Small chromosomal structural variants are detected by the meiotic machinery in C. elegans

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Abstract

Heterozygous chromosomal structural variants are widely present in many different organisms. These variants exist in many different forms, including copy number variants, insertions, deletions, and translocations and can be present in both homozygous and heterozygous forms. The widespread nature of structural variation begs the question as to what happens to heterozygous structural variants when homologous chromosomes pair during meiosis and if pairing could allow the genome to detect the presence of heterozygous variants. Here, we investigated whether homolog pairing during meiosis allows for the detection of heterozygous deficiencies in *C. elegans*. By determining Rad51 levels and kinetics in 28 heterozygous deficiency strains and 7 control strains, we demonstrate that heterozygous deficiencies as small as 34kb and as large as 2647kb displayed elevated Rad51 levels during meiotic prophase I and are, therefore, detected by the meiotic machinery. We also find a rough positive correlation between deficiency size and Rad51 response. We further demonstrate that many heterozygous deficiency strains show increased levels of leptotene-zygotene nuclei in early pachytene. This type of delay has previously been associated with the presence of unsynapsed chromosomal regions during *C. elegans* meiosis. Additionally, we find that homozygous viable translocations and inversions show a reduced Rad51

response when homozygous compared to when heterozygous. Thus, we suggest that detection of heterozygous deficiencies is dependent on pairing of homologous chromosomes. Finally, we discuss the implications of these findings given the widespread nature of structural variants in the genomes of a wide array of organisms.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 2

Chapter 3

1 INTRODUCTION

Overview of Chapter

The ability of the meiotic genome to detect chromosomal structural variants may play important roles in many different gene regulatory processes. Past studies have established that the genome is able to detect and mark large structural variants, such as entire unpaired chromosomes or translocations of entire chromosomes arms in a homolog pairing-dependent manner. Surprisingly, these studies have not addressed whether similar detection processes act upon small chromosomal structural variants, such as heterozygous deletions or insertions. In this introductory chapter, I will begin by describing the roles that chromosomal interactions play in a variety of contexts, including regulatory interactions between enhancers and promoters, homolog pairing and interactions in which it plays a key part, such as transvection, and, finally, the function of chromatin insulators in mediating chromosomal interactions. Next, as homolog pairing and synapsis are important factors in my work, I will examine the mechanics of both processes during meiosis. Finally, as various forms of structural variation are very prevalent in the human genome and could be subject to detection, I will address current evidence that suggests chromosomal structural variants are detected by the genome during meiosis in several different species, which is the focus of this dissertation.

Many types of chromosomal interactions play important roles in different organisms

Work done in many organisms has shown that interactions between chromosomes play important roles in gene regulation (BAXTER *et al.* 2002; DEAN 2011; FRASER 2006; HEERMANN 2011; KANTIDZE and RAZIN 2009; VANDRIEL and FRANSZ 2004). For the purpose of this introduction, I have divided the various forms of chromosomal interactions into four different types dependent upon the type of interaction and its function: 1) Regulatory interactions, 2) Homolog pairing and transvection, 3) Organization of the nucleus, and 4) Functions related to insulation, such as active chromosome hubs and chromosomal domain formation. I will address each of these types of interactions below.

1) Regulatory interactions

Long-range interactions have been thought to occur since the discovery that enhancers often lay megabases away from their respective promoters. I will discuss several examples of these interactions below: For instance, Ashe *et al.* demonstrated such an interaction at the *β-globin* locus in human cell lines. In this study, human noneyrthroid cell lines, which do not intrinsically express β-globin, were transiently transfected with a plasmid containing the *β-globin* gene. Interestingly, while the endogenous gene copies remained inactive, a direct interaction between the endogenous locus and the plasmid resulted in activation of the plasmid copy of the gene (1997).

Another example of long-range enhancer-promoter interactions occurs in *HeLa* cells at the *β-globin* locus, where intervening chromatin between the enhancer and promoter loops out, allowing for the enhancer to interact directly with the promoter as determined by chromosome confirmation capture (3C) (DEKKER 2006; NOLIS *et al.* 2009). Furthermore, if the endogenous enhancer-promoter system is replaced with the exogenous bacterial λ repressor system, looping still occurs and allows for restoration of enhancer function (NOLIS *et al.* 2009).

Long-range enhancer promoter interactions may occur in a tissue-specific manner. One example occurs at the cystic fibrosis transmembrane conductance regulator gene (CFTR), which is expressed primarily in human epithelial cells. Ott *et al*. (2009) used 3C technology to establish that a 10kb chromatin loop forms between the CFTR intronic enhancer and the CFTR promoter and allows for direct contact of the enhancer and promoter. This interaction was present in epithelial cell lines and absent from control non-epithelial cell lines, confirming tissue specificity (OTT *et al.* 2009).

Long-range enhancer promoter interactions are not limited to mammalian genomes. In fact, one of the first demonstrations of long-range interactions was in Drosophila: Cléard and colleagues demonstrated in *Drosophila melanogaster* that components of the Bithorax complex (BX-C) participate in long-range interactions. In this instance, the authors used Dam identification (DamID), a method that involves tethering of a methyltransferase to a region of the genome and

determination of direct interactions by examining the genome for areas of increased methylation that would be indicative of close proximity of the two regions, to demonstrate a long-range interaction. DamID results showed an interaction between the *Fab-7* boundary element to the *Abdominal-Bm* promoter ~35kb away. This interaction is enriched in Drosophila head tissue compared to the abdominal tissues, indicating a tissue-specific regulatory effect (CLÉARD *et al.* 2006).

Another example of long-range regulatory interactions in Drosophila occurs in the Antennapedia complex. Here, Calhoun and Levine identified a regulatory sequence greater than 40kb from its interacting promoter of the *scr* gene, whose function is not currently known. The authors propose the formation of a chromatin loop between these regions that allows for maintenance of this interaction and activation of transcription (CALHOUN and LEVINE 2003).

Loop structures have also been identified in *Saccharomyces cerevisae* using 3C. By modifying the standard 3C methodology to increase detection of short-range interactions, Singh, *et al*. (2009) were able to demonstrate that loops as small as 1kb form between promoters and terminators of several yeast genes. A later study utilized this 3C tweak to determine that looping occurs at the yeast *GAL10* gene after a cycle of activation and repression. Thus, looping may occur in yeast in order to establish transcriptional memory and may tether transcriptional and RNA processing machinery to the nuclear pore, allowing for rapid reactivation of transcription at genes looped in this manner (LAINE *et al.* 2009). Taken together,

these examples indicate the importance of long-distance chromosomal interactions in gene regulation in many different organisms.

2) Homolog pairing and transvection

Homolog pairing is the process by which maternal and paternal homologous chromosomes pair and interact with each other. Pairing occurs in the Drosophila soma, though the level of pairing is dependent upon the assay used and at which point in the cell cycle the assays were conducted (CSINK and HENIKOFF 1998; reviewed in DUNCAN 2002; FRITSCH *et al.* 2006; FUNG *et al.* 1998; GEMKOW *et al.* 1998; Reviewed in MCKEE 2004; METZ 1916; STEVENS 1907; STEVENS 1908; VAZQUEZ *et al.* 2002). For instance, Fung and colleagues demonstrated using fluorescence *in-situ* hybridization (FISH) that between 85-100% of sites studied along chromosome arm 2L were paired within 5 days of egg deposition (1998). Gemkow *et al.* showed, also using FISH, that homolog pairing of chromosome 3R reaches a stable level of approximately 70% in postgrastrulating embryos and never reaches the level of 100% pairing, indicative of an equilibrium with the unpaired state (1998). Csink and Henikoff utilized FISH to demonstrate that somatic pairing levels on chromosome 2R ranged from 80-90% during G1 to a minimum of 60-70% during late S phase/G2 (1998). Vazquez and colleagues used FISH combined with live cell imaging to determine that pairing of homologs reaches its highest point during G2, consistent with prior FISH analyses of homolog pairing (2002). Interestingly, the mechanisms that establish and/or maintain somatic homolog pairing are currently unknown; however, recent work has shown that disruption of *topoisomerase II* via

either chemical inhibitors or RNAi significantly reduces levels of pairing from over 80% to less than 60% in Drosophila cell culture (WILLIAMS *et al.* 2007).

Additionally, the Drosophila *gypsy* insulator and its associated proteins, Suppressor of Hairy Wing (Su(Hw)), Modifier of mdg4 (Mod(mdg4)), and Centrosomal Protein 190 (CP190), have been implicated in somatic homolog pairing. These proteins colocalize at sites along chromosomes that do not correspond to *gypsy* binding sites and are thought to promote clustering of chromatin into chromatin domains (BRASSET and VAURY 2005; CAPELSON 2004; COMET *et al.* 2011; GASZNER and FELSENFELD 2006; KRAVCHENKO *et al.* 2005; KRIVEGA *et al.* 2010; KUHN 2003; VALENZUELA and KAMAKAKA 2006). As expected, loss of Su(Hw) disrupts homolog pairing, as demonstrated by Fritsch, *et al.* (2006) using FISH probes for chromosome 3R in eye disc nuclei. This observation is consistent with evidence that CCCTC binding factor (CTCF), another insulator-associated protein in mammals, is important for establishing and maintaining interchromosomal interactions (DONOHOE *et al.* 2007; LING *et al.* 2006; YAFFE and TANAY 2011).

Transvection: One of the most well studied examples of interchromosomal interactions is transvection (Figure 1a). Transvection is a process whereby homologous chromosomes can interact with each other, producing changes to gene regulation and chromosome topology (CHEN *et al.* 2002; DUNCAN 2002; GELBART 1982; KENNISON and SOUTHWORTH 2002; LEWIS 1954; MATZKE *et al.* 2001; MORRIS *et al.* 1998; RASSOULZADEGAN *et al.* 2002; SANDHU *et al.* 2009; SOUTHWORTH and KENNISON

2002; WU and MORRIS 1999). The most well known type of transvection is enhancer action in trans, during which an enhancer on one homologous chromosome activates transcription of a promoter on the other homolog. This process typically occurs when one homolog has a disabled or deleted promoter, and the other homolog has either a disabled enhancer or lack thereof. Alternatively, mutation of core promoter elements can also influence whether an enhancer acts in cis or in trans (MORRIS *et al.* 2004).

There is genetic evidence for another form of transvection, in which there are homolog pairing-mediated changes to gene topology (Figure 1b). Here, the presence of structural heterology, such as a heterozygous insertion or deletion, disrupts local homolog pairing (MORRIS *et al.* 1998). A chromatin loop may form at the disruption due to the flanking forces of homolog pairing.

It is important to note that some forms of transvection are dependent on homolog pairing and that transvection can be disrupted when genomic rearrangements prevent pairing of the region of interest. This property of transvection was first demonstrated by Lewis in 1954, who found that X-ray induced rearrangements between the transvecting allele, in this instance, the Ultrabithorax gene, and the centromere disrupted transvection (LEWIS 1954). Additional studies of other loci that demonstrate transvection have seen similar disruption effects when chromosomal rearrangements affecting the transvecting loci are introduced. For instance, Geyer, *et al.* demonstrated that homolog pairing appears to be required for

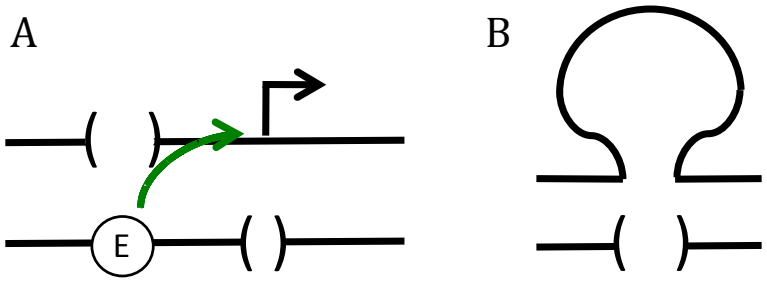

Figure 1. Examples of transvection in *Drosophila melanogaster.* 1a) Transvection via enhancer action in trans. The trans-acting enhancer is designated as " E." The enhancer activates transcription of a gene on the homologous chromosome. 1b) Homolog pairing-mediated changes to gene topology. Homolog pairing forces the chromatin opposite of a deficiency into a loop.

transvection at the *yellow* locus, as *yellow* alleles inserted at other loci do not result in transvection (1990). In this instance, when one of the *yellow* alleles undergoing transvection was inserted at ectopic locations throughout the genome, transvection was disrupted, suggesting that transvection at *yellow* requires homolog pairing.

Similarly, transvection at the *decaplentaplegic* (*dpp*) gene complex, which is important for proper wing development, is disrupted when rearrangements that disrupt homolog pairing are introduced (GELBART 1982). Here, the presence of heterozygous rearrangements of chromosome arm 2L reduces transvection. Consistent with these observations, double rearrangement heterozygotes, in which two rearrangements with similar breakpoints are combined, restore some level of pairing and, as a result, allow for transvection (GELBART 1982). The Drosophila *vestigial* (*vg*) locus behaves in a similar manner in regards to disruption of transvection. *Vestigial* is important for proper wing, haltere, and bristle

morphologies. At this locus, rearrangements between the centromere and the *vg* locus disrupt transvection. Like *dpp*, transvection is somewhat restored when two rearrangements with breakpoints on the same chromosome arm are placed in trans, provided that the two rearrangements result in chromosomes with similar sized arms (COULTHARD 2005). Lastly, the Drosophila *eyes absent* gene (*eya*) behaves in a similar manner to *dpp* and *vg*. Flies homozygous for mutant *eya* alleles display reduced or absent eyes. When structurally normal chromosomes are present at the *eya* locus, transvection is observed, as the resultant fly eyes are near normal-sized. However, rearrangements located between *eya* and its centromere result in reduced eye size, indicating that transvection is disrupted (LEISERSON *et al.* 1994). These and other studies of transvection highlight the importance of homolog pairing in gene regulation.

Proteins may also play a role in the regulation of transvection. For instance, the Drosophila protein Zeste affects some instances of transvection (MARIANI *et al.* 1985; PIRROTTA 1991; PIRROTTA *et al.* 1987). One example of such an interaction occurs at the *white* gene, which is responsible for proper red eye pigmentation, located on the X chromosome. Wild-type flies display red eyes; however, in a *zeste*^{1} mutant background, female flies carrying two copies of the *white* gene display reduced eye pigmentation, whereas females carrying only one copy of *white* display wild-type red eyes. In this instance, it is thought that hyperaggregation of the Zeste1 mutant protein may exert a pairing-sensitive silencing effect on the *white* gene (CHEN

and PIRROTTA 1993a; CHEN and PIRROTTA 1993b; DUNCAN 2002; JACK and JUDD 1979; ZACHAR *et al.* 1985). This is likely due to the fact that the *white* enhancer contains five Zeste binding sites, which allow aggregation of Zeste protein directly at the eye enhancer (BENSON and PIRROTTA 1988; CHEN *et al.* 1992; QIAN *et al.* 1992).

Interestingly, Zeste has also been implicated in long-range interactions at several loci in Drosophila (DORSETT 1999; LANEY and BIGGIN 1997; QIAN *et al.* 1992). In one case of transvection at *Ubx*, *zeste* is required for transactivation, but dispensable for cis-activation, indicating that Zeste plays a role in interchromosomal interactions, but does not appear to play a role in intrachromosomal interactions (GOLDSBOROUGH and KORNBERG 1996). In the case of pairing-sensitive silencing at *white*, the presence of interacting chromosomal elements on both homologs, self-agreggation of Zeste, and the ability of Zeste to interact with silencing maintenance complexes, such as PcG complexes, are essential for the silencing interaction to occur (BICKEL and PIRROTTA 1990; LANEY and BIGGIN 1997; QIAN *et al.* 1992; ROSEN *et al.* 1998).

More recent analyses have re-examined the effects of Zeste on transvection at *white* in the context of long-range interactions. Here, when the eye enhancer and *white* promoter are less than 3kb apart, Zeste is not required for enhancer-promoter functionality. However, when the eye enhancer and *white* promoter are separated by

more than 3kb or when the 8kb *yellow gene* is inserted in the intervening space, Zeste is required for *white* expression (KOSTYUCHENKO *et al.* 2009). These data further implicate Zeste in long-range interactions.

The abovementioned examples of transvection occur in Drosophila where homologous chromosomes are closely paired in all somatic tissues; however, most organisms do not display a high level of homologous chromosome pairing, with such interactions being transient and specific to certain loci. One example of a transient pairing interaction in mammalian cells happens during X chromosome inactivation (XCI). XCI is the process by which some organisms inactivate one X chromosome in XX females to compensate for differential dosage of the X between XX females and XY males (LYON 1999). XCI is regulated by three non-coding genes on the X chromosome: *Xite, Tsix,* and *Xist*, contained within what has been deemed the "X-inactivation center". On the inactive X, Xist RNA is expressed and forms an "Xist cloud" on the inactive chromatin. Xist RNA is then thought to recruit further silencing factors, such as the Polycomb Group (PcG) repressive complexes, which function to maintain X-inactivation in further generations. On the active X, expression of Xist is blocked by transcriptional interference from Tsix, allowing active chromatin to persist (AVNER and HEARD 2001; BROCKDORFF 2011; FAN and TRAN 2011; MOREY and AVNER 2011).

Intriguingly, homolog pairing plays an important role in the stochastic choice that determines which X is active and which is inactive (BROCKDORFF 2011; MARAHRENS 1999). Two studies utilized FISH probes near to the Xic to establish that two regions X chromosomes transiently pair during the onset of XCI (AUGUI *et al.* 2007; XU 2006). More recently, Masui and colleagues (2011) used live-cell imaging to confirm that transient X pairing occurs at the onset of XCI and that, post-pairing, *Tsix* expression is transiently monoallelic, allowing a window for Xist expression on the future inactive X chromosome. Thus, these data provide the first direct evidence that transient Xic pairing is important in the choice between the active and inactive X chromosomes.

Another transient interaction between homologs occurs during V(D)J recombination in B and T cell development in mammals. V(D)J recombination ensures that the immune system generates a random complement of novel antigen receptors responsible for detection of infectious agents. During this process, homologous IgG alleles pair in a process dependent on the associated V(D)J recombinase, RAG1/RAG2. When either RAG1 or RAG2 was deleted, pairing IgG alleles was reduced ten-fold. It is thought that this interaction constricts RAG-induced DNA cleavage to only one homologous allele, as the second allele is relocated to the different region of the nucleus post-cleavage (BRANDT *et al.* 2010).

Moreover, there is additional evidence for a somatic homolog pairing event in human renal oncocytoma. Koeman, *et al.* (2008) utilized chromosome painting

technology to discover that the human 19q chromosome arm is somatically paired in renal oncocytoma cells. Remarkably, pairing is associated with an upregulation of the EGLN2 gene, which is involved in the oxygen-sensing pathway and could be important for providing adequate blood and oxygen supplies to the growing tumors (KOEMAN *et al.* 2008). While this association is interesting, causality has not yet been determined.

Lastly, there is further evidence that transient homolog pairing interactions may be developmentally and cell cycle regulated. In one example, a deletion of a specific region of human chromosome 15, 15q11-13, has been previously associated with increased risk for autism spectrum disorders. Thatcher and colleagues (2005) demonstrated, using FISH probes to this region, that there is significantly increased association between the two homologous chromosomal regions in samples taken from infant through juvenile-aged brains. Interestingly, when these control samples from normal tissue were compared to those derived from tissue of individuals with autism spectrum disorders, the tissue from autistic individuals showed a significant decrease in homolog pairing of 15q11-13 (LEUNG *et al.* 2011; THATCHER 2005).

Further evidence suggests that transient pairing interactions between heterochromatic regions play a role in maintaining chromatid cohesion during meiosis. Here, major and minor satellites within the mouse centromere were shown to display distinct sets of interactions. The major and minor satellites from different chromosomes form distinct clusters within meiotic nuclei. Genomic regions

associated with clusters of major satellites replicated asynchronously and showed prolonged sister chromatid cohesion when compared to minor satellites (GUENATRI 2004). Thus, these data suggest that major satellite domains may be important for centromere cohesion and proper segregation of chromatids during meiosis.

3) Organization of the nucleus

The preponderance of cancer-associated rearrangements that have been found in many different isolates of the same cancer type have begged the question as to whether mammalian nuclei organize their chromosomes into distinct chromosomal domains. Early evidence in Giemsa-stained Chinese hamster ovary nuclei showed a globular chromatin structure, which suggested such an organization. More recent studies using multi-color FISH in human fibroblast nuclei provided direct evidence that, indeed, the genome is organized into chromosomal territories. The presence of territories may be important for determining partner selection during the formation of chromosomal rearrangements by enhancing the possibility of a rearrangement between chromosomes in neighboring domains. Given that different types of cancer typically display distinct rearrangements, it is likely that the nuclear localization of chromosome territories varies depending on cell type (CREMER and CREMER 2010; WIJCHERS and DE LAAT 2011).

Excitingly, chromosomal territories and long-range looping interactions have been shown to work in concert to regulate gene expression. For instance, the mouse Sonic hedgehog (Shh) gene is expressed in developing limb buds and is controlled by

an enhancer 1Mb upstream from its promoter. 3D-FISH and 3C analyses of this interaction demonstrated that the promoter and upstream enhancer form a 1Mb loop and contact each other in cells that are competent to express Shh. Interestingly, gene activation may be dependent on the location of the loop structure within chromatin territories within the nucleus. Specifically, when Shh expression is active, the Shh locus is found outside of its characteristic chromosome territory. This change in localization may correspond to recruitment of the Shh locus to an region of enhanced transcription outside of its chromosome territory (AMANO *et al.* 2009; FERRAI and POMBO 2009).

Similar chromosome looping and relocalization is seen at HOX gene clusters. In this instance, 3C has shown that silent HOX gene clusters display a characteristic arrangement of chromatin loop structures (FERRAIUOLO *et al.* 2010). As individual HOX genes are activated during specific stages of development, decondensation of the silenced conformation is observed during which activated genes loop out of their chromosome territory. Once transcription is down-regulated, looped out regions return to their original looped and clustered formation (CHAMBEYRON 2004; CHAMBEYRON 2005; FERRAIUOLO *et al.* 2010). Like activation of Shh, this relocalization of actively transcribed HOX genes to locations outside of their chromosome territories may represent recruitment to regions of actively transcribed chromatin within the nucleus.

4) Insulator Functions

A third, important type of chromosomal interaction is mediated by interactions between chromatin insulators. Insulators are chromosomal elements that mediate intra- and interchromosomal interactions and are involved in regulating transcription and nuclear architecture (reviewed in GERASIMOVA and CORCES 2001; GURUDATTA and CORCES 2009; KRIVEGA and DEAN 2011; KUHN and GEYER 2003; WALLACE *et al.* 2009; WOOD *et al.* 2011; YANG and CORCES 2011). Examples of insulator-mediated interactions have been discovered in many different organisms.

