chromosome arms (Rockman and Kruglyak, 2009). This overall pattern is observed on all chromosomes, although it is less pronounced on the X chromosome. Various meiotic mutants are known to disrupt the crossover distribution (Carlton et al., 2006; Meneely et al., 2012; Mets and Meyer, 2009; Nabeshima et al., 2004; Tsai et al., 2008; Wagner et al., 2010; Zetka and Rose, 1995). Recent work from our lab has shown that prolonging DSB formation by disrupting crossover formation on one or more chromosomes (see Chapter 2) causes an overall change in the chromosome-wide distribution of crossover events (Chitra Kotwaliwale and Abby Dernburg, unpublished). Analysis of the crossover events observed from the SNP mapping experiment described above showed that dsb-1^{SAQ} and dsb-1^{WT} hermaphrodites display similar overall patterns of crossover distribution (Figure
As has been previously described for wild-type animals, crossover frequency was elevated on chromosome arms and suppressed in the center of chromosomes (Figure 3.6). These results indicate that increasing the number of DSBs, without greatly perturbing the meiotic system or affecting the timing of DSB formation, does not disrupt the characteristic pattern of crossover distribution along chromosomes.

Discussion

A conserved negative feedback loop that keeps DSB number in check

I have shown that DSB-1, a protein required for meiotic DSB formation in *C. elegans*, is phosphorylated in a manner dependent on the formation of meiotic DSBs. The phosphorylation of DSB-1 is mediated by the DNA damage response kinases ATM and ATR. Furthermore, DSB-1 appears to be a direct target of ATM and ATR, as mutating the ATM/ATR consensus SQ motifs abolishes phosphorylation of DSB-1. A combination of genetic and cytological evidence suggest that nonphosphorylatable DSB-1 mutants, *dsb-1*<sup>SAQ</sup>, have increased levels of meiotic DSBs. These results provide evidence of a negative feedback loop in which ATM and ATR respond to meiotic DSBs by phosphorylating DSB-1, which in turn downregulates DSB formation, perhaps by inhibiting the DSB-promoting activity of DSB-1.

Studies in mouse, budding yeast, and fruit flies have also implicated a role for ATM and/or ATR in downregulating meiotic DSB formation (Carballo et al., 2013; Joyce et al., 2011; Lange et al., 2011; Zhang et al., 2011), suggesting that the negative feedback loop that I have described for *C. elegans* may be widely conserved across eukaryotes. In budding yeast, the target of Mec1/Tel1 (ATM/ATR) phosphorylation in response of meiotic DSBs was reported to be Rec114 (Carballo et al., 2013), an essential DSB factor. A homolog of Rec114 has been identified in mouse and various other eukaryotic organisms, although it appears to be absent from *C. elegans* as well as *Drosophila* (Kumar et al., 2010). My results demonstrate that during *C. elegans* meiosis, DSB-1 is a target of ATM and ATR, although DSB-1 is not conserved outside of *Caenorhabditis*. Taken together, these studies indicate that even though the molecular targets of ATM/ATR phosphorylation are not necessarily conserved, the overall negative feedback loop mediated by ATM and ATR to limit the number of meiotic DSBs is conserved across various eukaryotes.

*DSB-1*<sup>SAQ</sup> provides a way to increase DSB number without greatly perturbing the system

In previous studies, the level of DSBs has been artificially manipulated by ectopically inducing DSBs with gamma irradiation. However, these DSBs differ from endogenous meiotic DSBs for a number of important reasons. First, ectopic DSBs are not dependent on SPO-11, and may not be processed in the same way. SPO-11 remains covalently attached to the 5′-end of the DNA upon making a DSB, which requires a particular set of endonucleases to remove SPO-11 from
the DSB site (Keeney, 2001). Irradiation induced DSBs would not have SPO-11 attached to the broken DNA strand, and may therefore be processed in a different way. Additionally, the presence of SPO-11 and associated proteins may also affect the processing of SPO-11 induced DSBs. Second, artificial DSBs do not occur with the same genomic distribution as endogenous DSBs. Whereas ectopic DSBs are expected to be distributed randomly throughout the genome, endogenous meiotic DSBs are not randomly distributed, rather they are enriched on chromosome arms and tend to be correlated with certain genomic features and chromatin marks (Chitra Kotwaliwale and Abby Dernburg, unpublished). The excess DSBs produced in the dsb-1^{5AQ} mutant, however, are likely to follow a similar pattern of genomic distribution as those produced in wild-type animals, as the pattern of crossover distribution was similar. Third, whereas endogenous DSBs occur within a specific window of time during early meiotic prophase, ectopic DSBs occur throughout the entire worm and gonad. DSBs made outside of the normal time window, are likely to be repaired differently. For example, it is known that DSBs occurring during late meiotic prophase go through a different mode of repair than DSBs made earlier in the meiotic program (Hayashi et al., 2007). The DSBs produced in the dsb-1^{5AQ} mutant, however, appear to be made and repaired with the same timing as in wild-type animals, as the appearance and disappearance of RAD-51 foci was similar. Therefore, increasing the level of DSBs through gamma irradiation does not necessarily replicate the affects that may be observed if DSB levels are increased in a natural way. The dsb-1^{5AQ} mutant, therefore, is an invaluable tool for assessing the consequences of having elevated levels of DSBs, as the mode, genomic distribution, and timing of DSB formation are not likely to be altered.