One of the most well studied insulators is the *gypsy* insulator present in Drosophila, which has been shown to mediate several different types of interactions (GEYER and CORCES 1992). First, Gerasimova and Corces demonstrated that, using FISH probes targeted to known *gypsy* binding sites, *gypsy* insulators localized to specific clusters within the nucleus, deemed insulator bodies. Based upon the known locations of *gypsy* binding sites on Drosophila chromosomes, the authors postulated that interactions between *gypsy* sites within the insulator bodies would produce chromatin loops of approximately 250kb, due to looping out of regions between binding sites (GERASIMOVA *et al.* 2000; GERASIMOVA and CORCES 1998).

Additionally, further studies examining interactions between *gypsy* insulators demonstrated that *gypsy-gypsy* interactions generate loops ranging in size from 1.5kb to 150kb can be generated. These data suggest that insulator body structures are forming in nuclei and that, given the small size of the proposed loop structures,

steric hindrance is not a factor in loop formation (BYRD and CORCES 2003; CAI and SHEN 2001; MONGELARD and CORCES 2001; SAVITSKAYA *et al.* 2006).

Insulators may also play a role in maintaining higher order chromatin structure. For example, insulator-mediated chromatin loops may regulate transcription by isolating enhancers and respective enhancers into different chromatin domains. This separation may prevent enhancers from directly contacting promoters, eliminating transcription (BRASSET and VAURY 2005; MAEDA and KARCH 2007; WALLACE and FELSENFELD 2007).

Another example of insulators regulating genes through the creation of chromosome domains occurs in the Drosophila BX-C. BX-C contains three homeotic genes (*Ubx, abd-A,* and *Abd-B*) and an interval of approximately 300kb of regulatory elements (AKBARI *et al.* 2006; BENDER and HUDSON 2000; LEWIS 1978; MAEDA 2006; MAEDA and KARCH 2010; MCCALL and O'CONNOR 1994; SPITZ 2010). This region contains three insulators that function as boundary elements, and are thought to restrict expression of the three homeotic genes to the appropriate body segments within the developing fly via creation of differential chromatin domains that restrict the activity of enhancers and promoters to within the same loop structure (BARGES *et al.* 2000; GYURKOVICS *et al.* 1990; KARCH *et al.* 1994; MIHÁLY *et al.* 1997).

In an analogous manner, mammalian insulators may perform similar functions in maintaining nuclear architecture, the best studied of which is the

human CTCF insulator. For instance, at the paternally imprinted human *Igf2/H19* locus, 3C confirmed that *Igf2* promoters interact consistently with the appropriate endodermal enhancers in all instances examined. At *Igf2/H19,* the imprinting control region (ICR) regulates proper imprinting. Interestingly, a CTCF-mediated insulator present in the ICR interacts with inactive enhancers and promoters, forming loops that are required for maintenance of the maternal imprint at this locus (YOON *et al.* 2007).

Further evidence for the role of CTCF in maintaining higher order chromatin structure has been found at the α-globin locus. Here, carbon-copy chromosome conformation capture (5C), was utilized to build a three-dimensional map of interactions the locus. The data illustrate that two "chromatin-globules" form, concentrating active genes toward the center of the globules, and inactive genes toward the periphery, with paired CTCF sites playing a role in maintaining these structures (BAÙ *et al.* 2011). The concentration of active genes to the center of these globules may be an example of a "transcription factory," which is a region of the genome where actively transcribed genes are enriched, perhaps to facilitate access to transcription machinery.

More recent studies have expanded on previous work of CTCF-mediated interactions by using high-throughput techniques to develop genome-wide maps of chromatin interactions, called "interactomes." In one such study, the authors used chromatin interaction analysis by paired-end tag (ChIA-PET) sequencing to confirm

that CTCF forms chromatin domains and potentiates transcription via extensive interactions with promoters and enhancers (HANDOKO *et al.* 2011). A second study used a similar high-throughput mapping technology, Hi-C, to examine genome-wide patterns of CTCF interactions. Here, the data showed that, when regions from 0-5kb away from either side of a CTCF site are compared, there is increased CTCF binding to one side of the CTCF binding site. These data are consistent with the formation of CTCF-mediated chromatin domains (YAFFE and TANAY 2011).

The aforementioned examples of different types of chromosomal interactions highlight the importance of intra- and interchromosomal interactions in the regulation of genes and genomic architecture. Interestingly, though while the abovementioned interactions occur in somatic tissues, chromosomal interactions also play a very important role in meiotic cells.

Homolog pairing and synapsis in *C. elegans*

While somatic homolog pairing has been shown to occur in Diptera and transiently in other organisms, such as humans, meiotic homolog pairing has been shown to occur in during meiosis in most organisms. Meiosis is a process that ensures genetic diversity through regulation of recombination events and accurate chromosome segregation into gametes. Homolog pairing during meiosis precedes the formation of the synaptonemal complex (SC), a proteinaceous structure responsible for maintaining alignment of homologous chromosomes during meiosis. It is currently thought that homolog pairing begins during the leptotene-zygotene

stages of meiosis, whereas synapsis initiates during early pachytene stage and that homolog pairing and synapsis are somewhat independent processes (BARLOW and HULTÉN 1996; LOIDL *et al.* 1994; PAWLOWSKI and CANDE 2005; ROEDER 1997; SCHERTHAN *et al.* 1992; WEINER and KLECKNER 1994; XU *et al.* 1997). For the purposes of this dissertation, homolog pairing will refer to the presynaptic alignment of homologous chromosomes and synapsis will refer to the state during which homologous chromosomes are bound by the proteinaceous SC.

Homolog pairing is dependent on the dynamic movements of chromosomes within the nucleus. Such movements allow homologous chromosomes to find one another in the comparatively large nuclear compartment. Chromosomal movements are dependent on SUN and KASH-domain proteins (STARR 2011). KASH proteins interface with cytoskeletal networks, such as actin and microtubules, in the cytoplasm and their localization is restricted to the outer nuclear membrane. The SUN family of proteins is present on the inner nuclear membrane and serves to connect KASH proteins to chromosomes present within the nucleus. Taken together, the ultimate function of SUN/KASH proteins is to transfer mechanical motion from cytoskeletal networks outside of the nucleus through the nuclear membranes and into the nucleus, where the motion is transferred to chromosomes undergoing the search for a homologous partner; this transfer of motion is often accomplished via pairwise interactions between SUN and KASH proteins (BAUDRIMONT *et al.* 2010; HAQUE *et al.* 2006; MCGEE *et al.* 2006; MINN *et al.* 2009; TAPLEY *et al.* 2011).

In *C. elegans*, the SUN/KASH pair responsible for the transfer of movement from the cytoplasm into the nucleus consists of SUN-1 and ZYG-12 (SATO *et al.* 2009). It is thought that rapid chromosomal motion transferred in this manner is responsible for eliminating transient interactions between homologs that are not productive in establishing the correct homolog-homolog interactions and preventing inappropriate recombination events from proceeding (BAUDRIMONT *et al.* 2010; KOSZUL and KLECKNER 2009).

C. elegans is unique in the mechanism that it utilizes to synapse homologous chromosomes during meiosis. *C. elegans* chromosomes have genetically and molecularly defined regions at the end of each chromosome that stabilize pairing interactions and promote SC assembly. These regions are called the pairing centers (PCs) (MACQUEEN *et al.* 2002; MACQUEEN *et al.* 2005). Disruption of these regions via deletions or translocations leads to problems with synapsis (MCKIM *et al.* 1988; MCKIM *et al.* 1993; VILLENEUVE 1994; ZETKA and ROSE 1995; ZETKA and ROSE 1992). Further studies identified a series of zinc finger proteins that are recruited to the specific motifs at the PCs and play a central role in establishment and maintenance of homolog pairing and synapsis. Each protein localizes to the PCs of one or two sets of homologous chromosomes during prophase I: HIM-8 to the X chromosome, ZIM-1 to chromosomes II and III, ZIM-2 to chromosome V, and ZIM-3 to chromosomes I and IV (PHILLIPS and DERNBURG 2006b; PHILLIPS *et al.* 2009; PHILLIPS *et al.* 2005).

Additionally, two recent studies have shown that the polo kinases PLK-1 and PLK-2 are recruited to PCs to provide dynamic interactions with the nuclear membrane and the PC end of chromosomes. Here, PLK-2 is required for interhomolog interactions as disruption causes synapsis between non-homologous chromosomes. Furthermore, PLK-2 is responsible for phosphorylation of SUN-1 and formation of SUN-1/ZYG-12 interactions that transmit motion required for homolog pairing from the cytoskeleton into the nuclear compartment (HARPER *et al.* 2011; LABELLA *et al.* 2011). Moreover, *plk-2* is necessary for the cell cycle delay and apoptosis of nuclei with asynapsed chromosomes (HARPER *et al.* 2011).

Implications for homolog pairing in humans

As homolog pairing also plays important roles during meiosis, it is possible that similar loop structures are formed when chromosomal structural variation, such as a heterozygous deficiency or insertion, is present during synapsis. Interestingly, many human copy number variants (CNVs) exist in the genome as structural variations and may form similar structures during meiosis. Human CNVs are regions of the genome ranging in size from several base pairs up to several megabases that exist in variable copy number throughout the population (ALKAN *et al.* 2011; FREEMAN 2006; IAFRATE *et al.* 2004; LEE and SCHERER 2010; PERRY *et al.* 2008; SEBAT 2004). For instance, if you compare any two individuals, they are likely to differ in dozens or even hundreds of loci (ALKAN *et al.* 2009).

Since their discovery in 2004, CNVs have become an exciting topic in genetics due to their connections with an array of human diseases. To mention only several recent reports, CNVs have been associated with cancer (CAMPS *et al.* 2008; LEBRON *et al.* 2011; NORSKOV *et al.* 2010; SHLIEN *et al.* 2010; SPELEMAN *et al.* 2008; TRAN *et al.* 2011; YOSHIHARA *et al.* 2010), schizophrenia (BUIZER-VOSKAMP *et al.* 2011; MORENO-DE-LUCA *et al.* 2010), autism (BREMER *et al.* 2010; MORENO-DE-LUCA *et al.* 2010; SANDERS *et al.* 2011), Alzheimer disease (BROUWERS *et al.* 2011; KAY *et al.* 2010; SHAW *et al.* 2011; ZHANG *et al.* 2009a), and Crohn's disease (MCCARROLL and ALTSHULER 2007; MCCARROLL *et al.* 2008; ZHANG *et al.* 2009b) reviewed by (KUIPER *et al.* 2010; LEE and SCHERER 2010). Given that many CNVs exist within the population as deletions, insertions, and duplications (MCCARROLL *et al.* 2006; MULLANEY *et al.* 2010), CNVs may generate considerable genomic structural heterogeneity, which could be detected by the meiotic machinery when homologous chromosomes pair and synapse during prophase I.

Evidence for the detection of unpaired chromosomal regions during meiosis

Evidence for detection of unpaired chromosomal regions exists in many different organisms. Detection processes may be enriched during meiosis, as pairing of homologous chromosomes and synapsis allows for direct comparison of each pair of homologs. One example of detection processes is meiotic silencing of the sex chromosomes (MSCI). Here, the meiotic silencing process is able to detect the largely unpaired X and Y chromosomes.

Meiotic silencing of the sex chromosomes

The X and Y chromosomes present an interesting variation in the pairing process, as they are homologous across *only a small segment,* called the pseudoautosomal region and are, therefore, largely unpaired (Figure 2). We now know that meiotic homolog pairing also plays a role in gene regulation. This is illustrated by the phenomenon of meiotic silencing.

The history of our understanding of meiotic silencing begins with early observations of an intriguing structure in mammalian, marsupial, and insect nuclei called the sex body, a chromosomal domain which has since been shown to contain the sex chromosomes (MOHR 1916; OHNO and MAKINO 1961; PAINTER 1924; SOLARI 1974). Studies done in the years spanning 1891 through the 1970s gave this structure many different monikers, from intranuclear body to chromatin nucleolus and X-vesicle and, in fact, it was the earlier of these studies that allowed for the discovery of the X chromosome (HENKING 1891; LENHOSSEK 1898; MAKINO 1941; PAINTER 1924).

Later studies done in a variety of organisms during the 1950s further characterized the sex body, showing that the X and Y chromosomes are condensed and heterochromatinized during meiosis (OHNO and MAKINO 1961; SACHS 1954). Consistent with these observations, studies of ^{3}H-uridine incorporation done in the 1970s showed that transcription of the XY pair is greatly reduced during meiosis in the mouse (HENDERSON 1964; KIERSZENBAUM and TRES 1974a; KIERSZENBAUM and TRES

1974b; MONESI 1965; UTAKOJI 1966). More recently, advances in immunofluorescence staining technology have shown that the X and Y chromosomes become associated with numerous proteins, including Hormad1, BRCA1, ATR, γH2AX, H3K9me2 and UBR2, and are eventually transcriptionallysilenced. This process was named Meiotic Sex Chromosome Inactivation (MSCI) (AN *et al.* 2010; BAARENDS *et al.* 2005; CLOUTIER and TURNER 2010; KEEGAN *et al.* 1996; MOENS *et al.* 1999; SCULLY *et al.* 1997; SHIN *et al.* 2010; TURNER 2007; TURNER *et al.* 2004; TURNER *et al.* 2002; TURNER *et al.* 2006; ZHOU *et al.* 2008).

The first hypotheses as to why meiotic nuclei undergo MSCI were published in the 1970s. Initially, it was thought that the sex chromosomes are transcriptionally silenced because male fertility is dependent on the absence of X chromosome transcription (FOREJT 1982; LIFSCHYTZ and LINDSLEY 1974; LIFSCHYTZ and LINDSLEY 1972). A later study hypothesized that meiotic silencing reflects the state of sex chromosome pairing where, in the heterogametic sex, lack of robust pairing between the X and Y leads to silencing (JABLONKA and LAMB 1988). This hypothesis has held true; the minimal pairing of the X and Y chromosomes *is*, in fact, what induces the formation of the sex body, causing unpaired regions to accumulate marks associated with silenced chromatin (Figure 2) (AN *et al.* 2010; BAARENDS *et al.* 2005; SHIN *et al.* 2010; TURNER 2007; TURNER *et al.* 2000; TURNER *et al.* 2006; TURNER *et al.* 2005; ZHOU *et al.* 2008).

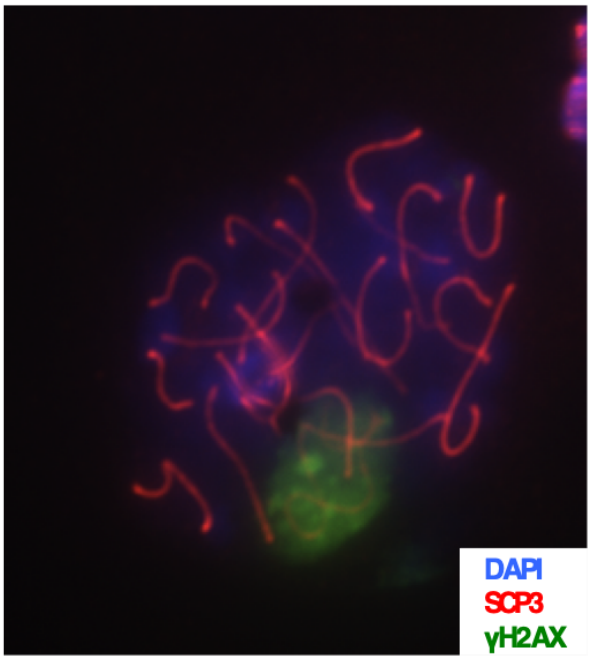

Figure 2. Mouse pachytene spermatocyte. The largely unpaired XY chromosome pair is enriched for γH2AX, indicative of a silencing response.

If the underlying basis for MSCI is the largely unpaired status of the sex chromosomes, one might predict that a process analogous to MSCI should occur on any unpaired chromosomal region, even if autosomal. Recent studies have confirmed this prediction; the silencing response is not restricted to the sex chromosomes, but also affects unpaired regions of the autosomes. Thus, in a fashion similar to that of the X and Y chromosomes, unpaired autosomal regions accumulate marks characteristic of silenced regions, including Hormad1, BRCA1, ATR, H3K9me2, UBR2, and γH2AX (Baarends *et al.* 2005; Ferguson *et al.* 2008; Fukuda *et al.* 2010; Homolka *et al.* 2007; Khalil *et al.* 2004; Mahadevaiah *et al.* 2008a; Manterola *et al.* 2009; Shin *et al.* 2010; Turner 2007; Turner *et al.* 2005). This process is called meiotic silencing of unpaired chromatin (MSUC) (Figure 3).

During MSUC, it is currently thought that BRCA1 is localized on unsynapsed chromosome axes during early pachytene and then recruits ATR (ataxia telangiectasia and Rad3 related) to the unsynapsed axes. ATR then phosphorylates histone H2AX, producing γH2AX, which is thought to initiate the silencing response. Additional proteins indicative of the silencing response, such as H3K9me2 appear

during mid-pachytene and persist through diplotene (Figure 3) (MAHADEVAIAH *et al.* 2008b; MANTEROLA *et al.* 2009; TURNER *et al.* 2004; TURNER *et al.* 2005). It is possible that meiotic silencing functions through mRNA intermediates that direct silencing proteins to unsynapsed regions, perhaps through the mouse Maelstrom protein (COSTA *et al.* 2006). However, the involvement of small RNAs in mammalian meiotic silencing responses has yet to be firmly established. A second silencing mechanism may exist independently of the BRCA1-mediated MSUC response. In this mechanism, UBR2, an E3 ubiquitin ligase, associates with unpaired chromosomal axes independent of BRCA1. UBR2 then ubiquitinylates histone H2A, potentially resulting in transcriptional silencing (AN *et al.* 2010; BAARENDS *et al.* 2005; ZHOU *et al.* 2008).

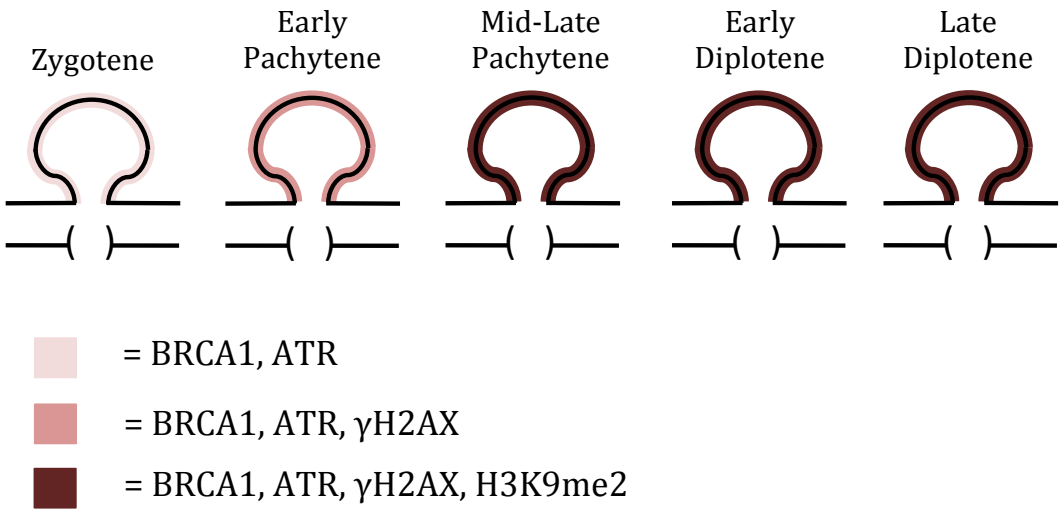

Figure 3. Meiotic silencing of unpaired chromatin (MSUC). During MSUC, unpaired chromosomal regions first accumulate BRCA1 and ATR. Upon entering pachytene, these regions further accumulate γH2AX and H3K9me2 and are transcriptionally silenced. Adapted from Manterola, *et al.* (2009).

While the early studies conducted by Turner and colleagues firmly established that the unpaired regions of the X and Y chromosomes undergo meiotic silencing, more recent work has confirmed that autosomal regions are subject to the same process. For instance, a heterozygous translocation in mice between chromosomes 16 and 17 showed elevated levels of BRCA1 and γH2AX, consistent with MSUC. Additionally, the rearranged chromosomes were shown to associate with the XY body and disrupted proper activation of the X chromosome, resulting in male sterility (HOMOLKA *et al.* 2007).

A second study examined meiosis in human carriers of a reciprocal translocation between chromosomes 8 and 13. In this study, immunofluorescence techniques showed that BRCA1 and γH2AX associated with unsynapsed regions of chromosomes containing the translocation. Additionally, analysis with an antibody for RNA polymerase II (PolII) illustrated that both the XY chromosome pair and the unpaired translocation chromosomes displayed no PolII staining, suggesting that both sets of chromosomes are silenced (FERGUSON *et al.* 2008).

Interestingly, the extent of asynapsis within meiotic nuclei plays a role in the integrity of the meiotic silencing response in mouse oocytes. When greater than ten percent of chromosomal axes are unsynapsed, BRCA1 and ATR recruitment to chromosome axes is substantially reduced. Furthermore, when greater than two to three unpaired autosomes were present in oocyte nuclei, MSUC is impaired as

shown by increased PolII staining in the XY body compared to nuclei with lower levels of asynapsis (KOUZNETSOVA *et al.* 2009).

Similarly, work done in *C. elegans* has shown that unpaired regions of the genome are enriched for silencing marks during meiosis. This process is also not limited to the single, unpaired X chromosome in XO males, but also marks autosomes that are unpaired due to mutations in genes that are essential for meiotic homolog pairing; for example, mutations in *zim-2* and *him-8*, which unpair chromosome V and the X chromosome, respectively, lead to meiotic silencing (BEAN *et al.* 2004; PHILLIPS and DERNBURG 2006a; PHILLIPS *et al.* 2005). The unpaired chromosomal regions are marked in early pachytene with histone modifications that are indicative of silenced chromatin, such as H3K9me2, and retain these marks until meiotic division (BEAN *et al.* 2004; KELLY and ARAMAYO 2007). Additionally, meiotic silencing in *C. elegans* requires components of a small RNA-mediated pathway, which could potentially be important for targeting of silencing proteins and markers to unsynapsed regions (MAINE *et al.* 2005; SHE *et al.* 2009).

Insights from Neurospora further implicate pairing as key step in meiotic silencing and detection of unpaired regions: MSUD and RIP

Our understanding of meiotic silencing in mice and *C. elegans* has benefited greatly from prior discoveries of pairing-mediated silencing phenomena in the fungus *Neurospora crassa*. One such phenomenon is meiotic silencing by unpaired DNA (MSUD). During MSUD, unpaired regions of the genome are aberrantly

transcribed and, ultimately, silenced during meiosis (Figure 4) (ALEXANDER *et al.* 2008; NAKAYASHIKI 2005; SHIU *et al.* 2001). Through elegant genetic studies using the ascospore maturation-1 gene, Aramayo and Metzenberg showed that copies of this gene lacking a pairing partner in meiosis were silenced (1996). Subsequently, Shiu and Metzenberg confirmed that MSUD affects only unpaired chromosomal regions (2001). MSUD is capable of silencing regions on the order of 500-100bp in length, highlighting the sensitivity of this process to the paired state of homologs.