Worms are resilient to an increase in DSB levels

My analysis of dsb-1^{5AQ} mutants shows that worms are resilient to an increase in DSB levels. Worms are able to tolerate a modest increase in DSBs without causing genomic instability. Additionally, the increase in DSB levels does not cause an increase in crossover number or alter the genomic distribution of crossover events, demonstrating that crossover interference and homeostasis is robust. Conversely, it has been shown that worms are able to tolerate a great reduction in the number of DSBs and still maintain the necessary number of crossovers (Rosu et al., 2011), similar to the robust crossover homeostasis observed in budding yeast (Martini et al., 2006). These studies show that organisms are able to display robust crossover homeostasis in the face of a large range in the number of recombination initiation events.

Difficulties in estimating DSB number

Here I provide evidence that the dsb-1^{5AQ} mutant displays an increase in the number of meiotic DSBs. I observed a 2-fold increase in the number of RAD-51 foci. However, the number of RAD-51 foci is not indicative of the number of meiotic DSBs for two important reasons. First, the number of RAD-51 foci observed in fixed animals represents a snapshot of the total DSBs that are being dynamically formed and processed over a period of time. Therefore the
relationship between the number of RAD-51 foci observed at any given time depends on the turnover of these recombination intermediates. Second, it is not known whether a single RAD-51 focus represents only one DSB. Studies from other organisms hint at the possibility that each RAD-51 focus may represent multiple DSBs. Unfortunately, more quantitative approaches for estimating DSB number such as the Spo11-oligo quantification approach (Neale and Keeney, 2009) have not yet been successful in *C. elegans*, although attempts have been made in our lab to get the system working. The failure of the Spo11-oligo approach in our lab has been due to the inability to successfully raise SPO-11 antibodies, or to affinity tag *spo-11*. Although I can say with confidence that the DSB level is increased in the *dsb-1*<sup>5AQ</sup> mutant, it is not clear how many more DSBs are made in this mutant compared to wild-type.
Materials and Methods

**Immunoblotting.** Lysate from 30-50 young adult hermaphrodites, picked at 24 hours post L4, was used for each lane. Gel electrophoresis was performed using 4-12% Novex NuPage gels (Invitrogen). Proteins were transferred to PVDF membrane. Guinea pig DSB-1 antibodies were used for immunoblotting endogenous DSB-1. Mouse anti-FLAG (Genescript, A00187) and rabbit anti-FLAG (Sigma F7425) was used to detect DSB-1-3xFLAG. HRP-conjugated secondary antibodies and ECL Western Blotting Substrate (Pierce) was used for detection.

**Irradiation experiments.** Young adult worms, approximately 24 hours post L4 at 20 °C, were irradiated with 10 Gy (1000 rad) of gamma irradiation with a Cs-137 source, unless otherwise specified. For some experiments animals were treated with 100 Gy (10,000 rad). For each experiment, unirradiated controls were treated identically to irradiated animals, other than exposure to radiation. For DSB-1 immunoblotting experiments, animals were lysed 1 hour post irradiation.

**Phospho-specific DSB-1 antibody.** A synthetic phospho-peptide (GSS{PSER}QPTSF{PSER}QPC) containing sequence corresponding to phosphorylated serines S137 and S143 of DSB-1 was generated by Biomatik. This peptide was used to immunize 3 rats and 1 chicken. Antibody was affinity purified against the phospho-peptide. The affinity purified antibody was then passed over a column containing the nonphosphorylated peptide (GSSSQPTSFSQPC) to remove any antibody that was not specific to the phosphorylated form of the peptide.

**SNP genotyping.** The *dsb-1* mosSCI alleles, *ieSi22* and *ieSi25*, were introgressed into the Hawaiian strain CB4856 with five successive crosses. After introgression large portions of chromosome II still retained Bristol N2 SNPs. *dsb-1(tm5034)* was separately introgressed into the Hawaiian strain with 8 successive crosses, and only a very small portion of chromosome IV surrounding the *dsb-1* locus retained Bristol N2 SNPs, allowing for the use of chromosome IV SNP information in the SNP genotyping experiments. *dsb-1^{WT}(ieSi22)* II; *dsb-1(tm5034)* IV and *dsb-1^{SAQ}(ieSi25)* II; *dsb-1(tm5034)* IV Hawaiian lines were then constructed. Hawaiian males were then crossed to Bristol hermaphrodites of the corresponding genotype to produce *dsb-1^{WT}(ieSi22)*; *dsb-1(tm5034)* or *dsb-1^{SAQ}(ieSi25)*; *dsb-1(tm5034)* F1s that were heterozygous for Bristol or Hawaiian SNPs. Five heterozygous F1 hermaphrodites were then crossed to wild-type Hawaiian males. F2 progeny from these crosses were then picked to individual plates. Genomic DNA was purified from nearly starved F2 plates. Genomic DNA samples were then analyzed by Illumina GoldenGate genotyping by the UC Davis genome facility.
Table 3.1. Analysis of DSB-1 phosphorylation in various mutant backgrounds. DSB-1 phosphorylation was analyzed by immunoblotting whole worm lysates from young adult hermaphrodites of the indicated genotypes. The middle column indicates whether or not DSB-1 was phosphorylated for the corresponding genotype. If DSB-1 was phosphorylated, but the level of phosphorylation was substantially different than in wild-type animals, it is indicated. DSB-1 phosphorylation was also analyzed in some mutants after induction of ectopic DSBs with 1 kRad or more of gamma irradiation (IR). The third column indicates whether DSB-1 phosphorylation was observed after irradiation, and if it differed from the level of phosphorylation observed under unirradiated conditions.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Viability (n)</th>
<th>% Males (n)</th>
<th>Brood Size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>98.3 (538)</td>
<td>0.0 (529)</td>
<td>294 (5)</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;WT&lt;/sup&gt; (ieSi22); dsb-1(tm5034)</td>
<td>91.2 (352)</td>
<td>0.0 (321)</td>
<td>297 (3)</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5AQ&lt;/sup&gt; (ieSi25); dsb-1(tm5034)</td>
<td>95.1 (841)</td>
<td>0.0 (800)</td>
<td>230 (9)</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5DQ&lt;/sup&gt; (ieSi26); dsb-1(tm5034)</td>
<td>94.5 (549)</td>
<td>1.0 (519)</td>
<td>223 (5)</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5EQ&lt;/sup&gt; (ieSi27); dsb-1(tm5034)</td>
<td>98.2 (273)</td>
<td>0.0 (268)</td>
<td>286 (3)</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;WT&lt;/sup&gt; (ieSi22)/+; dsb-1(tm5034)</td>
<td>93.6 (1799)</td>
<td>0.2 (1684)</td>
<td>-</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5AQ&lt;/sup&gt; (ieSi25)/+; dsb-1(tm5034)</td>
<td>94.8 (1328)</td>
<td>0.0 (1259)</td>
<td>-</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5DQ&lt;/sup&gt; (ieSi26)/+; dsb-1(tm5034)</td>
<td>95.0 (1304)</td>
<td>0.7 (1239)</td>
<td>-</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5EQ&lt;/sup&gt; (ieSi27)/+; dsb-1(tm5034)</td>
<td>92.0 (661)</td>
<td>0.5 (608)</td>
<td>-</td>
</tr>
<tr>
<td>ced-4</td>
<td>81.8 (1738)</td>
<td>0.1 (1422)</td>
<td>-</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5AQ&lt;/sup&gt; (ieSi25); ced-4; dsb-1(tm5034)</td>
<td>86.0 (4147)</td>
<td>0.0 (3566)</td>
<td>-</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5DQ&lt;/sup&gt; (ieSi26); ced-4; dsb-1(tm5034)</td>
<td>89.2 (1644)</td>
<td>0.0 (1466)</td>
<td>-</td>
</tr>
<tr>
<td>dsb-1&lt;sup&gt;5EQ&lt;/sup&gt; (ieSi27); ced-4; dsb-1(tm5034)</td>
<td>86.7 (1215)</td>
<td>0.1 (1053)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2. Progeny viability, incidence of males, and brood size from *dsb-1* phosphomutants. Quantification of viable and male self-progeny from hermaphrodites of indicated genotype, as well as average brood sizes, is shown. The numbers in parentheses indicate the number of fertilized eggs (for viability), adult progeny (for male counts), and broods (for brood size) counted.
Figure 3.1. A subset of DSB-1 is phosphorylated. Immunoblotting of DSB-1 revealed that a subset of DSB-1 runs as a slower migrating band by electrophoresis. (A) anti-FLAG immunoblots against a wild-type strain carrying untagged, endogenous DSB-1 and a strain carrying a DSB-1-3xFLAG transgene. Lysate from 50 animals, 24 hours post L4, was used for each lane. (B) Immunoblotting of endogenous DSB-1. Lysate from 50 animals, 24 hours post L4, was used for each lane. (C) Immunoprecipitated DSB-1-3xFLAG samples were treated with Lambda phosphatase (+ PPase), or no phosphatase (- PPase), prior to gel electrophoresis and immunoblotting. The phosphatase treated samples show a majority of DSB-1 to run as faster migrating band relative to the no phosphatase treatment lane.
Figure 3.2. DSB-1 phosphorylation is dependent on DSBs. (A-C)
Immunoblotting analysis of DSB-1 phosphorylation in animals of the indicated genotypes that were treated with 0, 10, or 100 Gy of gamma irradiation. Lysate from 50 animals, 24 hours post L4, was used for each lane. Animals were lysed 1 hour post irradiation. Bands marked 1 and 2 indicate the unphosphorylated and phosphorylated forms of DSB, respectively. Bands marked * are nonspecific bands that are present in dsb-1 mutants.
Figure 3.3. DSB-1 phosphorylation is dependent ATM and ATR. (A-C)
Immunoblotting analysis of DSB-1 phosphorylation in animals of the indicated genotypes that were treated with 0 or 10 Gy of gamma irradiation. Lysate from 50 animals, 24 hours post L4, was used for each lane. Animals were lysed 1 hour post irradiation. Bands marked 1 and 2 indicate the unphosphorylated and phosphorylated forms of DSB, respectively. Bands marked * are nonspecific bands that are present in dsb-1 mutants.
A