The Neurospora genome harbors a second pairing-mediated mechanism for silencing, repeat-induced point mutation (RIP), which silences duplicated chromosomal regions. During RIP, duplications are believed to pair and then undergo a random process of mutation in which C residues are changed into T, after which, the duplicated regions are marked by histone H3K9me3 (SELKER *et al.* 1987; TAMARU *et al.* 2003). RIP can recognize duplications as small as 400 bp in size, if the duplications are linked, or 1 kb, if the duplications are unlinked (WATTERS *et al.* 1999). Like MSUD, RIP speaks to the exquisite sensitivity that homolog pairing affords to the processes that it mediates.

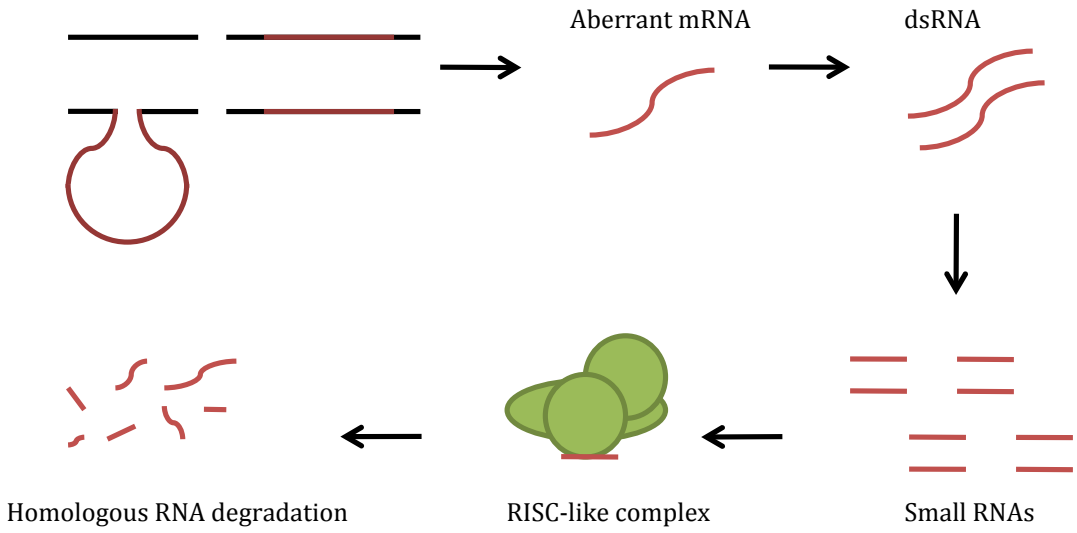

Figure 4. Meiotic silencing by unpaired DNA (MSUD). During MSUD, unpaired regions of chromosomes (shown in red) are aberrantly transcribed and produce complementary small RNAs, which target silencing machinery to the unpaired locus and any other homologous regions within the genome.

Preview of dissertation

In many different organisms, there is evidence for detection of unpaired chromosomal regions during meiosis. These phenomena range from MSUD and RIP in *N. crassa*, to MSCI and MSUC in *C. elegans* and mice. While these studies have demonstrated the ability of the meiotic genome to detect large structural variants, such as translocations and entire unpaired chromosomes, they have not addressed if smaller variants can be detected during meiosis. My work sought to resolve this this lack, utilizing a series heterozygous deficiencies in the *C. elegans* germline. Heterozygous deficiencies are an idea type of structural variant to study in this context, as they are present in a wide range of sizes and their presence can be

verified with quantitative technologies, such as quantitative PCR. Furthermore, the *C. elegans* germline is an excellent system for these analyses due to the ease at which gonads can be extracted and stained for various markers of detection. Moreover, the germline is a temporal-spatial gradient of progression through meiosis, allowing for easy evaluation of the various stages of meiosis.

Chapter 2 demonstrates the ability of the genome to detect large chromosomal structural changes. First, by comparing Rad51 response in homozygous viable translocations and an inversion, we show that homolog pairing appears to play a central role in detection of structural variants, as homozygous translocations and inversions show a reduced Rad51 response compared to their heterozygous counterparts. Secondly, by combining deficiencies with high and low Rad51 response, we demonstrate that the resultant double deficiency strains show an intermediate level of response, indicative of an epistatic interaction between deficiencies with high and low response. Lastly, we combined FISH using probes to deficiency loci with immunostaining for Rad51 foci. Our data show a low level of Rad51 localization at deficiency loci, indicating that mechanisms preventing colocalization may be in play.

Chapter 3 illustrates the ability of the *C. elegans* genome to detect heterozygous deficiencies ranging in size from approximately 34 kb to 2650 kb, using Rad51 and a delay in meiotic progression as readouts for detection. Additionally, our data indicate that there is a rough positive correlation between

deficiency size and Rad51 response and that this response is not chromosome-specific. Furthermore, the data suggest that a delay in meiotic progression may be an independent readout for detection of structural heterozygosities.

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2 Can meiotic cells detect chromosomal structural variants?

Matthew Jakubik conducted all experiments and data analysis.

INTRODUCTION

Homolog pairing is the process by which maternal and paternal homologous chromosomes pair each other. Pairing occurs in Drosophila somatic tissues (STEVENS 1907; STEVENS 1908; METZ 1916; FUNG *et al.* 1998; MCKEE 2004) and in the meiotic tissues of most organisms. Work done in many organisms has shown that interactions between homologous chromosomes can play important roles in gene regulation. One of the most well studied examples of such interactions is transvection. In transvection, homologous chromosomes interact with each other and as a result, produce changes to gene regulation and chromosome topology (LEWIS 1954; MORRIS *et al.* 1998; WU AND MORRIS 1999; DUNCAN 2002). One form of transvection is homolog pairing-mediated changes to gene topology. Here, the presence of structural heterology, such as a heterozygous insertion or deletion, disrupts local homolog pairing (MORRIS *et al.* 1998).

As homologous chromosome pairing is also an essential part of meiosis, we sought to determine if homolog pairing played a role in the ability of the *C. elegans* germline to detect structural variants using genetic assays reminiscent of the elegant genetic experiments that Ed Lewis used to discover transvection. To address this potential mechanistic detail, we assessed the ability of the genome to detect two homozygous viable translocations and a homozygous viable inversion in both homozygous and heterozygous states.

We next asked if smaller chromosomal structural variants such as heterozygous deletions would also provoke a response by the meiotic genome. Analogously, a chromatin loop may form at the site of a heterozygous deletion due to the forces of homolog pairing flanking the disrupted region; thus, forcing the unpaired chromatin into a loop structure. Evidence of loop formation has been shown in many different organisms and cell types using a variety of techniques to visualize chromosomal interactions within nuclei. Many early studies in this area focused on establishing how interactions among chromatin insulators shaped nuclear architecture and chromatin topology.

FISH performed on the *gypsy* insulator in Drosophila demonstrated that insulators clustered to specific regions within the nucleus. The authors postulated that the X chromosome, containing 80 *gypsy* insulator sites, would produce clusters of 16 individual insulators, called insulator bodies. The resultant chromatin loops occurring between insulators would be approximately 250kb in length (GERASIMOVA AND CORCES 1998; GERASIMOVA *et al.* 2000). Further characterization of *gypsy* interactions using different configurations of two or three copies of the insulator and a reporter gene provided further genetic evidence that loops as small as 1.5kb can form and affect gene expression, suggesting that steric hindrance does not appear to be a factor in loop formation (CAI AND SHEN 2001; MONGELARD AND CORCES 2001; SAVITSKAYA *et al.* 2006). Additional studies used three FISH probes of approximately 150kb to visualize larger loop structures formed by *gypsy* interactions, thus confirming their presence (BYRD AND CORCES 2003).

Another area in which chromosomal loops have been shown to be highly important is in enhancer-promoter interactions. While it was originally thought that enhancers facilitated transcription by loading transcription factors onto chromatin upstream of their respective promoters, recent work has indicated that promoters and enhancers may directly interact with each other, often via DNA-protein interactions (DE LAAT 2003; KUHN 2003; FRASER 2006; WALLACE AND FELSENFELD 2007; SCHOENFELDER *et al.* 2010; ONG AND CORCES 2011). A study examining enhancer-promoter interactions at the Drosophila *white* gene, which is responsible for red eye pigmentation, has shown that, when the *white* enhancer is separated from the promoter by 3kb of spacer DNA, interactions between the enhancer and promoter require the presence of the *zeste* protein, which may be involved in many long-distance interactions in flies (KOSTYUCHENKO *et al.* 2009).

Though genetic evidence may provide some information into long distance interactions, newer biochemical methods have shed even more light on these processes. Chromosome Conformation Capture (3C), a PCR-based method for discovering long distance interactions, has been used to identify many enhancer-promoter interactions (DEKKER 2002). One such study used 3C to identify four regulatory elements within a 460kb interval of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in tissue culture cells. These elements interacted with the CFTR promoter by forming loops ranging in size from 20-200kb (GHELDOF *et al.* 2010). Another study used 3D FISH and 3C to demonstrate that limb bud development is regulated by an enhancer promoter loop that brings Sonic

hedgehog (Shh) into direct contact with an enhancer located 1Mb upstream. Interestingly, this loop only forms in tissues competent for Shh expression, restricting activation of Shh to the appropriate cells during development, suggesting that loops may form in a tissue-specific manner (AMANO *et al.* 2009). Further recent studies have used multiplexed methods, such as Chromatin Interaction Analysis by Paired-End Tag sequencing (ChIA-PET) to identify upwards of 30,000 long range interactions mediated by the following marks of enhancers, promoters, or regulatory elements: H3K4me1, H3K4me2, H3K4me3, H3K27ac, POLR2A, and RAD21 (Hiedari 2014).

Chromosomal loops may play other roles in the genome, such as maintaining higher order chromatin structure. For instance, it is thought that loops are important for insulator activity as they can isolate an enhancer and its corresponding promoter into distinct chromosomal domains (BRASSET AND VAURY 2005; MAEDA AND KARCH 2007; WALLACE AND FELSENFELD 2007). Studies of the Drosophila Bithorax Complex (BX-C) have shed some light into how insulators may function as boundary elements. BX-C contains three homeotic genes (*Ubx, abd-A,* and *Abd-B*) and an interval of approximately 300kb of regulatory elements (LEWIS 1978; MCCALL AND O'CONNOR 1994; BENDER AND HUDSON 2000; AKBARI *et al.* 2006; MAEDA 2006). This region contains three insulators that function as boundary elements, and are thought to restrict expression of the three homeotic genes to the appropriate body segments within the adult fly (GYURKOVICS *et al.* 1990; KARCH *et al.* 1994; MIHÁLY *et al.* 1997; BARGES *et al.* 2000). Additional studies have shown that

Fab-7, one of these boundary elements, interacts with *Abd-B* only in segments where this gene is silenced, implying the formation of discreet chromatin loops controlling gene expression in different tissues (SIPOS AND GYURKOVICS 2005; CLÉARD *et al.* 2006; LANZUOLO *et al.* 2007). Taken together, all of the aforementioned studies demonstrate the chromatin loops may be a common feature of the genome of many different organisms and may play an important role in gene regulation.

Interestingly, loop structures may form at human CNVs, which are regions of the genome ranging in size from several base pairs up to several megabase pairs that exist in variable copy number throughout the population (IAFRATE *et al.* 2004; SEBAT 2004; FREEMAN 2006; PERRY *et al.* 2008; LEE AND SCHERER 2010; ALKAN *et al.* 2011). Excitingly, since their discovery in 2004, CNVs have been shown to play an important part in the pathology of many diseases and have been shown to be a novel mechanism for generating necessary variation in the genome {McCarroll, 2007 #362}. To mention only several recent reports, CNVs have been associated with cancer (CAMPS *et al.* 2008; SPELEMAN *et al.* 2008; NORSKOV *et al.* 2010; SHLIEN *et al.* 2010; YOSHIHARA *et al.* 2010; LEBRON *et al.* 2011; TRAN *et al.* 2011), schizophrenia (MORENO-DE-LUCA *et al.* 2010; BUIZER-VOSKAMP *et al.* 2011), autism (BREMER *et al.* 2010; MORENO-DE-LUCA *et al.* 2010; SANDERS *et al.* 2011), Alzheimer disease (ZHANG *et al.* 2009b; KAY *et al.* 2010; BROUWERS *et al.* 2011; SHAW *et al.* 2011), and Crohn's disease (MCCARROLL AND ALTSHULER 2007; MCCARROLL *et al.* 2008; ZHANG *et al.* 2009a) reviewed by (KUIPER *et al.* 2010; LEE AND SCHERER 2010). CNVs may generate considerable genomic structural heterogeneity due to their propensity to exist as

deletions, insertions, and duplications (McCarroll *et al.* 2006; Mullaney *et al.* 2010). The meiotic machinery may detect the ensuing structural heterogeneity when homologous chromosomes pair and synapse during prophase I. Here, we investigated whether small, chromosomal structural variants are detected during meiosis in the *C. elegans* germline. Our data show that structural variants similar in size to human CNVs are indeed detected in the meiotic genome.

RESULTS

Heterozygous translocations and inversions show reduced RAD-51 response when made homozygous

Our model predicts that the genome can detect structural heterozygosity. As such, one facet of the model is that when heterozygous structural changes to the genome are made homozygous, there is no longer any basis for detection by the genome. This prediction is reminiscent of the studies conducted by Ed Lewis that demonstrated the basis for transvection. Lewis found that rearrangements with breakpoints located between the Bithorax complex and the centromere disrupted transvection (Lewis 1954). Studies of other loci that demonstrated transvection found similar pairing requirements, e.g transvection at *decapentaplegic, vestigial,* and *eyes absent* is dependent on homolog pairing. In these three instances, when rearrangements that disturb homolog pairing at these loci are introduced, transvection is weakened or eliminated (Coulthard 2005; Gelbart 1982; Leiserson *et al.* 1994).

To investigate whether homolog pairing is important for detection of chromosomal structural variants, we studied two homozygous viable translocations (eT1(III;V) and hT2(I;III)) and a homozygous viable inversion of approximately 3.3Mb in length on chromosome I (hIn1) . Hermaphrodites heterozygous for each translocation and inversion, respectively, were crossed to the corresponding males in order to generate homozygous offspring. Both homozygous and heterozygous versions of each variant were stained with an anti-RAD-51 antibody (Novus Biologicals) and levels of RAD-51 foci were quantitated. All three strains displayed significantly increased levels of RAD-51 foci ($P<0.05$) in several zones when their heterozygous versions were compared to RAD-51 levels in control nuclei. Strikingly, a statistically significant reduction in RAD-51 levels was observed when these strains were made homozygous for their respective structural variants (Figure 4).

In particular, when heterozygous, eT1(III,V) showed significantly elevated RAD-51 levels in zones 4 through 8, whereas when it was made homozygous, levels of RAD-51 foci were not significantly different than controls in all zones (Mann-Whitney U-test, $P<0.05$). The second translocation, hT2(I,III), showed significantly elevated levels of RAD-51 in zones 5 through 8 when heterozygous, but elevated levels only in zones 5 and 6 when homozygous ($P<0.05$). The homozygous viable chromosome I inversion, hInI, displayed significantly increased levels of foci in zones 5 through 8 when heterozygous, whereas it displayed significantly higher levels of foci in only zones 4 and 6 when made homozygous. Given that RAD-51 levels did not completely return to control levels in the homozygous state , it is possible that the

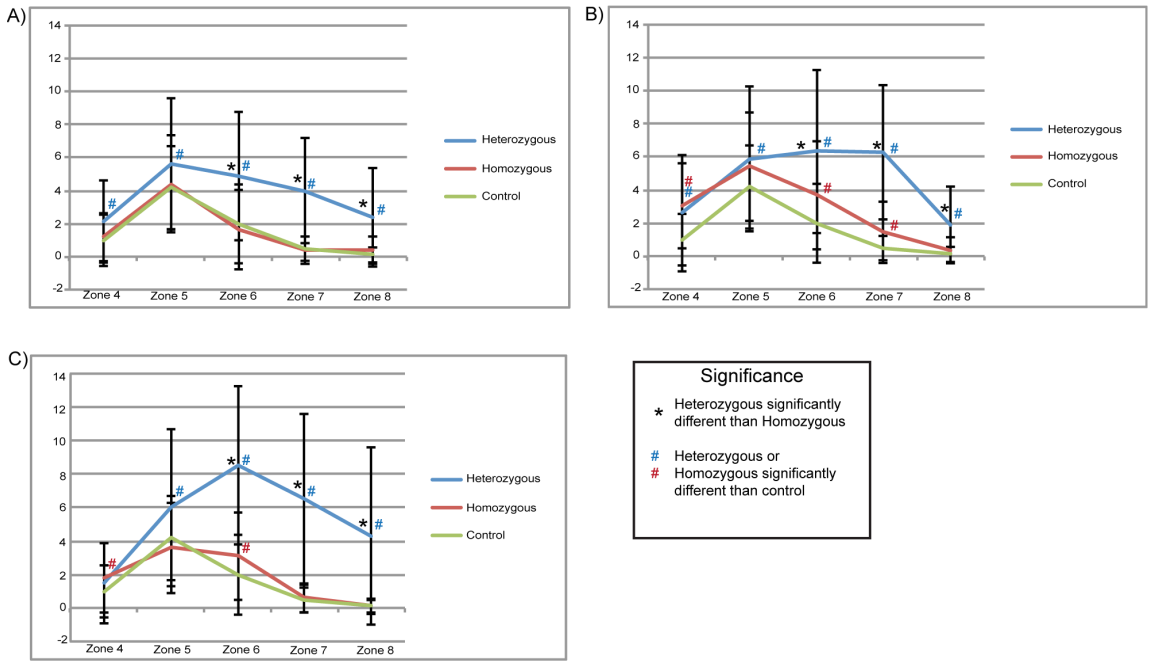

Figure 4. Graphs of translocations and inversions in heterozygous and homozygous states compared to controls. A) eT1(III;V). This translocation displays significantly higher levels of RAD-51 foci in all zones when its heterozygous state than when made homozygous. When homozygous, eT1 RAD-51 levels are not significantly different than controls. B) hT2(I;III). hT2 similarly exhibits statistically significantly higher levels of RAD-51 foci when in a heterozygous state. These levels decrease in the homozygous state, but remain statistically significantly higher than controls in zones 4, 6, and 7. C) hIn1. In its heterozygous state, hIn1 has significantly higher levels of RAD-51 foci in all zones 5 through 8 compared to controls. When hIn1 is made homozygous, RAD-51 levels are no longer significantly different than controls in zones 6, 7, and 8; however, levels remain significantly different than controls in zones 4 and 5. (Mann-Whitney U-test, $p<0.05$)

meiotic cell can detect and mark other aspects of structural variants, such as the breakpoints, and that these marks are carried through meiosis and could affect the offspring. These data imply that homolog pairing is the basis for detection of structural heterozygosities.

Small, heterozygous deficiencies alter RAD-51 levels and kinetics during meiotic prophase I

Design of experiment

Selection of assays: As our study examines the overall ability of the meiotic genome to detect heterozygosity, the readout for detection could be one or more of several different possibilities. Six different assays were investigated as readouts for detection of small, heterozygous deficiencies. Below, I will describe one of the six different assays that were considered as possible readouts. The remaining five assays will be discussed in Chapter 3 of this dissertation.

We began by examining the kinetics of *C. elegans* RAD-51, a protein involved in homology searching during double strand break (DSB) repair (SUNG 1994). We initially chose to focus on RAD-51 due to three previous studies. The first study characterized the behavior and kinetics of *C. elegans* RAD-51 in both wild-type strains and in the presence of heterozygous translocations. The second examined the effects of heterozygous insertions on crossover distribution, a potential downstream effect of altered RAD-51 kinetics (HAMMARLUND *et al.* 2005). The third determined RAD-51 kinetics in the context of disrupted synapsis (COLAIÁCOVO *et al.* 2003). These studies are described below.

Alpi, *et al.* established the kinetics and behavior of RAD-51 in *C. elegans* by using immunofluorescence to determine that RAD-51 levels increase during leptotene and zygotene stages, reach a peak during mid-pachytene stage, and then decrease through the diplotene stage. Interestingly, in meiotic nuclei with one

heterozygous translocation (eT1(III;V) or hT1(I;V)), the authors noted that levels of RAD-51 foci were higher than in wild-type nuclei and presented in clusters, suggesting that RAD-51 foci formed abundantly on unsynapsed chromosome axes (2003).

Hammerlund and colleagues sought to determine if large breaks in sequence homology altered crossover distribution and number in *C. elegans* by studying two large insertions on the X chromosome and two large insertions on chromosome V. In all cases, crossover number was not affected; however, crossover distribution was altered, with more crossovers occurring on the side of the chromosome with the PC. This implies that heterosynapsis, synapsis between nonhomologous regions, disrupts crossovers on the distal side of the chromosomes (HAMMARLUND *et al.* 2005). Alterations to the number and distributions of crossovers suggest that proteins important earlier in the meiotic homologous recombination pathway, such as RAD-51, may similarly display altered kinetics and localization in the presence of heterologous regions.

Colaiácovo and colleagues examined the effects of disrupted synapsis on strand exchange and recombination by mutating components of the synaptonemal complex (SC). Wild-type gonads have low levels of RAD-51 throughout pachytene and show very few remaining foci in late pachytene. When SYP-2, a structural component of the central region of the SC, is mutated, synapsis is impaired, resulting in increased levels of RAD-51 that persist into late pachytene, likely due to the

inability of these nuclei to repair DSBs using the homologous chromosome as a template. This result suggests that RAD-51 may be involved in sensing unsynapsed chromosome axes.

Similarly, a more recent study has shown that SAD-6, a potential SNF2-family protein is required for efficient MSUD response in *N. crassa*. Importantly, SAD-6 is closely related to RAD-54, a protein involved homologous recombination, and may spatially constrain the search for homologous chromatin to regions near double strand breaks (Samarajeewa 2014). This study further supports the idea that proteins involved in homologous recombination or DSB repair may play a role in the detection of unpaired chromatin.

Taken together, these studies suggest that RAD-51 is a potential candidate to assess structurally heterozygous regions for detection by the meiotic machinery, as heterozygous variants lack homologous partners off of which to repair. Importantly, RAD-51 is loaded onto meiotic chromosomes beginning in early pachytene and persists until late pachytene, making it an excellent marker for the entire pachytene stage of meiotic prophase I.

Selection of deficiencies: In this study, we sought to determine if small, structural variants could be detected by the meiotic nucleus in *C. elegans*. While there are many types of structural variants, including translocations, inversions, and so on, our studies focused on heterozygous deficiencies. Heterozygous deficiencies

were chosen for several important reasons, which can be placed into one of two categories explained below: 1) Prior reports concerning detection of structural variants, and 2) Size of available variants.