B

C

D
Figure 3.4. The nonphosphorylatable DSB-1 mutant has increased levels of DSBs. (A) Immunofluorescence staining of RAD-51 was used as a cytological marker of early recombination intermediates and sites of meiotic DSBs in early pachytene nuclei. \( \text{dsb-1}^{\text{SAQ}} \) mutants show increased levels of RAD-51 foci relative to wild-type. (B-D) Quantification of viable and male self-progeny for the indicated genotypes. (B) Whole broods from 4-5 hermaphrodites were counted for each genotype. \( \text{dsb-2} \) animals show a decrease in progeny viability and an increase in percentage of male progeny compared to wild-type animals. This defect is partially rescued in \( \text{dsb-1}^{\text{SAQ}} \text{dsb-2} \); \( \text{dsb-1}(\text{tm5034}) \) animals. (C) A different representation of the same data for progeny viability reported in (B), showing the viability in 12-hour embryo laying intervals. Time represents hours post L4 at 20 °C. \( \text{dsb-2} \) animals show a reduction in progeny viability with age. \( \text{dsb-1}^{\text{SAQ}} \text{dsb-2} \); \( \text{dsb-1}(\text{tm5034}) \) animals also show a slight reduction in progeny viability with age. (D) Embryos from 8-10 hermaphrodites laid 0-30 hours post L4 (around 300 embryos for each genotype) were counted for \( \text{him-17} \) and \( \text{dsb-1}^{\text{SAQ}} \); \( \text{dsb-1}(\text{tm5034}) \); \( \text{him-17} \) animals. \( \text{him-17} \) animals show a decrease in progeny viability and an increase in percentage of male progeny compared to wild-type animals. This defect is partially rescued in \( \text{dsb-1}^{\text{SAQ}} \); \( \text{dsb-1}(\text{tm5034}) \); \( \text{him-17} \) animals.
Figure 3.5. *dsb-1* phosphomutants do not display meiotic defects or genomic instability. (A-C) Quantification of viable self-progeny for the indicated genotypes is shown. Homozygous *dsb-1* phosphomutants display similar levels of viability as wild-type or control animals. For each bar the number in parentheses indicates the total number of fertilized eggs counted.
Figure 3.6. Increasing DSB levels does not affect crossover distribution. Array based SNP genotyping was used to map crossover events in \( dsb-1^{5AQ} \) (ieSi25); \( dsb-1^{WT} \) (ieSi22); \( dsb-1^{tm5034} \) hermaphrodites that were heterozygous for Hawaiian and Bristol SNPs. A total of 33 and 48 F2 individuals were genotyped for \( dsb-1^{WT} \) and \( dsb-1^{5AQ} \), respectively. The total number of crossovers observed for each chromosome for \( dsb-1^{WT} \) and \( dsb-1^{5AQ} \), respectively, are as follows: 17 and 18 crossovers for Chr I; 15 and 26 crossovers for Chr III; 17 and 21 crossovers for Chr IV; 20 and 24 crossovers for Chr V; 15 and 21 crossovers for Chr X.
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