1) A wide array of deficiencies is available from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota, making it easy to acquire the strains of interest. More importantly, heterozygous deficiencies can be validated using qPCR, whereas some types of structural variants, such as translocations and inversions, cannot be so easily validated. Finally, there is previously established evidence that deficiencies on *C. elegans* chromosome V alter levels of recombination to the right of their locations, possibly due to the presence of an unpaired loop altering crossover distribution (ROSENBLUTH *et al.* 1990). These data provide preliminary evidence that the meiotic nucleus may detect and respond to the presence of a deficiency, in this instance, by altering the recombinational landscape of the surrounding chromosome.

2) The second important consideration in choosing which type of structural variant to utilize was the size range of each class of variant available for study. Here, we utilized a series of heterozygous deficiencies, ranging in size from approximately 1kb to 2.6Mb. We chose this size range because prior studies of human CNVs have shown that large CNVs, ranging in size from 500bp to over 2Mbp, depending on the disease studied, are enriched in individuals with obesity, intellectual disability, and schizophrenia (BOCHUKOVA *et al.* 2010; WANG *et al.* 2010; GIRIRAJAN *et al.* 2011) [ADD

Brand… Doyle 2014]. It is possible that the size of these CNVs plays a role in their respective disease associations. Thus, based on the previously available evidence for detection of deficiencies and their availability within the size range of human CNVs, heterozygous deficiencies were chosen for further analysis.

Unfortunately, the vast majority of deficiencies in the *C. elegans* genome have not been molecularly defined. However, we were able to estimate the sizes of our deficiencies based on preexisting complementation data from www.wormbase.org, allowing us to resolve minimum and maximum possible sizes based on complementation tests conducted with previously mapped genes inside and outside of the deficiency breakpoints, respectively (Figure 5).

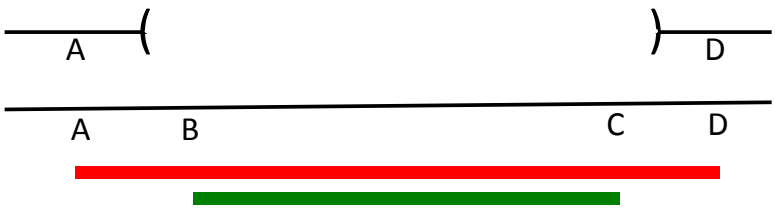

Figure 5. Deficiencies sizes were determined by complementation tests of genes inside and outside of the deficiency breakpoints. Genes A and D are located outside of the breakpoints and genes B and C are located within the deficient region. Maximum complementation size is indicated by the red bar and minimum complementation size is shown by the green bar. Minimum deficiency sizes were used to order heterozygous deficiency strains when possible. If a breakpoint did not have both internal and external complementation data, only the available data were used to estimate deficiency size.

For instance, for the MT2180 strain, the minimum and maximum sizes determined by complementation tests are 675kb and 1006kb, respectively. Henceforth, to be conservative, strains will be referred to by their minimum complementation size in this dissertation (See Materials and Methods, Table 5). Though we were unable to determine a precise size for each deficiency, a comparison of the maximum and minimum possible sizes for each deficiency show that both cover the same overall size range.

Our study began by examining the list of hundreds of deficiency strains available from the CGC. Initially, we chose 40 heterozygous deficiency strains to examine; however, 10 strains were balanced over a translocation or inversion. As the presence of another structural variation in addition to the heterozygous deficiency would confound our analysis, the translocations or inversions were crossed out of these strains prior to analysis. If this was not possible, the strains were not used for analysis.

Furthermore, given that meiotic homolog pairing in *C. elegans* initiates at genetically and molecularly defined regions, called "pairing centers" or "PCs," five deficiency strains with breakpoints within PC regions were excluded from analysis as disruption of these regions has been previously shown to affect synapsis (McKim *et al.* 1988; Zetka and Rose 1992; McKim *et al.* 1993; Villeneuve 1994; Zetka and Rose 1995; MacQueen *et al.* 2005; Phillips *et al.* 2005; Phillips and Dernburg 2006; Phillips *et al.* 2009). The remaining deficiency strains are not known to harbor any

additional confounding structural rearrangements, such as translocations or free duplications, as determined by information available for each strain on WormBase and in the literature.

Importantly, the use of deficiencies allowed for the investigation of genome-wide differences in detection. For instance, it is possible that there are differences in response between sex chromosomes and autosomes, between different regions of the autosomes, or between different autosomes themselves. Thus, the set of deficiencies chosen for analysis contained deficiencies on all chromosomes and in different chromosomal regions, wherever possible. Our study examines 25 heterozygous deficiency strains with the following genomic distribution: On the X chromosome, we examined four strains, with deficiencies ranging in size from 7.5kb to 187kb. On chromosome I, we examined eight deficiencies ranging in size from 34kb to 1552kb. On chromosome II, we studied six deficiencies ranging in size from approximately 1kb to 2643kb. On the third chromosome, we examined seven deficiencies ranging in size from 197kb to 2351kb. On the fourth chromosome, we studied two deficiencies of 359kb and 2083kb. On the fifth chromosome, we examined one deficiency of 279kb in size.

Organization of the gonad: As the *C. elegans* gonad represents a temporal-spatial gradient of progression through meiosis, we divided the gonad into eight zones, corresponding to the different stages of meiotic prophase I (Figure 6). Zones 1 and 2 represent the premeiotic tip containing nuclei that have not yet entered into

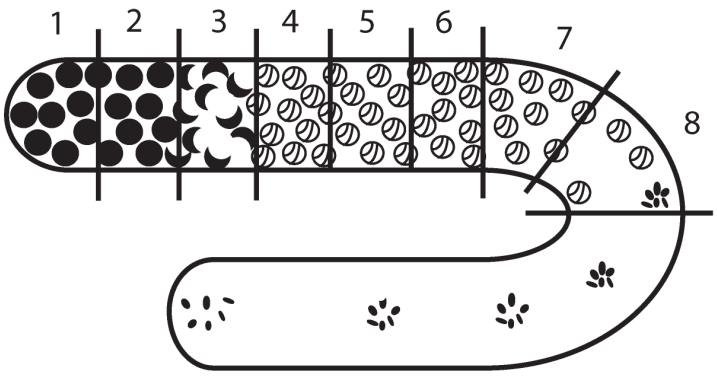

Figure 6. Zones of the *C. elegans* gonad used for analysis of RAD-51 kinetics and pachytene delay. Zones represent the stages of meiotic prophase I as follows: Zones 1 and 2 – Premeiotic stage, Zone 3 – Leptotene/ Zygotene stages, Zone 4 – Early Pachytene, Zone 5 – Mid-pachytene, Zones 6 and 7 – Late pachytene, and Zone 8 – Diplotene.

meiosis. Zone 3 is the transition zone, containing nuclei in the leptotene and zygotene stages that are transitioning into meiosis. Zone 4 is the early pachytene stage. Zone 5 represents the mid-pachytene stage. Zones 6 and 7 encompass late pachytene. Lastly, zone 8 is the diplotene stage. These divisions allow for temporal investigation of RAD-51 kinetics. We scored three to four gonads per heterozygous deficiency strain, for a total of approximately 70 total nuclei per zone in zones 4 through 7, and approximately 45 total nuclei in zone 8.

Determination of control values: As our heterozygous deficiency strains were created in many different laboratories, using N2 as our only control strain would not adequately control for the diversity of the genetic backgrounds of our experimental strains. Thus, we examined five randomly chosen control strains, in addition to N2, that are not known to carry any structural variants or any mutations that affect meiosis (Table 1). The strains and mutations are as follows: CB364 *dpy-18(e364)III*, MT1655 *bli-6(n776)IV*, BW983 *bli-4(e937) unc-37(e262)I*, CB193 *unc-29(e193)I*, and CB1091 *unc-13(e1091)I*. All strains were ultimately derived from N2 and have been

outcrossed at least one time by their respective laboratories. All controls were averaged and then used in statistical analysis and comparison to heterozygous deficiency strains, though the comparison to N2 alone is also shown below. Levels of RAD-51 foci in control strains were generally not statistically different from N2; however, all five randomly chosen strains showed significantly different ($P<0.05$) levels of RAD-51 in pachytene stage in one or two zones. Specifically, BW983, MT1655, and CB364 displayed zones with significantly lower levels of RAD-51, whereas CB193 and CB1091 displayed zones with significantly higher levels of foci. Interestingly, no control strain displayed statistically significant changes to RAD-51 levels in two consecutive zones (Table 1). Taken together, it is likely that these small differences represent normal stochastic variation present when comparing any two strains.

			RAD-51 FOCI									
			Zone 4		Zone 5		Zone 6		Zone 7		Zone 8	
Strain	Chr	N gonads	N nuclei	Mean	N nuclei	Mean	N nuclei	Mean	N nuclei	Mean	N nuclei	Mean
Avg. Cntr	n/a	n/a	n/a	1.0	n/a	4.2	n/a	2.8	n/a	0.6	n/a	0.3
N2	n/a	4	83	0.7	71	3.3	65	3.5	59	0.5	52	0.5
BW983	I	4	87	1.3	59	5.0	64	3.4	43	0.3	49	0.1
MT1655	IV	4	84	0.9	69	5.1	63	2.0	51	0.2	30	0.1
CB364	III	4	78	0.6	47	2.5	47	1.2	49	0.1	32	0.0
CB195	V	4	89	1.4	81	4.9	66	2.2	53	0.8	24	0.2
CB1091	I	4	91	1.1	85	4.3	46	4.3	53	1.0	38	1.0

Table 1. Quantification of RAD-51 levels in Control Strains. N gonads represents the number of gonads scored for each genotype and N nuclei represents the number of nuclei scored per genotype per zone. Mean number of RAD-51 foci/ nucleus is displayed for each zone of the gonad. Cells are highlighted red if the mean number of foci is significantly higher than N2 and highlighted blue if the mean number of foci is significantly lower than N2 ($P<0.05$, two-tailed Mann-Whitney test). Control strains (colored purple) display minimal statistically significant variation when compared to N2, suggesting a small background level of stochastic variation in RAD-51 levels in strains not known to harbor any chromosomal structural variants.

RAD-51 kinetics and levels are altered in heterozygous deficiency strains

Kinetics and levels of RAD-51 were analyzed in 25 heterozygous deficiency strains (Figure 7) using a commercially available rabbit α-RAD-51 antibody (Novus Biologicals).

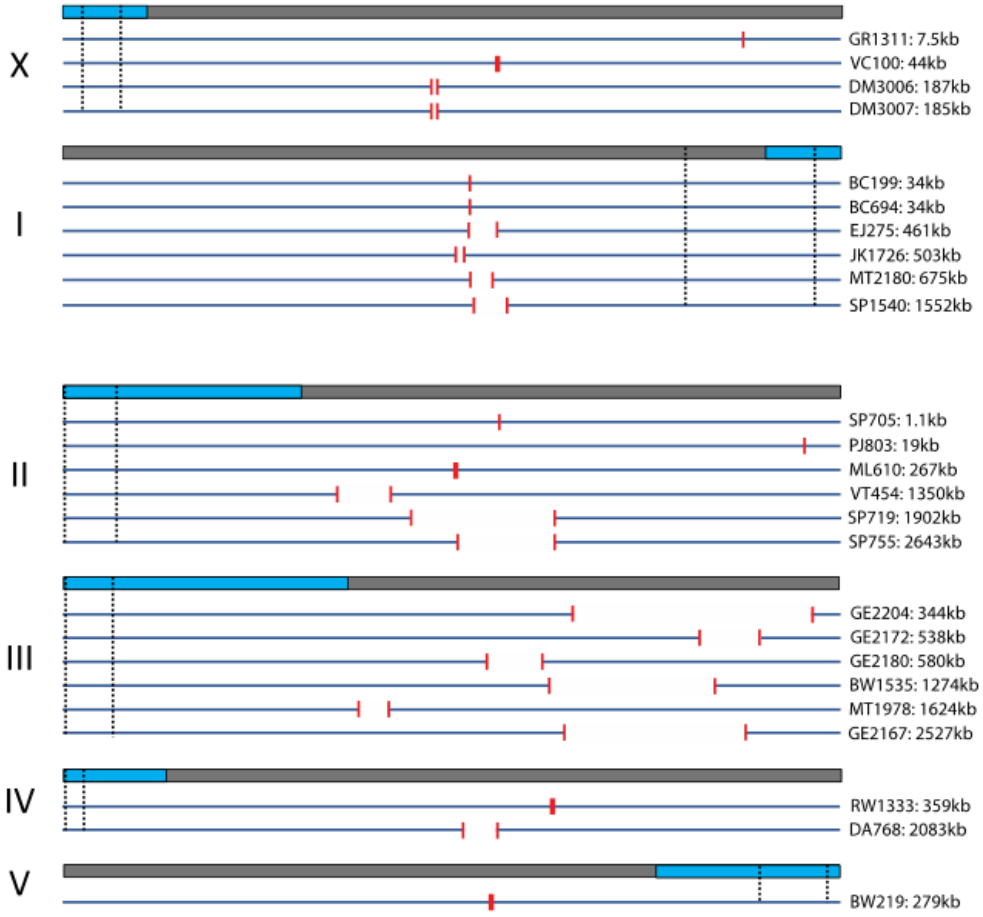

Figure 7. Genetic map of deficiencies. The *C. elegans* chromosomes are shown as gray bars. The genetically defined pairing centers are highlighted on the chromosomes in blue. Dashed vertical lines represent regions of the chromosomes enriched for pairing center protein binding sites. Deficiencies are ordered by increasing size on each individual chromosome and deficiency genetic boundaries are indicated by vertical red bars.

Hermaphrodites were picked during the L4 stage and dissected 21-24 hours later, in the young adult stage. This selection method produced age-matched worms, which is required for comparison of different strains. Between three and four gonads were scored for each deficiency strain. If three intact gonads were not recovered in a single preparation, the process was repeated a second time in order to obtain at least three gonads.

We began analysis by comparing levels of RAD-51 of the heterozygous deficiency strains and additional control strains to N2 alone. Analysis of RAD-51 kinetics using α-RAD-51 antibodies in N2 hermaphrodite germlines showed that levels of RAD-51 foci increased during early pachytene and reached a peak during mid-pachytene with 93% of nuclei containing at least one RAD-51 focus (Table 2). On average, control nuclei showed 4.2 RAD-51 foci per nucleus (n=412) in zone 5. Foci began to dissipate during late pachytene and were almost completely absent by the diplotene stage.

Average numbers of foci per nucleus in N2 were 2.8 (n=351), 0.6 (n=308), and 0.3 (n=225) in zones 6, 7, and 8, respectively. These results are consistent with levels of RAD-51 foci seen in previous studies, confirming antibody specificity (COLAIÁCOVO *et al.* 2003; SMOLIKOV *et al.* 2008; NOTTKE *et al.* 2011).

				RAD-51 FOCI				
				Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N	Min. Size (kb)	Mean	Mean	Mean	Mean	Mean
N2	n/a	4	n/a	0.7	3.3	3.5	0.5	0.5
BW983	n/a	4	n/a	1.3	5.0	3.4	0.3	0.1
MT1655	n/a	4	n/a	0.9	5.1	2.0	0.2	0.1
CB364	n/a	4	n/a	0.6	2.5	1.2	0.1	0.0
CB195	n/a	4	n/a	1.4	4.9	2.2	0.8	0.2
CB1095	n/a	4	n/a	1.1	4.3	4.3	1.0	1.0
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJ803	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	484	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2
MT1401	X(Df),I	4	2000	5.1	10.3	5.9	2.6	0.2
AF1	X,I	4	n/a	3.3	7.6	10.6	6.6	1.5
him-8	*him-8,X*	4	n/a	2.5	5.8	7.5	10.1	9.2

Table 2. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to N2. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or by molecular mapping. Mean number of RAD-51 foci is shown for each deficiency strain. Control strains are highlighted in purple. Cells are highlighted in red if the mean number of RAD-51 foci is significantly higher than N2 and highlighted in blue if the RAD-51 level is significantly lower than N2 (P<0.05, Mann-Whitney U-Test). Strains highlighted in gold represent positive control strains. "MT1401" contains a 2Mb heterozygous deficiency balanced over a translocation (szT1), "AFI" is a large heterozygous translocation (szT1), and "X" represents a strain with the *him-8(e1459)* allele, which causes unpairing of the X chromosome in approximately 40% of meiotic nuclei. Strains with heterozygous deficiencies greater than 19kb show a general perturbation of RAD-51 levels and kinetics. Additionally, there is a overall trend of increased perturbation of RAD-51 levels as the size of the heterozygous deficiencies increases.

We first asked, “Does the presence of a heterozygous deficiency strain alter RAD-51 levels and kinetics?” To answer this question, we compared the levels of RAD-51 foci in 25 heterozygous deficiency strains to N2 using a Mann-Whitney U-test. Strikingly, 17 out of 25, or 68%, of the heterozygous deficiency strains showed at least two consecutive zones in which RAD-51 foci levels were significantly different than N2. This is in stark contrast to control strains, of which no strain was significantly different than N2 in more than one consecutive zone. In heterozygous deficiency strains, RAD-51 was loaded onto chromosomes with similar kinetics to control strains. When the heterozygous deficiency strains were compared the following outcomes were observed: two heterozygous deficiency strains (1.1kb and 461kb) showed no significant differences compared to N2, six strains (7.5kb, 185kb, 187kb, 267kb, 279kb, and 359kb) showed significantly lower levels of RAD-51 foci in at least one zone, seven strains (with 44kb, 197kb, 503kb, 549kb, 1350kb, 1624kb, and 2351kb deficiencies) displayed significantly higher levels of foci in at least one zone, and the remaining ten strains (with 19kb, 34kb, 34kb, 484kb, 675kb, 924kb, 1552kb, 1902kb, 2083kb, and 2643kb deficiencies) displayed a mixture of significantly higher and significantly lower levels of RAD-51 in different zones. If both significantly higher and significantly lower levels of foci are considered together, there is a general trend of a greater level of response as the size of the heterozygous deficiency increases. However, as previously stated, in order to better account for the diversity of genetic backgrounds present among the heterozygous deficiency strains, a comparison to the average of N2 and five randomly chosen control strains was used for further analyses.

Upon comparison to the averaged control strains, the following outcomes were seen: all heterozygous deficiency strains showed a significant difference compared to N2 in at least one zone. Eight strains (with 1.1kb, 7.5kb, 185kb, 187kb, 267kb, 279kb, 359kb, and 461kb deficiencies) showed significantly lower levels of RAD-51 foci in at least one zone. Eight strains (with 44kb, 197kb, 503kb, 1552kb, 1624kb, 1902kb, 2351kb, and 2643kb deficiencies) displayed significantly higher levels of foci in at least one zone. The remaining ten strains (with 19kb, 34kb, 34kb, 344kb, 549kb, 675kb, 924kb, 1350kb and 2083kb deficiencies) displayed a mixture of significantly higher and significantly lower levels of RAD-51 in different zones (Table 4, $P<0.05$). Overall, strains with deficiencies less than 500kb tended show significantly reduced levels of RAD-51 foci in several zones and strains with deficiencies larger than 500kb tended to show significantly increased levels of RAD-51 foci in several zones.

Of the fourteen strains with deficiencies less than 500bp, four strains showed a level of response similar to those with larger deficiencies, e.g. the 19kb, 185kb, and 344kb deficiency strains showed a response in four zones and one of the 34kb deficiency strains displayed a response in all five zones. This suggests that the meiotic machinery can detect very small deficiencies. This is surprising given the prevalence of small structural changes, such as CNVs, within the *C. elegans* genome, which affect over 5% of the genome, ranging in size from 12.8kb to 1kb on the autosomes (MAYDAN *et al.* 2010). The presence of this natural structural variation

may account for the low level of statistically significant variation in RAD-51 foci seen among control strains (Table 1).

Intriguingly, five strains showed a response in only one zone: Strains with deficiencies of 1.1kb, 7.5kb, 279kb, and 461kb displayed significantly lower levels of RAD-51 foci in mid-to-late pachytene, while the 1902kb deficiency strain showed significantly increased foci in early pachytene. It is possible that haploinsufficiency of a gene required for the RAD-51 pathway may be responsible for the differences in RAD-51 kinetics observed in these strains.

We next asked, "Does there appear to be a size-dependent cut-off for detection?" Indeed, we do see an increased response in strains with heterozygous deficiencies greater than 500kb. For instance, strains with deficiencies greater than 500kb showed an average of 3.4 zones of significant difference, where as deficiencies smaller than 500kb displayed an average of 2.6 zones of significant difference compared to control strains ($P<0.05$, Table 3). These data indicate that there appears to be an increase in response to deficiencies greater than 500kb.

Our next question was, "Is there a correlation between size and level of response?" It appears as though there is a rough correlation between increasing deficiency size and increasing level of response. For example, a deficiency of 2351kb shows significantly increased RAD-51 foci levels ($P<0.05$) throughout all zones representing meiotic prophase I compared to a strain carrying a heterozygous

				RAD-51 FOCI				
				Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N	Min. Size (kb)	Mean	Mean	Mean	Mean	Mean
Avg. Cntr	n/a	n/a	n/a	1.0	4.2	2.8	0.6	0.3
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJB03	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	344	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2
MT1401	X(Df),I	4	2000	5.1	10.3	5.9	2.6	0.2
AF1	X,I	4	n/a	3.3	7.6	10.6	6.6	1.5
him-8	*him-8,X*	4	n/a	2.5	5.8	7.5	10.1	9.2

Table 3. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to average of controls. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or molecular mapping. Mean number of RAD-51 foci is shown for each deficiency strain. The average of 5 control strains is highlighted in purple and used for subsequent statistical analysis. Cells are highlighted in orange if the mean number of RAD-51 foci is significantly higher than the control strains and highlighted in blue if the RAD-51 level is significantly lower than control strains (P<0.05, Mann-Whitney U-Test). Strains highlighted in gold represent positive control strains. "MT1401" contains a 2Mb heterozygous deficiency balanced over a translocation (szT1), "AFI" is a large heterozygous translocation (szT1), and "X" represents a strain with the *him-8(e1459)* allele, which causes unpairing of the X chromosome in approximately 40% of meiotic nuclei. Strains with heterozygous deficiencies greater than 19kb show a perturbation of RAD-51 levels and kinetics throughout pachytene. Additionally, there is a general trend of increased perturbation as the size of the heterozygous deficiencies increases.

deficiency of 279kb, which only shows a response in a single zone.

Another important question is, "Is detection chromosome specific?" Detection does not appear to be chromosome-specific as elevated RAD-51 levels were seen for deficiencies present on all chromosomes. For instance, we examined heterozygous deficiencies on all five autosomes and the X chromosome. 25 out of 25 total deficiency strains examined demonstrated a significant change ($P<0.05$) in at least one gonadal zone. Thus, these data suggest that detection is not specific to any chromosome.

Interestingly, RAD-51 levels are significantly below wild-type averages in seven out of twenty-five strains during early pachytene; these strains have deficiencies of 19kb, 34kb, 185kb, 267kb, 359kb, 675kb, and 2083kb in size. There are two possible explanations for a reduction in RAD-51 levels in early pachytene. First, heterozygous deficiencies may be detected during earlier meiotic stages, such as leptotene/zygotene, during which homolog pairing is established (MACQUEEN *et al.* 2002), causing delayed synapsis and a corresponding delay in RAD-51 loading. Alternatively, the presence of a heterozygous deficiency may itself delay loading of RAD-51 onto chromosomes.

DISCUSSION

Analysis of homozygous viable translocations and a homozygous viable inversion indicate suggest that homolog pairing plays an important role in detection of structural variants

To gain insight into the genetic processes that play a role in detection of chromosomal structural variants, we examined the RAD-51 response of two homozygous viable translocations and one homozygous viable inversion in both the heterozygous and homozygous states. In all three cases, heterozygous structural variants showed increased levels of RAD-51 foci compared to their homozygous counterparts ($P<0.05$). This result suggests that pairing of homologous chromosomes plays an important role in the detection of chromosomal structural variants during meiosis. The fact that homolog pairing is important for the detection process is not surprising, as heterozygous regions are still subject to programmed double strand breaks during meiosis, though they lack a homologous partner off of which to repair.

Interestingly, a previous study characterizing the role of RAD-51 during meiosis in *C. elegans* found that RAD-51 foci persist on chromosomes heterozygous for translocations (ALPI *et al.* 2003). However, this study did not compare heterozygous translocations to homozygous viable translocations, likely due to the fact that most translocations are not homozygous viable. Additionally, RAD-51 kinetics were not evaluated in a quantitative manner as they were in our study.

From studies done in Drosophila, mice, and human cell lines, it has become apparent that chromosome loops of varying sizes form in the genomes of a diverse array of organisms and play important roles in gene regulation and nuclear architecture (MORRIS *et al.* 1998; GERASIMOVA *et al.* 2000; CAI AND SHEN 2001; MONGELARD AND CORCES 2001; SAVITSKAYA *et al.* 2006; AMANO *et al.* 2009; GHELDOF *et al.* 2010). However, no studies have yet characterized the behavior of putative loops during meiosis, particularly loops within the size range of human CNVs. Here, we use the powerful cytological tools available in the *C. elegans* germline to address this lack. Our analysis of 25 heterozygous deficiency strains allowed us to determine that the meiotic genome can detect the presence of small, heterozygous structural variants.

Very small heterozygous deficiencies are detected

One of the most striking discoveries of our study is that very small heterozygous deficiencies, on the order of tens of kilobases, appear to be detected by the meiotic nucleus, as evidenced by changes to RAD-51 levels and kinetics during prophase. This finding has profound implications for the genomes of countless different species, as various types of small structural variations are common to many genomes. This is particularly important when considering the many types of repetitive DNA, which make up approximately 40% of the human genome. First, one

type of variable number tandem repeat, the human minisatellite, has a unit size ≥10bp (BOIS 2003; GEMAYEL *et al.* 2010). As minisatellites are variable in copy number, it is possible that structural heterogeneities similar in size to the smallest deficiencies detected in this study are quite common during meiosis and may consequently be detected by the genome. Additionally, the repeats that make up satellite DNA, the main component of centromeric DNA and heterochromatin, range in size from 25bp for Satellite 1 to 171bp for the α repeat in humans (PLOHL *et al.* 2008; KANIZAY AND DAWE 2009). This size of entire sets of repeats is within the size range detected in our study, indicating that the genome may detect variations in these regions during meiosis if the copy number differs between homologous chromosomes. Since heterochromatic and repetitive regions are thought to be involved in chromosome pairing (TAKEO *et al.* 2011; TSAI *et al.* 2011), perhaps the presence of unpaired loops in these regions allows for more facile sequence comparison between homologs, as the looped out chromatin may be more easily accessible.

Most interestingly, the size of many human CNVs is within the range of detection established in our study and these CNVs are often present as insertions and deletions (MCCARROLL *et al.* 2006; MULLANEY *et al.* 2010). Thus, it is possible that CNVs are detected by the meiotic genome in humans. In this instance, detection could be important for several different reasons. First, detected CNVs may be marked or silenced, which could have implications for CNV-associated diseases in later generations, as these changes may be transmitted to offspring. Secondly, the

detection process may be involved in regulation of processes responsible for CNV copy number change, such as recombination. Additionally, detection of putative chromatin loops at satellites and CNVs may allow the genome to detect the overall level of synapsis during meiosis, and could be linked to a synapsis checkpoint.

Detection of structural variation may control transposition

It is important for the meiotic genome to suppress movement of transposable elements during gametogenesis in order to preserve genomic integrity (KIDWELL AND LISCH 2001; CASTAÑEDA *et al.* 2011). Detection of heterozygous genomic regions may have arisen as way for the genome to prevent transposition. Potential evidence for this mechanism has been found in *Neurospora crassa* in a process deemed meiotic silencing by unpaired DNA (MSUD) (ARAMAYO AND METZENBERG 1996; SHIU *et al.* 2001; SHIU AND METZENBERG 2002). During MSUD, structurally heterozygous regions of the genome and all homologous regions, as small as 500-1000bp, are silenced through an RNAi-like mechanism (ALEXANDER *et al.* 2008; BARDIYA *et al.* 2008; XIAO *et al.* 2010). As a result of this mechanism, the *N. crassa* genome has very few transposon insertions compared to higher organisms (KELLY AND ARAMAYO 2007).

Evidence of other detection mechanisms in mammals and *C. elegans*

Interestingly, mammalian genomes have been shown to house similar detection mechanisms. The most notable is meiotic silencing of unpaired chromatin (MSUC). During MSUC, Turner and colleagues showed that large regions of unsynapsed chromatin, such as entire chromosomes or large translocations, are

enriched for proteins and chromatin modifications thought to be associated with silenced chromatin (KHALIL *et al.* 2004; SCHIMENTI 2005; TURNER *et al.* 2005; TURNER *et al.* 2006; TURNER 2007; BURGOYNE *et al.* 2009; WOJTASZ *et al.* 2009; AN *et al.* 2010; CLOUTIER AND TURNER 2010; FUKUDA *et al.* 2010). Along similar lines, mouse pachytene spermatocytes with several unsynapsed chromosomal axes display increased levels of apoptosis compared to wild-type spermatocytes, possibly due to altered distribution of MSUC proteins, including BRCA1 (MAHADEVAIAH *et al.* 2008). The detection mechanisms discovered in this study may exist to monitor global levels of synapsis to ensure genome integrity. Unfortunately, all previously conducted studies have not examined the effects of MSUC on small structural variations.

Additional studies conducted in *C. elegans* show a similar process, which targets large, unpaired regions with marks associated with silenced chromatin, such as H3K9me2 (KELLY *et al.* 2002; BEAN *et al.* 2004; KELLY AND ARAMAYO 2007). This mechanism, like MSUD, is thought to involve the production of small RNAs (MAINE *et al.* 2005; SHE *et al.* 2009). Additional evidence for a genome defense mechanism in *C. elegans* can be seen during the creation of lines containing transgenic DNA. Here, arrays of transgenic DNA are silenced over several generations, indicating a potential mechanism for preventing expression and proliferation of foreign DNA (KELLY *et al.* 1997; KELLY AND FIRE 1998; SEYDOUX AND STROME 1999; PRAITIS *et al.* 2001; TOWBIN *et al.* 2011). We attempted to determine levels of H3K9me2, but given the small size of the heterozygous deficiencies we examined compared to the overall

size of the genome, immunofluorescence combined with FISH lacked the required resolution to determine if deficiency regions were enriched for silencing marks.

MATERIALS AND METHODS

Genetics

C. elegans strains were cultured at 20°C under standard conditions described by Brenner (1974). The Bristol N2 strain was used as a control during RAD-51 stainings to confirm that strains were analyzed at the correct developmental time point. Five additional strains not known to carry any chromosomal rearrangements or mutations that affect meiosis were randomly chosen from stocks available at the Caenorhabditis Genetics Center at the University of Minnesota and were analyzed and average RAD-51 levels of these five strains and N2 were utilized for statistical analysis of RAD-51 levels and for evaluation of pachytene delay. The five additional control strains are as follows: CB364 *dpy-18(e364)III*, MT1655 *bli-6(n776)IV*, BW983 *bli-4(e937) unc-37(e262)I*, CB195 *unc-42(e270) sma-1(e30)V*, and CB1091 *unc-13(e1091)I*. Strains with chromosomal rearrangements used to evaluate detection were kindly provided by the Caenorhabditis Genetics Center and are shown in Tables 2 and 5. Strains were maintained by selecting the appropriate phenotypes for maintenance of the heterozygous deficiencies.

Specifically, the wild-type homologous chromosome opposite the deficiencies is marked with a recessive marker, such as *unc* (uncoordinated), *dpy* (dumpy), or *bli* (blistered), near a deficiency breakpoint. Thus, when a deficiency is lost, the

recessive maker becomes homozygous, allowing for maintenance of the deficiencies by selecting worms with the phenotype indicative of the heterozygous deficiency state. Furthermore, none of the deficiencies used in this study are homozygous viable, eliminating the Df/Df class of progeny from consideration.

As strains can sometimes spontaneously lose their deficiencies due to recombination events, qPCR comparing regions external and internal to the deficiency was performed, with the expected external:internal ratio near 2.0 (Table 4). All strains tested showed an external:internal ratio near 2.0, indicating that none had lost deficiencies.

Deficiency Strain	Copy Number In : Out	Ratio In : Out
187kb	2287 : 4805	2.10
461kb	633 : 1190	1.88
503kb	1847 : 4135	2.24
549kb	459 : 1069	2.33
675kb	946 : 1693	1.79
1552kb	559 : 1090	1.95
1624kb	912 : 1760	1.93
2083kb	755 : 1638	2.17
2647kb	2219 : 3928	1.77

Table 4. Quantitative PCR to confirm presence of deficiencies. Deficiency strains are ordered in increasing size. qPCR copy number from probes inside and outside the deficiencies are listed, respectively. A ratio of ~2.0 is expected if the deficiencies are present.

Immunostaining and DAPI analysis

DAPI staining, immunostaining, and analysis of resultant nuclei were carried out as in Colaiácovo, *et al.* (2003); however, zones 1 through 8 were imaged and analyzed. In brief, young adult worms were dissected 21-24 hours post-L4 stage. Slides were freeze cracked on dry ice and then fixed in methanol at -20°C for 1 minute. Slides were then fixed with 4% formaldehyde for 30 minutes at RT. Next, slides were washed in PBST for 5 minutes and then blocked for at least 1 hour in 0.5% bovine serum albumin in PBST. After blocking, primary antibody was added to the slides, which were then incubated overnight at RT. On the following day, slides were washed twice in PBST for 5 minutes. Secondary antibody was added and slides incubated for 2 hours at RT. Following 3 x 5 minute washes in PBST, slides were stained with DAPI, destained for 1 hour and then passed through 10mM Tris pH 8.0 with 0.1% Tween before mounting with Vectashield (Vector Laboratories). Three to four gonads were analyzed for each genotype. Statistical analysis comparing control strains and experiment strains were done using the two-tailed Mann-Whitney test at the 95% confidence interval. Antibodies were used at the following dilutions: guinea pig α-Rad51 (1:200), rabbit α-Rad51 (COLAIÁCOVO *et al.* 2003) (1:200), rabbit α-Rad51 (Novus Biologicals) (1:10,000), rabbit α-pChk-1 (Santa Cruz Biologicals) (1:50), mouse α-uH2a (Millipore) (1:100), rabbit α-H3K9me2 (Upstate) (1:10,000). Secondary antibodies were all used at 1:100 (Jackson Immunologicals).

Imaging and Microscopy

Immunofluorescence images were collected on two different microscope setups. Heterozygous deficiency, translocation, and inversion strains were imaged on an IX-83 microscope (Olympus) and a cooled CCD camera (model XM10; Olympus). Image sections were 0.20μm. Images were deconvolved using and RAD-51 foci were scored using CellSens software (Olympus). All other images were collected using an IX-70 microscope (Olympus) and a cooled CCD camera (model CH350; Roper Scientific) controlled by the DeltaVision system (Applied Precision). Image sections were 0.20μm. Images were deconvolved using SoftWorx 3.0 as in Allard and Colaiácovo (2010). RAD-51 foci were scored using SoftWorx versions 1.3 and 2.0. Levels of H3K9me2 staining were determined using ImageJ software (NIH).

Slides were imaged within four weeks of production. To control for potential degradation over time, N2, the 2412kb deficiency, and the 2083kb deficiency were imaged during week one and again during week four. RAD-51 levels in all zones were compared between the week one and week four images. No significant change was found in levels of RAD-51 foci (Mann-Whitney U-test $P<0.05$), confirming that imaging within a four week time period did not affect the levels of RAD-51 foci visible on the slides.

Quantitative Analysis of Germ Cell Apoptosis

Germ line corpses were scored in adult hermaphrodites between 20 and 24 hours post-L4, using acridine orange as described in Kelly, *et al*. on an Olympus BX-60 microscope at 60X magnification (2000).

Quantitative PCR analysis

Primers to regions internal and external to deficiency loci were designed using Primer3 software (ROZEN AND SKALETSKY 2000). Genomic DNA was extracted from 6 plates of mixed age animals for each genotype of interest and analyzed using KAPA SYBR® FAST universal 2X qPCR master mix (KAPA Biosystems). Each analysis was performed in triplicate.

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3 Further insights into the detection of small chromosomal structural variants during meiosis

Matthew Jakubik conducted all experiments and data analysis.

INTRODUCTION

The pairing of homologous chromosomes plays an important role in gene regulation in many different organisms. Homolog pairing-mediated phenomena have been well studied in *Drosophila melanogaster*, where homologous chromosomes are intimately paired in all somatic tissues (STEVENS 1907; STEVENS 1908; METZ 1916; CSINK AND HENIKOFF 1998; FUNG *et al.* 1998; GEMKOW *et al.* 1998; reviewed in DUNCAN 2002; VAZQUEZ *et al.* 2002; Reviewed in MCKEE 2004; FRITSCH *et al.* 2006). Somatic homolog pairing allowed for the discovery of transvection by Ed Lewis. Transvection is a process in which the interaction of homologous chromosomes can produce changes to gene regulation and chromosome topology (LEWIS 1954; MORRIS *et al.* 1998; WU AND MORRIS 1999; DUNCAN 2002).

Lewis firmly established that homolog pairing is required for transvection at the Drosophila Bithorax Complex. Using X-rays to induce chromosomal rearrangements, Lewis was able to deduce that breakpoints located between the centromere of Drosophila chromosome III and BX-C resulted in loss of transvection, defining a "critical region" for transvection at the Ultrabithorax gene (LEWIS 1954). Similar pairing requirements have been identified at other genes shown to display transvection in Drosophila. For instance, Geyer, *et al.* determined that homolog pairing appears to be required for transvection at the *yellow* locus, as *yellow* alleles inserted at other loci do not result in transvection (1990). Similarly, transvection at the *decapentaplegic* gene complex is disrupted when rearrangements that disrupt homolog pairing are introduced (GELBART 1982). These and other studies of

transvection highlight the important role that homolog pairing may play in gene regulation.

Additionally, we combined FISH to deficiency loci with RAD-51 immunostaining to determine if RAD-51 was present at deficiency loci.

RESULTS

As our study examines the overall ability of the meiotic genome to detect heterozygous deficiencies, the readout for detection could be one or more of several different possibilities. Below, I will describe the five different assays that were considered as possible readouts. Of those considered, three assays did not produce results consistent with detection of deficiencies. Excitingly, two readouts proved to be potential assays for evaluating the ability of the genome to detect structural variation.

2. Apoptosis: As prior studies have shown that asynapsis can lead to elevated levels of germ cell apoptosis, we next investigated whether heterozygous deficiency strains also displayed higher levels of germline apoptosis when their average number of germline corpses were compared to the average level of apoptotic corpses among all of the controls. 32% of heterozygous deficiency strains show statistically different numbers of apoptotic corpses when compared to controls using Mann-Whitney U Tests.

3. Delayed entry into pachytene: Our RAD-51 data led us to investigate if heterozygous deficiency strains showed a delay in progression through meiosis, as it has previously been established that large disruptions of synapsis cause extension of the leptotene/zygotene stages of prophase I and are detected by the genome (PHILLIPS *et al.* 2005; CARLTON *et al.* 2006; JARAMILLO-LAMBERT AND ENGEBRECHT 2009; JARAMILLO-LAMBERT *et al.* 2010). For instance, in a *him-8* mutant background, which results in X chromosome asynapsis, Phillips and colleagues found that nuclei with leptotene-zygotene morphology persisted into mid-to-late pachytene (2005). Furthermore, additional studies of *him-8* mutants demonstrated that defects in synapsis of the X chromosome resulted in a genome-wide delay of progression through meiosis. In our study, only the 461kb deficiency strain showed a significant transition zone delay when compared to the average of controls (Mann-Whitney U-test, $P<0.05$).

4-5. uH2A, pCHK-1: Given that past studies in *C. elegans* and other organisms have also found other potential markers of unpaired chromatin, we also examined deficiency strains for localization of ubiquitinylated histone H2A (uH2A), and activation of CHK-1 (BEAN *et al.* 2004; TURNER *et al.* 2004; TURNER *et al.* 2005; TURNER *et al.* 2006; TURNER 2007; BURGOYNE *et al.* 2009; JARAMILLO-LAMBERT AND ENGEBRECHT 2009; AN *et al.* 2010; CLOUTIER AND TURNER 2010; JARAMILLO-LAMBERT *et al.* 2010; ROYO *et al.* 2010).

It has previously been shown that phosphorylated CHK-1 (pCHK-1) accumulates in nuclei that have activated the DNA damage response checkpoint (MOSER *et al.* 2009; ALLARD AND COLAIÁCOVO 2010). No accumulation of pCHK-1 was seen in the nuclei of heterozygous deficiency strains, indicating that the DNA damage response checkpoint was not activated in these strains (data not shown). Thus, relocalization of pCHK-1 was not an effective readout for detection of structural heterogeneity.

A final potential readout is the accumulation of ubiquitinated H2A on chromatin. Previous studies done in mice have implicated uH2A in a BRCA1-independent mechanism capable of detecting unpaired chromatin (AN *et al.* 2010; CLOUTIER AND TURNER 2010). Unfortunately, the commercially available anti-uH2A antibody used in prior studies did not react with the *C. elegans* protein, so we were unable to determine if uH2A is involved in the detection of heterozygous deficiencies.

Heterozygous deficiency strains show altered levels of germline apoptosis

Controls and heterozygous deficiency strains were stained with acridine orange and the numbers of apoptotic germline nuclei were quantified (COLAIACOVO 2003). Control strains showed an average of 1.5 apoptotic nuclei/gonad. 32% of heterozygous deficiency strains displayed altered levels of apoptotic nuclei (specifically, 1.1kb, 7.5kb, 34kb, 1350kb, 1552kb, 1902kb, 2083kb and 2351kb deficiency strains). This number is higher than would be expected due to stochastic

variation alone, as only 20% (1/5) of control strains had elevated levels of apoptosis when compared to N2 alone. Interestingly, the 1552kb and 2351kb strains were the only two strains to display significantly lower levels of germline apoptosis (0.4/ gonad and 0.7/gonad, respectively) than the average of controls (1.5/gonad) and are the only strains to display such behavior. It is possible that the haploinsufficient region present in these two strains reduced the dosage of a gene required for proper regulation of germline apoptosis.

Heterozygous deficiency strains do not show extended transition zones

The occasional reduction of RAD-51 levels in early pachytene led us to examine whether heterozygous deficiencies could delay entry into the pachytene stage of meiosis, as evidenced by the presence of nuclear morphologies consistent with nuclei in the leptotene/zygotene stages, also called transition zone nuclei, in early to mid-pachytene stages. Transition zone nuclei have a characteristic polarized spatial organization, or crescent shape, (Figure 8, inset), allowing for facile distinction from pachytene nuclei. The number of transition zone nuclei in early pachytene was quantified (Figure 8). Control strains typically contain between zero and four transition zone figures in zone 4, representing 1-14% of total early pachytene nuclei, or 9.0% on average. Zone 4 in heterozygous deficiency strains displayed between zero and seven transition zone

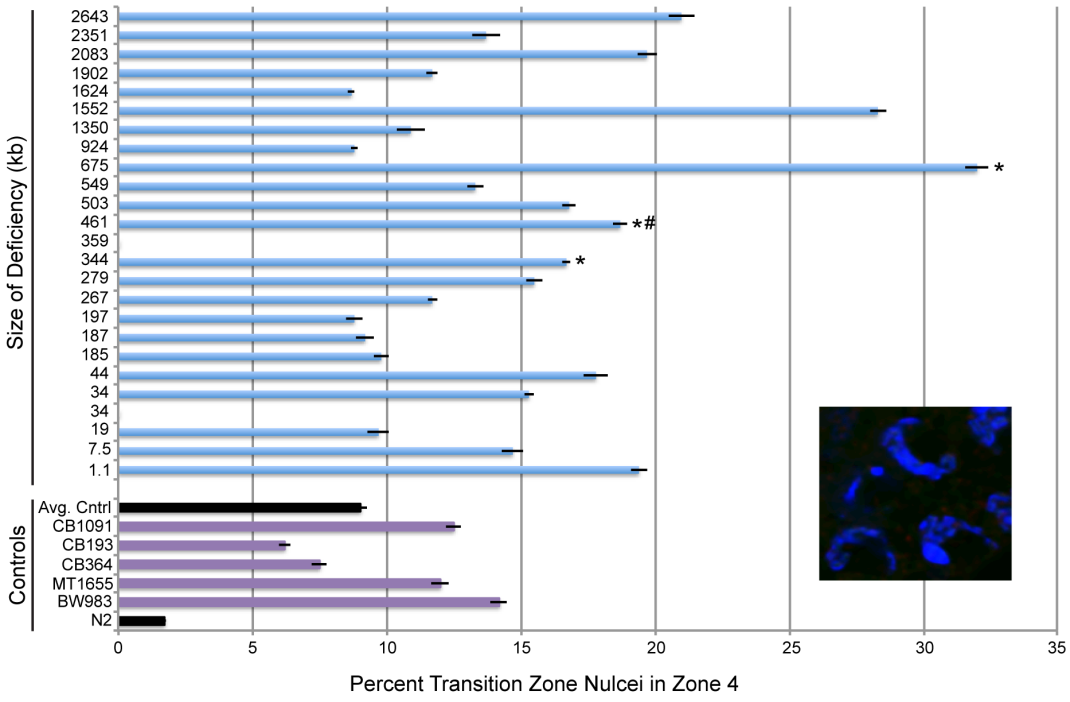

Figure 8. Heterozygous deficiency strains show a small, but observable trend of increased levels of leptotene-zygotene nuclei in early pachytene, though only 3 strains are statistically significantly different. Control strains are shown in purple and black. Heterozygous deficiency strains are shown in blue and ordered by increasing size from bottom to top. Percent of early pachytene nuclei displaying leptotene-zygotene morphology is shown. Error bars represent standard error of the mean. Inset shows characteristic crescent morphology of leptotene-zygotene nuclei. "*" indicates significant difference between N2 and a heterozygous deficiency strain (P<0.05). "#" indicates a significant difference between the average of controls and a heterozygous deficiency strain (P<0.05).

figures, corresponding to between 0% and 28% of early pachytene nuclei, on average 14%. There does not appear to be a correlation between the size of a deficiency and the level of delay. For instance, transition zone nuclei represented 18, 16, and 28.3% of zone 4 nuclei in strains that were heterozygous for deficiencies that were 44kb, 279kb, and 1552kb in size, respectively. However, transition zone nuclei with deficiencies of 34kb, 359kb, and 924kb showed approximately 0, 0, and

9% of transition zone figures in zone 4. Three strains with 675kb, 461kb, and 344kb heterozygous deficiencies, respectively, showed statistically significantly elevated levels of transition zone nuclei when compared to N2 alone. Only the 461kb deficiency strain displayed significantly elevated levels of transition zone nuclei when compared to the average levels of control strains. These data indicate that the presence of a heterozygous deficiency doesn't significantly affect delay the leptotene and zygotene stages.

Synapsis is not grossly disrupted in heterozygous deficiency strains

As previous studies have associated transition zone delays with large areas of unsynapsed chromatin, I examined strains for disrupted synapsis. To this end, the 924, 1624, and 1902kb heterozygous deficiency strains were co-stained for SYP-1 and HTP-1/2, a synaptonemal complex central element and axial element, respectively (MACQUEEN *et al.* 2002; COUTEAU AND ZETKA 2005; MARTINEZ-PEREZ AND VILLENEUVE 2005; MARTINEZ-PEREZ *et al.* 2008). Synapsis did not appear to be grossly disrupted in 176 mid-to-late pachytene nuclei examined, suggesting that synapsis proceeds normally in heterozygous deficiency strains. Alternatively, the small size of the deficiencies relative to the entire genome coupled with the limited resolution of immunofluorescence and microscopy may have prevented visual detection of very small, unsynapsed regions.

Deficiency strains show perturbed H3K9me2 levels

Previous studies done in *C. elegans* and mouse germlines demonstrated that unpaired entire chromosomes and free duplications, are detected by the meiotic nucleus and are enriched for proteins and chromatin modifications indicative of silenced chromosomal regions, including H3K9me2 (KELLY *et al.* 2002; BEAN *et al.* 2004) (KHALIL *et al.* 2004; SCHIMENTI 2005; TURNER *et al.* 2005; TURNER *et al.* 2006; TURNER 2007; BURGOYNE *et al.* 2009; WOJTASZ *et al.* 2009; AN *et al.* 2010; CLOUTIER AND TURNER 2010; FUKUDA *et al.* 2010). As Bean and colleagues had already established that the unpaired X chromosome in XO males is enriched for H3K9me2, we investigated if our chosen deficiency strains affected levels of H3K9me2 staining in meiotic nuclei. Heterozygous deficiency strains were stained with an antibody for H3K9me2 and average fluorescence intensity of the H3K9me2 signal within each germline nucleus was measured using ImageJ software. All strains accumulated H3K9me2, confirming that deficiency strains did not prevent H3K9me2 protein from being produced or targeted to chromatin. Deficiency strains show a general perturbation of average H3K9me2 intensity throughout prophase I and increased variability in staining intensities compared to wild-type strains (Figure 9).

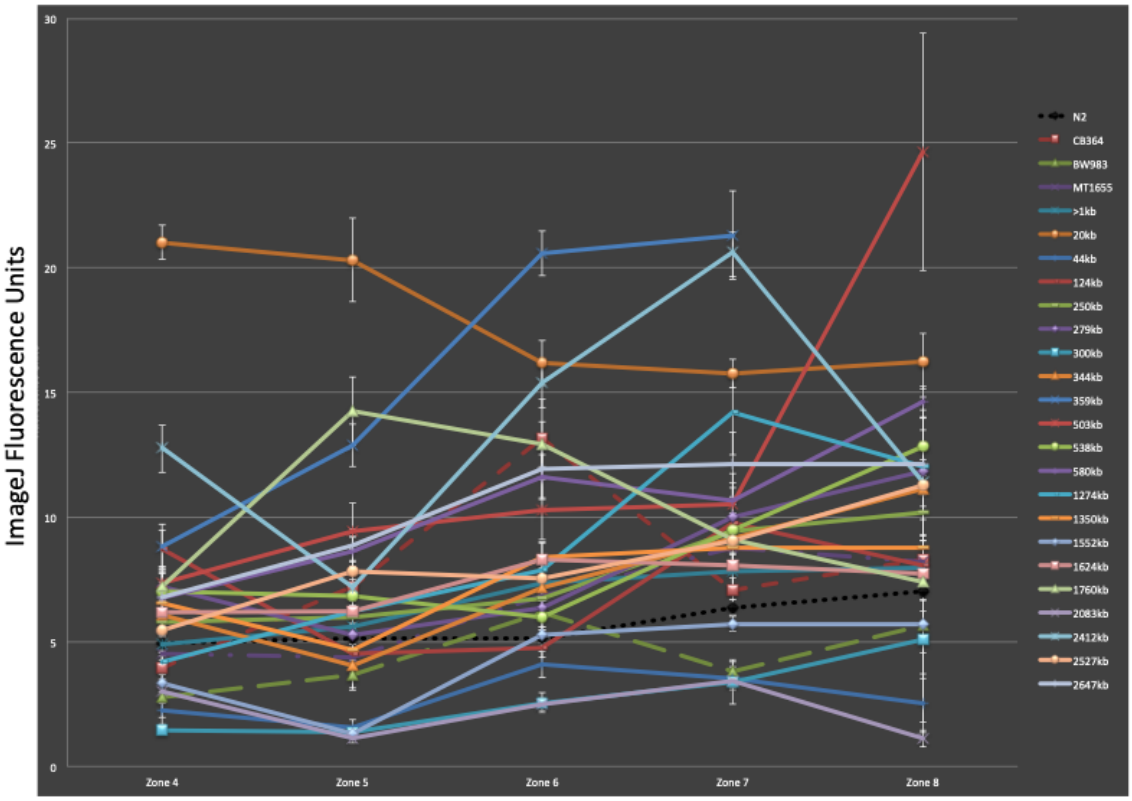

Figure 9. Quantitation of H3K9me2 levels in heterozygous deficiencies. Average levels of H3K9me2 staining was measured in meiotic nuclei using ImageJ software. Average levels for three to four gonads per deficiency strain are plotted above using ImageJ fluorescence units. Heterozygous deficiency strains show variable and disrupted H3K9me2 levels.

Spearman Rank Correlation Coefficient Tests Show No Correlation Between Readouts of Detection

As our data suggest that detection of heterozygous deficiencies could occur through several different mechanisms, we sought to determine if there was a correlation between different readouts. Spearman Rank Correlation Coefficient tests, which measure the dependence between two variables, were conducted to determine if there was correspondence between the three readouts for detection of

structural changes (Table 5). Comparison of RAD-51 levels with levels of transition zone delay resulted in rho values ranging from -0.116 to 0.267. No zones showed significant correlation (P<0.05, Student's t-test). Comparison of RAD-51 levels to levels of apoptosis produced rho values of -0.046 to 0.069. No zones showed statistically significant correlations according to Student's t-test. Lastly, comparison of levels of apoptosis to transition zone delay demonstrated no correspondence with a rho value of -0.146 and a P value of 0.477 (Student's t-test). Taken together, these test results show that three potential readouts for detection of chromosomal variants (levels of RAD-51 foci, levels of germline apoptosis, and transition zone delay) show no correlation.

A. Correlation between RAD-51 levels and germline apoptosis.

	Zone%	Zone%	Zone%	Zone%	Zone%
rho	0.058	0.036	0.069	(0.046	0.049
P%alue	0.779	0.862	0.738	0.824	0.811

B. Correlation between RAD-51 levels and transition zone delay.

	Zone%	Zone%	Zone%	Zone%	Zone%
rho	0.047	0.267	'0.044	0.039	'0.116
P%alue	0.821	0.187	0.832	0.848	0.573

C. Correlation between transition zone delay and germline apoptosis.

rho	!0.146
P%alue	0.477

Table 5. Spearman's Rank Correlation Coefficient Analysis for Readouts of Detection. A) Correlation between RAD-51 levels and levels of germline apoptosis. B) Correlation between RAD-51 levels and levels of transition zone delay. C) Correlation between transition zone delay and levels of germline apoptosis. Correlation value (ρ, rho) and P values are zone for all comparisons. No measurements show statistically significant correlations (P<0.05, Student's t-test).

There is minimal overlap between deficiency loci and RAD-51 signal

We next asked, "Is there overlap between RAD-51 foci and the deficiency loci discussed in Chapter 2?" Our model predicts that unpaired chromatin loops may form at deficiency loci. The topological nature of such loops may make them more easily accessible to proteins involved in the formation of double strand breaks and their repair. Furthermore, breaks may persist on the looped out chromatin as it does not have a homologous partner off of which to repair when heterozygous. To examine this, we dissected gonads and co-stained with FISH probes to the deficiency region and a RAD-51 antibody. Strains were considered to have overlapping RAD-51 and FISH probes when fluorescent spots from both signals coincided in deconvolved images. Strains displayed between 0 and 20% overlap throughout prophase I (Figure 10). For instance, the 187kb heterozygous deficiency strain showed the highest level of overlap with 17.3% overlap in zone 4, 6.4% in zone 5, 17.9% in zone 6, 19.9% in zone 7, and 9.5% in zone 8.

The strain with a 461kb heterozygous deficiency, showed overlap levels of 3.6%, 0%, 2.8%, 0.8%, and 0% in zones 4 through 8, respectively. Based upon these data, we conclude that, while we do see a low level of overlap between signals, overall, RAD-51 levels are altered throughout the nucleus. Furthermore, the data do not display a clear sized-based trend, i.e. larger deficiencies do not display higher coincidence of RAD-51 foci at their respective deficiency loci.

A control was performed to determine that our FISH probes were specific for the deficiency loci. Here, two adjacent probes targeted to a deficiency locus were differentially labeled and overlap was determined (Figure 11, Table 6).

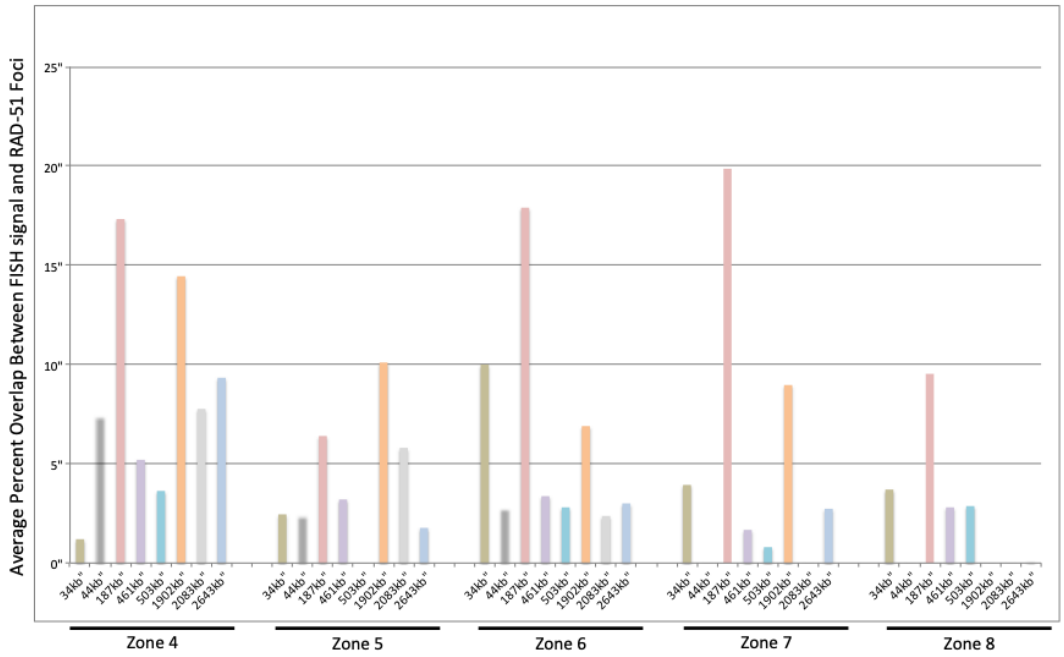

Figure 10. Average percent overlap between FISH probes to deficiency loci and RAD-51 antibody staining. Strains are color-coded according size of heterozygous deficiency. Three gonads were scored per deficiency strain. Strains typically displayed between 0% and 10% overlap, indicating that RAD-51 foci did not always appear at the deficiency locus and that changes to RAD-51 foci levels affected chromatin on a global level.

In the strain with a 2647kb heterozygous deficiency (n=128 nuclei), 87.5% of nuclei were co- labeled with overlapping probes. In the strain heterozygous for the 1624kb deficiency (n=118 nuclei), 83.1% of nuclei were co-labeled with overlapping probes (Table 8). No nuclei, in either instance, were seen with more than one focus per probe in each nucleus. These data confirm that FISH probes were specific to the

given loci. In the strain heterozygous for the 1624kb deficiency (n=118 nuclei), 83.1% of nuclei were co-labeled with overlapping probes (Table 6). No nuclei, in either instance, were seen with more than one focus per probe in each nucleus. These data confirm that FISH probes were specific to the given loci.

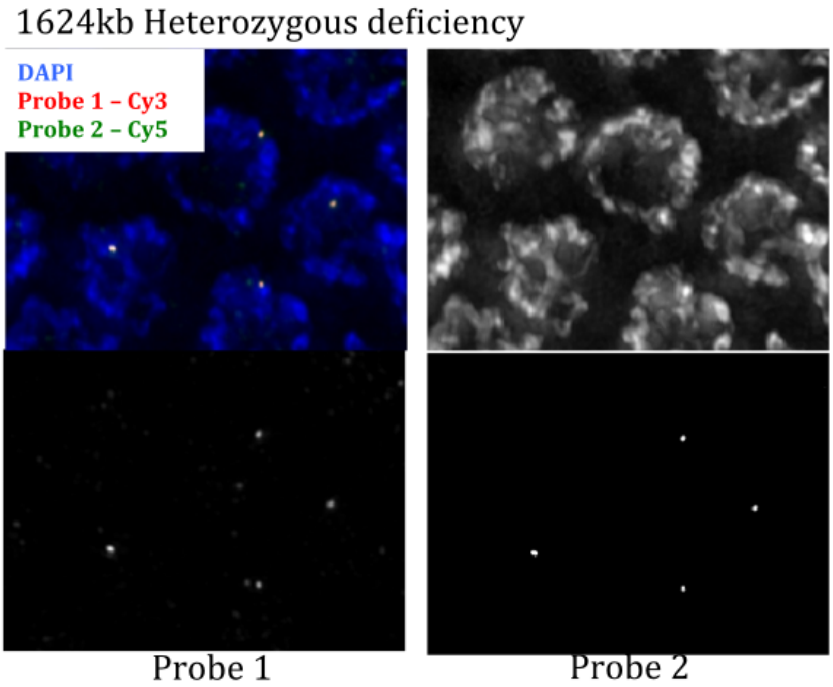

Figure 11. FISH control experiment to confirm FISH probe specificity. Adjacent FISH probes targeted to deficiency locus were differentially labeled and used to stain gonads. Colocalization of probes confirms FISH specificity for the deficiency locus.

Strain	Number of Nuclei Scored	Nuclei With Probe Overlap	Nuclei Without Probe Overlap
2643kb Deficiency	128	87.5%	12.5%
1624kb Deficiency	118	83.1%	16.9%

Table 6. Quantification adjacent probe overlap for dual FISH control experiment. Two adjacent FISH probes were targeted to deficiency loci to confirm FISH probe specificity. Between 83.1% and 87.5% nuclei examined showed overlapping FISH signals, confirming probe specificity. The remaining nuclei, 12.5% and 16.9%, respectively, did not show any FISH probe signal.

DISCUSSION

Pachytene delay may allow for proper homolog pairing and synapsis

Our study demonstrated that several heterozygous deficiency strains showed increased levels of transition zone nuclei present in zone 4 (early pachytene), representing up to 21% of total nuclei in zone 4. This is similar to previous studies showing that other meiotic mutations that disrupt synapsis lead to an analogous, but more severe lengthening of the transition zone (MACQUEEN *et al.* 2002; COLAIÁCOVO *et al.* 2003; COUTEAU AND ZETKA 2005; MARTINEZ-PEREZ AND VILLENEUVE 2005; MARTINEZ-PEREZ *et al.* 2008). As homolog pairing begins in the transition zone, it is possible that this delay in entry into early pachytene occurs to allow homologs with heterozygosity sufficient time to correctly synapse. Should synapsis fail, there is evidence for a pairing checkpoint that is activated during early pachytene in *C. elegans* (BHALLA AND DERNBURG 2005). However, as this checkpoint is activated specifically by unpaired PCs, it is unlikely that it, per se, would play a role in the

transition zone extension caused by unsynapsed regions that do not disrupt pairing center activity.

Limited correlation among readouts suggests the presence of independent detection mechanisms

Spearman rank correlation coefficient tests comparing the different readouts did not show correlation between the readouts. This lack of correlation has led us to believe that multiple mechanisms of detection may be at play. It is possible that different deficiencies are surveyed by different mechanisms depending on their size or location within the genome. The presence of multiple mechanisms may explain, for instance, why some strains with large deficiencies do not show consistently elevated levels of RAD-51 foci throughout pachytene, but do show a large number of transition zone nuclei in zone 4. Furthermore, the lack of correlation with increased response in readouts and deficiency size could also be explained by the existence of multiple detection mechanisms.

RAD-51 does not localize to deficiency loci at high levels

Our ImmunoFISH data showed limited overlap between RAD-51 signals and FISH probes to deficiency loci, ranging from 0% to 20% overlap. This is consistent with our prior observation that presence of a heterozygous deficiency on the X chromosome does not guarantee the presence of a RAD-51 focus on the X chromosome. The lack of correspondence could be explained by several different possibilities.

First, it is possible that the presence of heterozygosity alters RAD-51 distribution on the affected chromosome. This may occur via a process similar to chromosome interference, a process that limits the number of crossovers between bivalents (FOSS *et al.* 1993; HILLERS 2004; KLECKNER *et al.* 2004). In *C. elegans*, chromosome interference limits the number of crossovers to one per bivalent (HODGKIN *et al.* 1979; MENEELY *et al.* 2002; NABESHIMA 2004; HAYASHI *et al.* 2010). Interestingly, it has been shown that heterozygous insertions limit local crossovers to less than half of normal levels without affecting overall interference (HAMMARLUND *et al.* 2005). Analogously, a heterozygous deficiency may have the same effect on crossover distribution, perhaps by limiting local double strand breaks. Thus, as RAD-51 plays a role in double strand break resolution, its localization to deficiency loci may be inherently inhibited by the presence of the deficiency. This possibility is further supported by evidence that several deficiencies on *C. elegans* chromosome V suppress recombination distal to their chromosomal location (ROSENBLUTH *et al.* 1990).

Another possible reason for the lack of correlation between deficiency loci and RAD-51 signals is that the condensed size of the homolog containing the deficiency may be smaller, resulting in an unpaired region on the end of the wild-type homolog (Susanna Mlynarczyk-Evans, personal communication. Figure 11). Thus, RAD-51 may also preferentially localize to this potentially unsynapsed region.

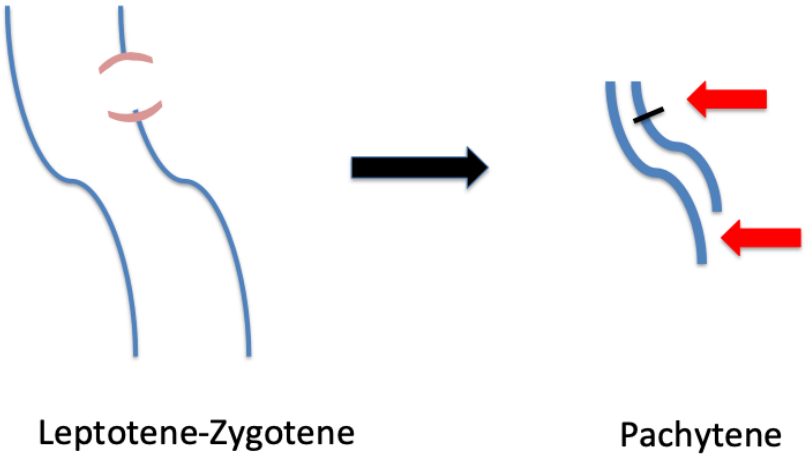

Figure 11. Model for RAD-51 localization in heterozygous deficiency strains. As chromosomes progress from leptotene-zygotene into pachytene, they continue to condense. Chromosomes harboring a deficiency may be smaller than wild type chromosomes due to condensation. This difference in size may produce an unsynapsed region on the wild type chromosome following synapsis. RAD-51 may then be targeted to both the deficiency region and to this additional unsynapsed region (red arrows), resulting in fewer RAD-51 foci at deficiency loci than expected.

MATERIALS AND METHODS

Genetics

C. elegans strains were cultured at 20°C under standard descriptions described by Brenner (1974). The Bristol N2 strain was used a control during Rad51 stainings to confirm that strains were analyzed at the correct developmental time point. Six additional strains not known to carry any known chromosomal rearrangements were randomly chosen from lab stocks and stocks available at the Caenorhabditis Genetics Center at the University of Minnesota and were analyzed and average Rad51 levels of these six strains and N2 were utilized for statistical analysis of Rad51 levels and for evaluation of pachytene delay. The six additional

control strains are as follows: CB364 *dpy-18(e364)III*, MT1655 *bli-6(n776)IV*, BW983 *bli-4(e937) unc-37(e262)I*, CB1951 *unc-42(e270) sma-1(e30)V*, CB1095 *unc-13(e1091)I*, and CB4845 *unc-119(e2498)III*. Strains with heterozygous deficiencies used to evaluate detection were kindly obtained from the Caenorhabditis Genetics Center. Strains were maintained by selecting the appropriate phenotypes for maintenance of the heterozygous deficiencies.

Immunostaining and DAPI analysis

DAPI staining, immunostaining, and analysis of resultant nuclei were carried out as in Colaiácovo, *et al.* (2003), except that zones 1 through 8 were imaged and analyzed. In brief, young adult worms were dissected 21-24 hrs post-L4. Slides were freeze cracked on dry ice and then fixed in methanol at -20°C for one minute. Slides were then fixed with 4% formaldehyde for 30 min. at RT. Next, slides were washed in PBST for 5 min. and then blocked for at least 1 hour in 0.5% bovine serum albumin in PBST. After blocking, primary antibody was added to the slides, which were then incubated overnight at RT. On the following day, slides were washed twice in PBST for 5 min. Secondary antibody was added and slides incubated for 2 hrs. at RT. Following three five-minute washes in PBST, slides were stained with DAPI, destained for 1 hr. and then passed through 10mM Tris pH 8.0 with 0.1% Tween before mounting with Vectashield (Vector Laboratories). Three to four gonads were analyzed for each genotype. Statistical analysis comparing control strains and experiment strains were done using the two-tailed Mann-Whitney test at the 95% confidence interval. Antibodies were used at the following dilutions:

guinea pig α-Rad51 (1:200), rabbit α-Rad51 (COLAIÁCOVO *et al.* 2003) (1:200), rabbit α-Rad51 (Novus Biologicals) (1:10,000), rabbit α-pChk-1 (Santa Cruz Biologicals) (1:50), mouse α-uH2a (Millipore) (1:100), rabbit α-H3K9me2 (Upstate) (1:10,000). Secondary antibodies were all used at 1:100 (Jackson Immunologicals).

ImmunoFISH analyses

ImmunoFISH studies were conducted as in Smolikov (2008). Probes were generated from commercially available fosmids (Table 9, Source Bioscience).

Imaging and Microscopy

Immunofluorescence images were collected using an IX-70 microscope (Olympus) and a cooled CCD camera (model CH350; Roper Scientific) controlled by the DeltaVision system (Applied Precision). Image sections were 0.20μm. Images

Strain	Fosmid(s) Used to Generate FISH Probes
BC199	WRM0630cD12, WRM069bE90
BC694	WRM0630cD12, WRM069bE90
GR1311	WRM0624dD12, WRM064cF08
KR2838	WRM064cE02, WRM0629bH05
EJ275	WRM0630aA07
JK1726	WRM0634bE04
SP719	WRM0620dH06, WRM0638cD09
DA768	WRM0612aH07
SP755	WRM0620dH06, WRM0638cD09

Table 9. List of fosmids used to generate FISH probes.

were deconvolved using SoftWorx 3.0 as in Allard and Colaiácovo (2010). Rad51 foci were scored using SoftWorx versions 1.3 and 2.0. Levels of H3K9me2 staining were determined using ImageJ software (NIH).

Crosses to generate double heterozygous deficiency strains

Crosses were conducted in several steps as follows (Figure 12): 1) Strains were generated to be double heterozygous for recessive markers present on the wild-type chromosomes opposing the two deficiencies of interest. Here, males of one homozygous mutant strain were generated by heat shock at 32°C for 4 hours. Males were then mated to hermaphrodites homozygous for the second marker overnight at 20°C. Mated hermaphrodites were singled onto individual plates and only plates with greater than 50% males were used. F2 progeny homozygous for both recessive markers were then isolated based on their phenotype. For instance,

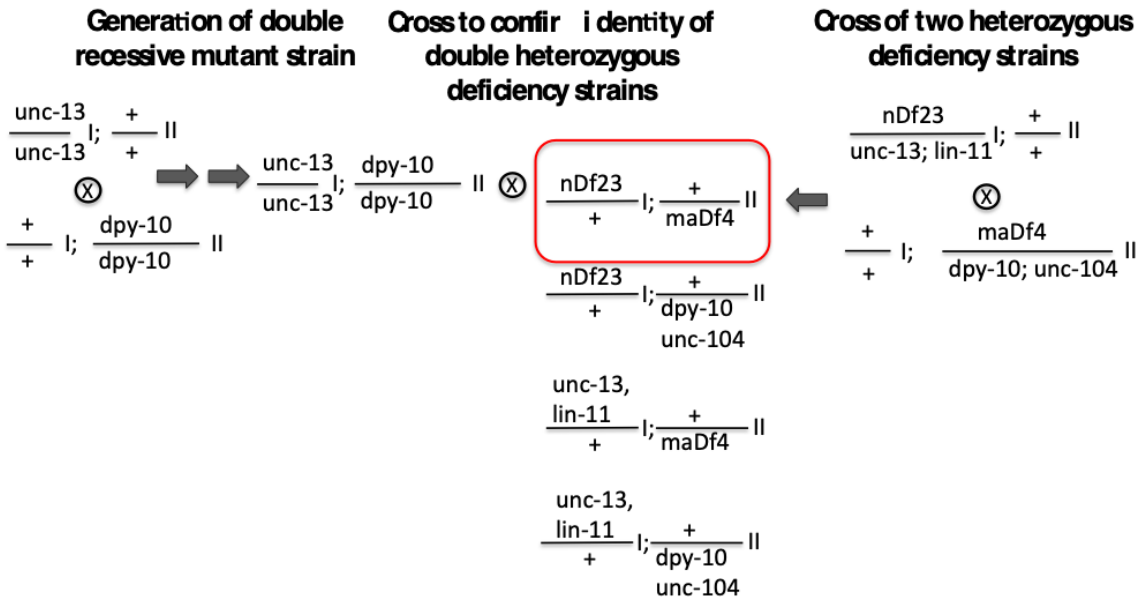

Figure 12. Example of cross scheme utilized to generate double heterozygous deficiency strains. First, as on the left, a double recessive mutant strain was generated. Second, as on the right, two heterozygous deficiencies were crossed, producing the four classes present in the center. These four classes were then mated to the double recessive mutant strain. The double heterozygous deficiency strain (highlighted in red) will produce wild type offspring when crossed to the double mutant and it can be selected for further analysis.

in the cross to generate a double homozygous marker strain for the cross combining the 1350kb and 1006kb deficiency strains, the resultant marker strain had the following genotype: unc-13(e1091)/unc-13(e1091) I; dpy-10(e128)/dpy-10(e128) II.

2) The two respective heterozygous deficiency strains were crossed as above. Mated hermaphrodites were singled onto individual plates and only plates containing more than 50% males were used for further crosses. For instance, the cross to combine the 1350kb (maDf4/dpy-10(e128), unc-104(e1265) II) and 1006kb (nDf23/unc-13(e1091), lin-11(n566) I) deficiency strains produced the following classes of offspring: A) nDf23/+ I; +/maDf4 II, B) nDf23/+ I; +/ dpy-10(e128), unc-104(e1265) II, C) unc-13(e1091), lin-11(n566)/+ I; +/maDf4 II, and D) unc-13(e1091), lin-11(n566)/+ I; +/dpy-10(e128), unc-104(e1265) II.

3) Since the classes of progeny generated in step 2 are indistinguishable by phenotype, males of these four classes were crossed to the double homozygous marker strain generated in step 1. Only class a produced wild-type offspring, indicating that it contained both deficiencies. Hermaphrodites that generated class A were then dissected and used in subsequent immunostaining analyses.

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4 Summary and Perspectives

Chromosomal structural variants, such as SNPs, CNVs, translocations, insertions and deletions are widespread in the genomes of many different organisms. Many prior studies have shown that such variants can be detected when homologous chromosomes pair during meiosis through a variety of mechanisms, such as MSCI, MSUD, or MSUC, in a wide array of organisms (AN *et al.* 2010; ARAMAYO and METZENBERG 1996; BAARENDS *et al.* 2005; BEAN *et al.* 2004; CLOUTIER and TURNER 2010; KEEGAN *et al.* 1996; MOENS *et al.* 1999; SCULLY *et al.* 1997; SHIN *et al.* 2010; SHIU *et al.* 2001; TURNER 2007; TURNER *et al.* 2004; TURNER *et al.* 2002; TURNER *et al.* 2006; ZHOU *et al.* 2008). Importantly, these studies have only addressed the ability of the genome to detect large variants, such as translocations of entire chromosome arms or entire unpaired chromosomes. The goal of my thesis was to determine if small structural variants also underwent detection processes.

Our data illustrate that small, heterozygous deficiencies, ranging in size from approximately 34kb to over 2.6Mb are detected in the meiotic germline of *C. elegans* (Chapter 2). Detection occurs via altered levels and kinetics of Rad51 foci and by an extension of the leptotene-zygotene stage of meiosis. Our data are consistent with previous studies examining the effects of much larger disruptions to homolog pairing and synapsis (ALPI *et al.* 2003; COLAIÁCOVO *et al.* 2003; PHILLIPS and DERNBURG 2006).

Additionally, we determined that detection occurs through disruption of homolog pairing by examining two homozygous viable translocations and a homozygous viable inversion, in both their respective homozygous and heterozygous states (Chapter 3). Here, we saw that the Rad51 response was reduced when variants were made homozygous, suggesting that detection is dependent on homolog pairing. Additionally, *C. elegans* strains combining two heterozygous deficiencies demonstrated an intermediate effect; indicative of an epistatic interaction occurring when more than one deficiency is present. Lastly, by combining Rad51 antibody staining and FISH probes targeted toward deficiency loci, we showed that there is relatively low colocalization of Rad51 at heterozygous deficiency loci.

Future studies

The current available tools and knowledge of possible detection mechanisms in *C. elegans* leave much to be discovered about additional possible mechanisms for detection of structural variants. Future studies should address the mechanistic details of detection. For instance, more accurate localization of detection marks should be addressed by examining detection mechanisms with tools such as chromatin immunoprecipitation-sequencing (ChIP-seq). Additionally, it is possible that additional proteins, such as yet undiscovered histone variants or other potential components of the meiotic machinery, could be important factors in the detection of small structural variants. It will also be important for future studies to search for

similar detection mechanisms operating in other organisms, such as mice or humans.

Our evidence that heterozygous regions ranging in size from 34kb to 2.6Mb are detected by the meiotic genome has especially interesting implications for some types of structural variation shown to be present in the human genome, in particular CNVs. CNVs typically range in size from several kilobases to several megabases and occur commonly as both deletion and insertion variants (ALKAN *et al.* 2011; FREEMAN 2006; IAFRATE *et al.* 2004; LEE and SCHERER 2010; MCCARROLL *et al.* 2006; MULLANEY *et al.* 2010; PERRY *et al.* 2008; SEBAT 2004). This is precisely the size range that we examined for detection in our studies. Furthermore, several studies have found that large CNVs, ranging in size from greater than 500bp to 2Mb, are enriched in individuals with obesity and schizophrenia (BOCHUKOVA *et al.* 2010; GIRIRAJAN *et al.* 2011; WANG *et al.* 2010). Our data show that heterozygous deficiencies within this size range show an increased detection response compared deficiencies less than 250kb in size. Thus, given our evidence, it is possible that CNVs are routinely detected within the meiotic genome when they are present in a heterozygous state, i.e. a deletion CNV allele in trans to a wild type allele.

If CNVs are detected during human meiosis, there are profound implications for human disease. For instance, if a deletion or insertion CNV allele lies in trans to a wild type copy, it is possible that homolog pairing of the surrounding homozygous

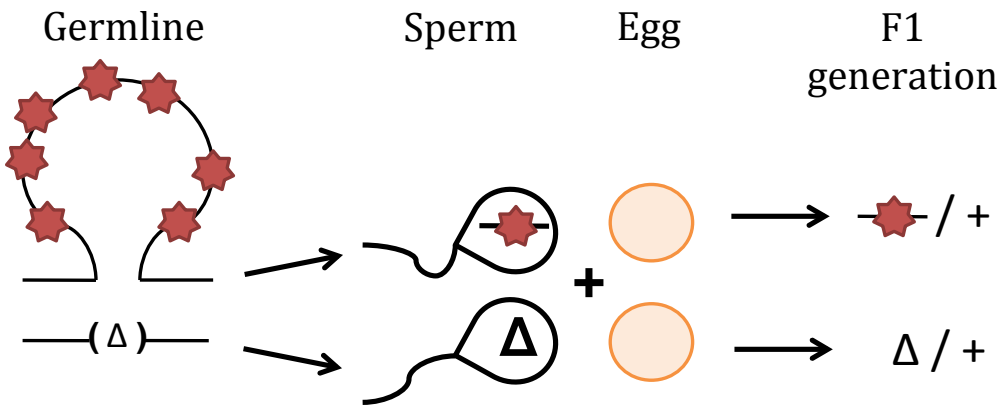

Figure 13. Implications of Detection of Heterozygous Structural Variants in Humans. Small, unsynapsed regions of the human genome may accumulate marks if they are detected during meiosis. Chromatin with these marks may be packaged into sperm or eggs and passed on to the F1 generation and could affect gene expression in the F1. Red star indicates marks placed on chromatin by the detection mechanisms.

DNA will force the wild type CNV allele into an unpaired chromatin loop (Figure 13). The meiotic machinery may detect and then mark this loop. Both homologs are then passed on to the F1 generation. If the marks placed on the chromatin by the detection process are retained on the wild type CNV allele, it is possible that gene regulation may then be altered in the F1. For instance, if the wild type CNV allele was marked with silencing marks, such as H3K9me2, then the wild type allele may behave in a similar manner to a deletion allele.

Interestingly, our results and the above model generate additional concerns as to how disease association studies are carried out. Currently, such studies attempt to correlate a disease phenotype with the presence of a CNV deletion allele in individuals carrying that deletion allele. Our model suggests that the disease phenotype may be present in offspring regardless of the allele inherited (Figure 14). Thus, it is important for future disease association studies to determine if parental heterozygosity for CNV alleles correlates to disease phenotypes in the F1 generation.

Our data (Chapters 2 and 3) have also led us to question if homolog pairing during meiosis may serve a purpose in addition to assuring accurate segregation and gametogenesis. In particular, we suggest that meiotic homolog pairing allows for a direct comparison of homologous chromosomes and, thus, may function as a mechanism by which the genome can sense the level of structural variation within the population. During such a comparison, CNVs would make an excellent measure of diversity, as individuals typically have hundreds of CNVs in a wide range of sizes distributed throughout the genome; these structural variants would allow for excellent coverage and comparison of the entire genome. Figure 15 illustrates a model in which meiotic pairing acts to achieve or maintain an advantageous level of variation within the population. In the first scenario, two homologs, with an " advantageous" level of variation, pair during meiosis, and detecting no need to increase or decrease variation fail to trigger any down stream mechanisms to alter the level of structural variation (Figure 15B). In a second scenario, the level of variation is below a certain threshold.

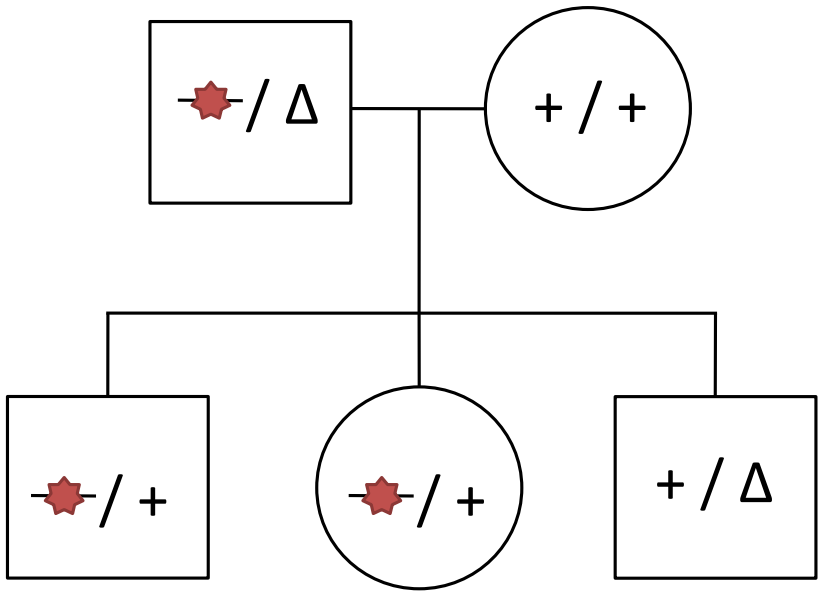

Figure 14. Pedigree Illustrating the Implications of Detection of Heterozygous Structural Variants in Humans. In this example, the male parent is heterozygous for a structural variant that is detected and marked by the meiotic machinery. Both the unmarked allele and the deletion alleles are passed on to the F1 generation. Disease phenotype may not depend on which allele is inherited, but rather the heterozygous state in the parental generation. Red star indicates marks placed on chromatin by the detection mechanisms.

Thus, mechanisms work to increase heterogeneity, possibly by increasing the rate at which mutations accumulate (Figure 15C). In the third scenario, the variation is too great resulting in one or more of several outcomes: an increase in DNA repair, an increase in the fidelity of this repair, or lastly, germ cell apoptosis (Figure 15A). The latter option would result in a type of meiotic drive, skewing the

meiotic outcome away from gametes with a level of diversity deemed too high. Taken together, the different responses to levels of variation could function at a population level to maintain a consistent and advantageous level of genetic difference among the members of the population.

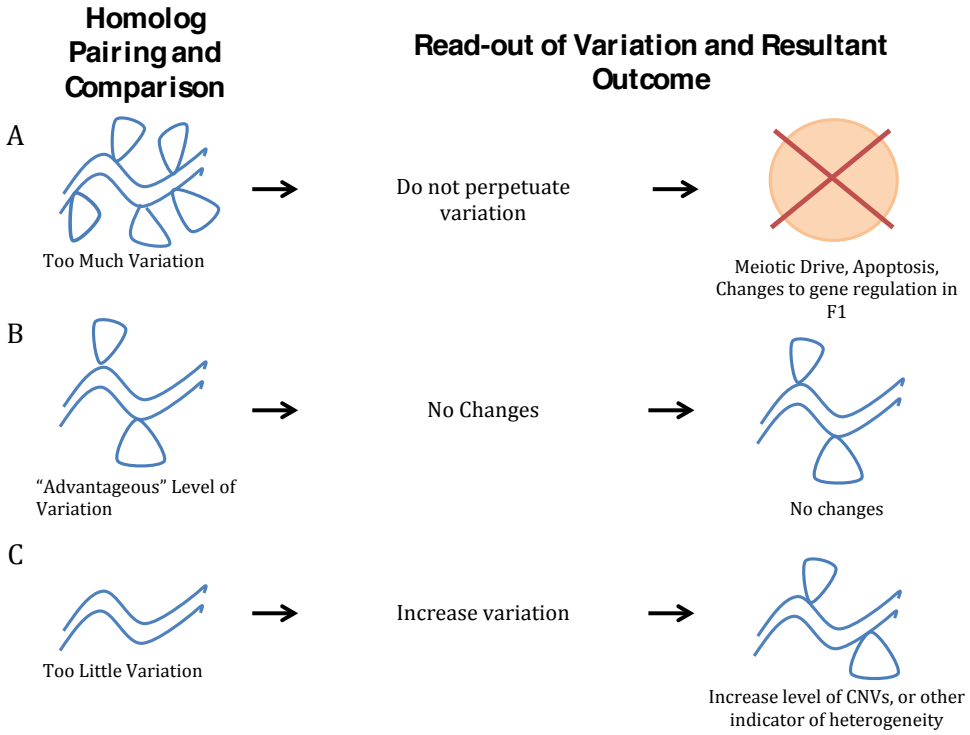

Figure 15. Homolog Pairing During Meiosis May Allow for Comparison of Genomic Variation Within the Population. A) Detection mechanisms find that genomic variation is above a certain threshold. Variation is not passed on to the F1 generation. B) Detection mechanisms find that variation is within an " advantageous" range and meiosis progresses normally. C) Variation is found to be below the "advantageous" level and mechanisms to increase variation are activated.

In conclusion, our studies have shown that the meiotic genome is competent for detecting small chromosomal structural variants. This finding is particularly important as our understanding of the role structural variants play in disease continues to improve. Given that many diseases have been associated with structural changes to the genome, it will be important for future studies to take our results into consideration when examining the connection between structural variation and disease. Lastly, our model in Appendix A suggests that meiosis may function to maintain an advantageous level of variation within the population in addition to its role in ensuring proper generation of gametes. It will be interesting to see if future studies address this possibility.

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Appendix A

This appendix includes text and discussion from a proposed manuscript: Copy Number Variation and Chromosome Pairing: Interactions and Implications for Human Disease and Evolution with authors Jakubik M. E. and Wu, C-t.

Copy Number Variation and Chromosome Pairing:
Interactions and Implications for Human Disease and Evolution

Human genomes sport a high level of structural variation, including single nucleotide polymorphisms (SNPs), translocations, inversions, and copy number variations (CNVs), the latter consisting of thousands of genomic regions present in variable copy number in the population. First documented in a systematic genome-wide fashion seven years ago and now one of the 'hottest' topics in human genetics, CNVs range in size from a megabase or more to a lower limit defined only by the constraints of wet bench and bioinformatic technology (ALKAN *et al.* 2011; FREEMAN 2006; IAFRATE *et al.* 2004; LEE and SCHERER 2010; PERRY *et al.* 2008; SEBAT 2004). Take any two individuals, and chances are that they will differ by scores, if not hundreds, of CNVs. One individual will have up to dozens (ALKAN *et al.* 2009) of a genomic region that is present in two copies in another individual. Choose another genomic region, and some individuals will be missing that region entirely. Take closely related individuals, even identical twins, and they may well differ in copy number in one or more regions (BRUDER *et al.* 2008). For that matter, sample two tissues from a single individual, and there is a reasonable chance that the two samples will differ by *de novo* CNVs, also called somatic copy number alterations (SCNAs; (BEROUKHIM *et al.* 2010)) in recognition of their somatic origin (MKRTCHYAN *et al.* 2010a; MKRTCHYAN *et al.* 2010b; PIOTROWSKI *et al.* 2008).

Augmenting the interest of researchers in CNVs has been the potential of these genetic features to explain human variation and disease. Citing just a fraction

of recent reports, CNVs have been associated with cancer (CAMPS *et al.* 2008; LEBRON *et al.* 2011; NORSKOV *et al.* 2010; SHLIEN *et al.* 2010; SPELEMAN *et al.* 2008; TRAN *et al.* 2011; YOSHIHARA *et al.* 2010), schizophrenia (BUIZER-VOSKAMP *et al.* 2011; MORENO-DE-LUCA *et al.* 2010), autism (BREMER *et al.* 2010; MORENO-DE-LUCA *et al.* 2010; SANDERS *et al.* 2011), Alzheimer disease (BROUWERS *et al.* 2011; KAY *et al.* 2010; SHAW *et al.* 2011; ZHANG *et al.* 2009a), and Crohn's disease (MCCARROLL and ALTSHULER 2007; MCCARROLL *et al.* 2008; ZHANG *et al.* 2009b) reviewed by (KUIPER *et al.* 2010; LEE and SCHERER 2010). In general, these association studies identify causative CNVs by positive correlations between the presence of a particular CNV and presentation of a disease trait. Here, we consider CNVs in light of meiotic forms of gene regulation that are driven by homolog pairing and predict that presentation of disease may not necessarily correlate with presence of the causative CNV for some CNV-associated diseases. Below, we provide a brief introduction to meiotic silencing, a form of meiotic gene regulation, and then present arguments for our prediction, highlighting the well-recognized potential of homolog pairing to effect changes in gene expression. We conclude with a more general speculation about the role of homolog pairing in genome evolution.

Meiotic silencing of the sex chromosomes

Meiosis is a type of cell division that reduces the number of chromosomes by half while maintaining genome complexity (Figure 16), permitting, for example, diploid organisms to generate haploid gametes. This reductional process is possible because each chromosome is paired end-to-end with its homologous partner early

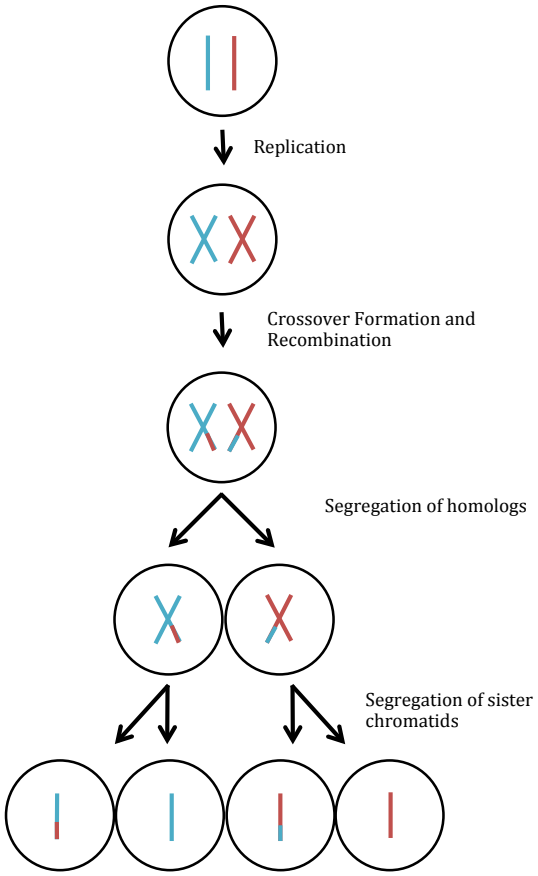


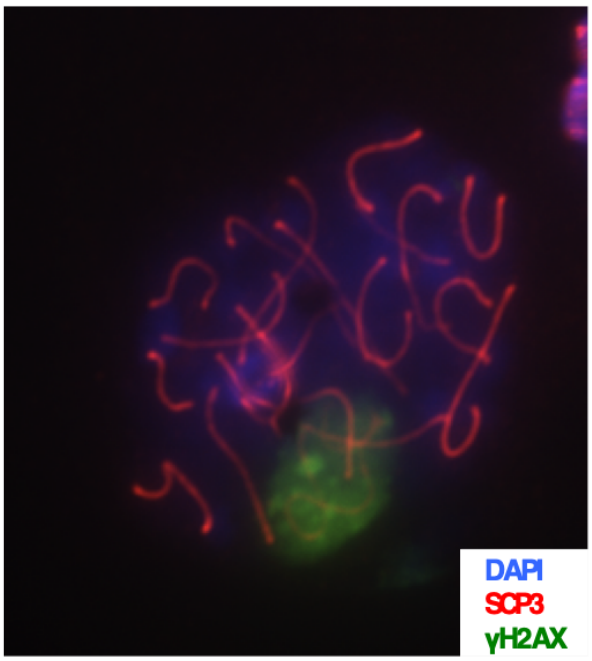
Figure 16. Events Involved in Meiosis. During meiosis, chromosomes are first duplicated, producing sister chromatids. Sister chromatids undergo crossover formation and recombination. Homologous chromosomes and sister chromatids segregate, producing four haploid gametes. Red and blue lines represent a pair of homologous chromosomes.

on in meiosis, during the pachytene stage, after which the two are permanently separated into daughter cells. Pairing, then, is an elegant solution to the challenge of splitting a diploid genome neatly and equally into two. It requires no counting or roll call of genetic material. Instead, it requires only that homologous chromosomes pair and then separate from each other. The sex chromosomes, the X and Y, present an

interesting variation in the pairing process, as they are homologous across *only a small segment,* called the pseudoautosomal region, which, however, is sufficient to cause the X and Y to segregate away from each other (Figure 17). We now know that meiotic homolog pairing also plays a role in gene regulation. This is illustrated by the phenomenon of meiotic silencing.

The history of our understanding of meiotic silencing begins with early observations of an intriguing structure in mammalian, marsupial, and insect nuclei called the sex body, a chromosomal domain which has since been shown to contain the sex chromosomes (MOHR 1916; OHNO and MAKINO 1961; PAINTER 1924; SOLARI 1974). Studies done in the years spanning 1891 through the 1970s gave this structure many different monikers, from intranuclear body to chromatin nucleolus and X-vesicle and, in fact, it was the earlier of these studies that allowed for the discovery of the X chromosome (HENKING 1891; LENHOSSEK 1898; MAKINO 1941; PAINTER 1924).

Later studies done in a variety of organisms during the 1950s further characterized the sex body, showing that the X and Y chromosomes are condensed and heterochromatinized during meiosis (OHNO and MAKINO 1961; SACHS 1954). Consistent with these observations, studies of ^{3}H-uridine incorporation done in the 1970s showed that transcription of the XY pair is greatly reduced during meiosis in the mouse (HENDERSON 1964; KIERSZENBAUM and TRES 1974a; KIERSZENBAUM and TRES 1974b; MONESI 1965; UTAKOJI 1966). More recently, advances in



immunofluorescence staining technology have shown that the X and Y chromosomes become associated with numerous proteins, including Hormad1, BRCA1, ATR, γH2AX, H3K9me2 and UBR2, and are eventually transcriptionally silenced. This process was named Meiotic Sex Chromosome Inactivation (MSCI) (An *et al.* 2010; Baarends *et al.* 2005; Cloutier and Turner 2010; Keegan *et al.* 1996; Moens *et al.* 1999; Scully *et al.* 1997; Shin *et al.* 2010; Turner 2007b; Turner *et al.* 2004; Turner *et al.* 2002; Turner *et al.* 2006; Zhou *et al.* 2008).

The first hypotheses as to why meiotic nuclei undergo MSCI were published in the 1970s. Initially, it was thought that the sex chromosomes are transcriptionally silenced because male fertility is dependent on the absence of X chromosome transcription (Forejt 1982; Lifschytz and Lindsley 1974; Lifschytz and Lindsley 1972). A later study hypothesized that meiotic silencing reflects the state of sex chromosome pairing where, in the heterogametic sex, lack of robust pairing between the X and Y leads to silencing (Jablonka and Lamb 1988). This hypothesis has held true; the minimal pairing of the X and Y chromosomes *is*, in fact, what induces the formation of the sex body, causing unpaired regions to accumulate

marks associated with silenced chromatin (Figure 17) (AN *et al.* 2010; BAARENDS *et al.* 2005; SHIN *et al.* 2010; TURNER 2007b; TURNER *et al.* 2000; TURNER *et al.* 2006; TURNER *et al.* 2005; ZHOU *et al.* 2008).

If the underlying basis for MSCI is the mostly unpaired status of the sex chromosomes, one might predict that a process analogous to MSCI will also occur on unpaired autosomal regions. Recent studies have confirmed this prediction; the meiotic machinery can recognize unpaired autosomes as well as unpaired sex chromosomes; the unpairing of entire autosomes or large segments thereof through aneuploidy and rearrangements leads to the accumulation of marks characteristic of silenced regions, including Hormad1, BRCA1, ATR, H3K9me2, UBR2, and γH2AX (BAARENDS *et al.* 2005; FUKUDA *et al.* 2010; KHALIL *et al.* 2004; MAHADEVAIAH *et al.* 2008; MAHADEVAIAH *et al.* 2001; MANTEROLA *et al.* 2009; REINHOLDT *et al.* 2009; SHIN *et al.* 2010; TURNER 2007b; TURNER *et al.* 2005). This process is called meiotic silencing of unpaired chromatin (Figure 18, MSUC, pronounced in our lab as MSUCh).

Similar observations have been made in the nematode, *C. elegans*. Here, the meiotic process targets H3k9me2 preferentially to the single unpaired X chromosome of XO males well as any X chromosome or autosome that is unpaired due to mutations in genes essential for meiotic homolog pairing; for example, mutations in *him-8* and *zim-2,* lead to the unpairing and marking of the X (BEAN *et al.* 2004; KELLY *et al.* 2002; PHILLIPS *et al.* 2005) and chromosome V (PHILLIPS and DERNBURG 2006), respectively, while mutations in *him-3*, which encodes a core

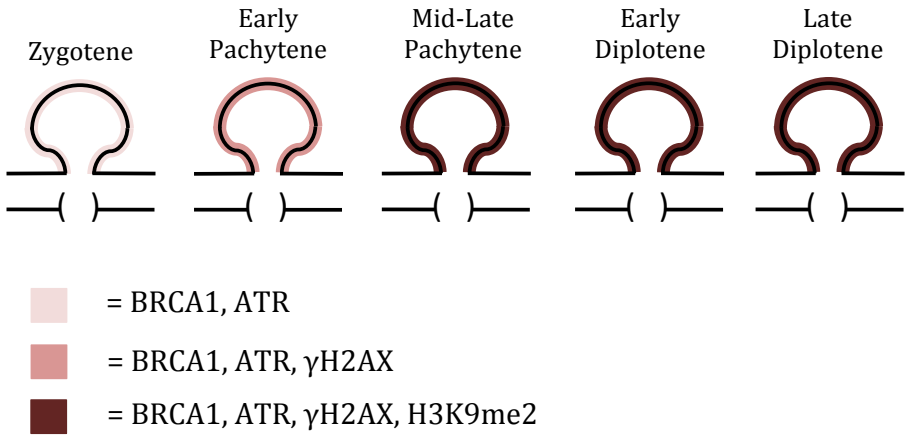


Figure 18. Meiotic silencing of unpaired chromatin (MSUC). During MSUC, unpaired chromosomal regions first accumulate BRCA1 and ATR. Upon entering pachytene, these regions further accumulate γH2AX and H3K9me2 and are transcriptionally silenced. Adapted from Manterola, *et al.* (2009).

component of the synaptonemal complex, result in the marking of both the X and autosomes (BEAN *et al.* 2004).

Insights from Neurospora and Drosophila that further implicate pairing as key step in meiotic silencing: MSUD, RIP, and transvection

Our understanding of meiotic silencing in mice and *C. elegans* has benefited greatly from prior discoveries of pairing-mediated silencing phenomena in the fungus *Neurospora crassa*. One such phenomenon is meiotic silencing by unpaired DNA (MSUD), in which, regions of the genome that fail to pair during meiosis are aberrantly transcribed and, ultimately, silenced (Figure 19) (ARAMAYO and METZENBERG 1996; NAKAYASHIKI 2005b; SHIU *et al.* 2001). Through elegant genetic studies of the ascospore maturation-1 gene, Aramayo and Metzenberg (1996)

showed that copies of this gene lacking a pairing partner in meiosis were silenced. Subsequently, Shiu and Metzenberg (2001) confirmed that MSUD affects only unpaired chromosomal regions. MSUD is capable of silencing regions as small as several kilobases or less (LEE *et al.* 2004), highlighting the sensitivity of this process to the paired state of homologs (reviewed in KELLY and ARAMAYO 2007; NAKAYASHIKI 2005a; SINGH *et al.* 2009).

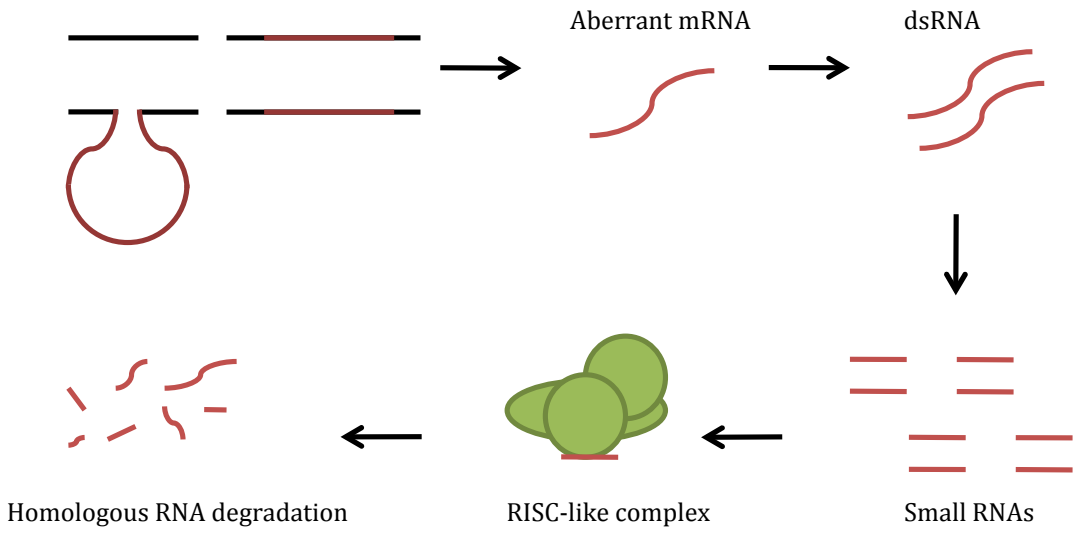


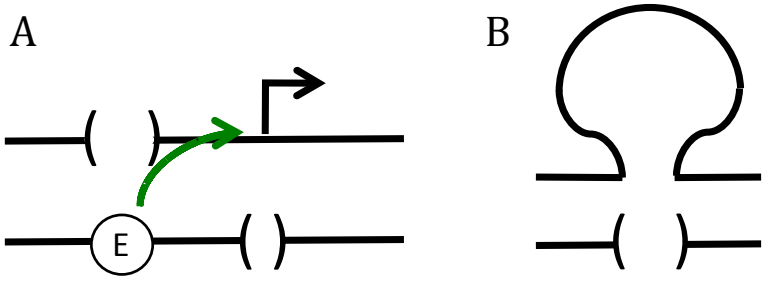
Figure 19. Meiotic silencing by unpaired DNA (MSUD). During MSUD, unpaired regions of chromosomes (shown in red) are aberrantly transcribed and produce complementary small RNAs, which target silencing machinery to the unpaired locus and any other homologous regions within the genome.

The Neurospora genome harbors a second pairing-mediated mechanism for silencing, repeat-induced point mutation (RIP), which silences duplicated

chromosomal regions. During RIP, duplications are believed to pair and then undergo a random process of mutation in which C residues are changed into T, after which the duplicated regions are marked by histone H3K9me3 (SELKER *et al.* 1987; TAMARU *et al.* 2003). RIP can recognize duplications as small as 500 bp in size, if the duplications are linked, or 1 kb, if the duplications are unlinked (WATTERS *et al.* 1999). Like MSUD, RIP speaks to the exquisite sensitivity that homolog pairing affords to processes that it mediates.

When meiotic silencing was discovered in Neurospora, it was initially termed "meiotic transvection." This name was chosen because the involvement of homolog pairing in MSUD is reminiscent of the phenomenon of transvection. (ARAMAYO and METZENBERG 1996). First described by Ed Lewis in (1954), transvection has now been observed in a number of species and can be most simply defined as a class of processes that render homologous chromosomal regions sensitive to the state of pairing (reviewed in DUNCAN 2002; GRANT-DOWNTON and DICKINSON 2004; HENIKOFF and COMAI 1998; KENNISON and SOUTHWORTH 2002; PIRROTTA 1999; WU and MORRIS 1999). It comes in many flavors and names and is most widely known for its capacity to activate or repress gene expression (CHEN *et al.* 2002; DUNCAN 2002; KENNISON and SOUTHWORTH 2002; MATZKE *et al.* 2001; RASSOULZADEGAN *et al.* 2002; SANDHU *et al.* 2009; SOUTHWORTH and KENNISON 2002). One of the better understood mechanisms leading to gene activation involves enhancers acting in trans on the promoter of their homolog lying on a separate chromosome (Figure 20A), while in a second form, the pairing of chromosomes

regions that are homologous but structurally dissimilar is believed to influence gene activity through the generation of specific gene topologies, such as chromatin loops (Figure 20B) (MORRIS *et al.* 1998). In as much as MSCI and MSUD involve homolog pairing, they are examples of transvection that lead to gene silencing, while RIP may well represent an extraordinary form of transvection that leads to gene mutation. Transvection effects have also been hypothesized to occur in humans, wherein the disruption or induction of interhomolog interactions has been postulated to correlate with the severity of disease phenotypes



The effects of genetic phenomena can span multiple generations

Thus far, we have discussed the importance of genetic processes at a single point in time. However, it is equally important to consider the evidence that the outcome of these processes in one generation may be remembered in future generations and effect changes on the genome. Below, we will describe several intergenerational effects that demonstrate the heritability of genetic processes:

Examples of intergenerational phenomena include imprinted X inactivation in mouse extraembryonic tissues and in marsupial embryos. In both cases, it is believed that the paternal X chromosome pre-selected to be inactivated during

spermatogenesis and is marked accordingly (AVNER and HEARD 2001; HUYNH and LEE 2003; LUCCHESI *et al.* 2005; LYON 1999; OKAMOTO *et al.* 2005; PAYER and LEE 2008; WUTZ and GRIBNAU 2007). Imprinting of the X chromosome is not limited to mice and marsupials.

Parental imprinting can also occur in Drosophila. Here, Maggert and Golic (2002) determined that the Drosophila Y chromosome carries a chromosome-wide imprint by showing that the expression of Y-linked transgenes is dependent on the parent of origin of the Y chromosome. Interestingly, the parental origin of the Y chromosome affects sex chromosome also affects dosage compensation in Drosophila XY males, where the rate of transcription of the single X is doubled as compared to the rate in XX females (GELBART and KURODA 2009; LUCCHESI *et al.* 2005; STRAUB and BECKER 2011). In this system, dosage compensation components are recruited to the Y chromosome by two non-coding RNAs (ncRNAs), without which the males die during embryogenesis (AKHTAR and BECKER 2000; MELLER *et al.* 1997; SMITH *et al.* 2000). Surprisingly, however, this lethality is suppressed when the Y chromosome is maternally transmitted, indicating that there is an imprint on the Y chromosome that exerts an effect on dosage compensation and male survival (MENON and MELLER 2009).

Similar to meiotic silencing, the intergenerational effects of meiotic processes are not constrained to the sex chromosomes and may be realized through interactions between different chromosomes in the process of trans-generational

epistasis. For example, in mice, several parental modifier mutations interact with a mutant allele of *Dnd1*^{Ter}, a gene involved in microRNA-based silencing. Male offspring that do not inherit any of the parental mutations show increased risk of testicular germ cell tumors, regardless of which parent transmitted the modifier mutation (LAM *et al.* 2007).

Are there consequences of unpairing during meiosis beyond meiotic silencing?

Not surprisingly, abnormalities in the process of meiotic silencing can lead to a reduction in fertility. This outcome has been observed when there is excessive autosomal unpairing. In these situations, silencing factors are believed to be titrated away from the normally inactive sex chromosomes, resulting in aberrant sex chromosome transcription and, ultimately, increased germline apoptosis (KOUZNETSOVA *et al.* 2009; MAHADEVAIAH *et al.* 2008; TURNER 2007a). Thus, meiotic silencing phenomena may play a role in fertility if a high level of unpairing disrupts normal meiotic silencing and, resultantly, meiotic gene regulation.

The impact of meiotic silencing can also extend into the next generation. Indeed, of the two X chromosomes of a *C. elegans* embryo arising from an XO × XX cross, the X derived from the XO male lags behind the X derived from the XX hermaphrodite in acquiring activating marks, apparently due to the lack of a pairing partner in the XO germ-line. This potential for intergenerational consequences of meiotic silencing is not surprising, as there is a long history of intergenerational effects, including the many examples of parental imprinting (BARTOLOMEI and

FERGUSON-SMITH 2011; MENON and MELLER 2010) and the recent observations of transgenerational effects (DAXINGER and WHITELAW 2010; FURUHASHI and KELLY 2010; MENON and MELLER 2010; NADEAU 2009; PASZKOWSKI and GROSSNIKLAUS 2011; YOUNGSON and WHITELAW 2008). Of particular relevance would be the behavior of *C. elegans* deficient for spr-5, a lysine specific demethylase. Such mutant animals accumulate H3K4me2 in germline nuclei, in a manner that becomes more pronounced from one generation to the next, resulting in progressive embryonic lethality and germline apoptosis (ARICO *et al.* 2011; KATZ *et al.* 2009; NOTTKE *et al.* 2011). Another potential consequence of meiotic unpairing, therefore, could be altered gene regulation and phenotypic changes in subsequent generations.

Do small structural variants of chromatin have implications for the heritability of human disease?

In brief, the gene regulatory effects of pairing-mediated processes are widespread and potent, occurring in meiotic and nonmeiotic cells in Neurospora, Drosophila, *C. elegans*, and mammals. Prior to my studies, the meiotic effects outside of Neurospora had been observed only in the presence of large structural disruptions that affected entire, or large portions of, chromosomes. My studies in *C. elegans* raise the possibility that meiotic pairing-mediated processes may also be able to act on small heterozygous deletions or insertions.

Given the many examples of processes that act on meiotic chromosomes, the meiotic genome may detect and respond to CNVs and other chromosomal structural

variants, as has also been suggested by Carmen Sapienza. If so, what are the outcomes of detection? One possible response would be a marking of the detected allele and/or its wild-type counterpart with epigenetic marks (Figure 21). Following completion of meiosis, both the marked and unmarked alleles would be

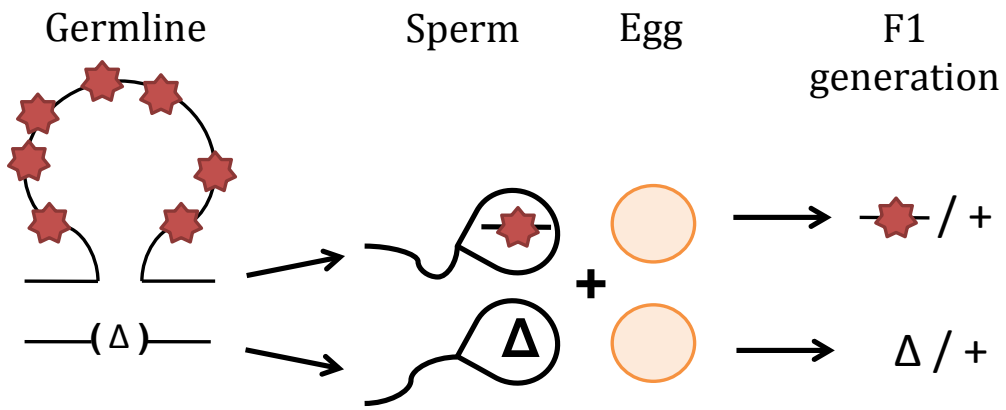


Figure 21. Implications of Detection of Heterozygous Structural Variants in Humans. Small, unsynapsed regions of the human genome may accumulate marks if they are detected during meiosis. Chromatin with these marks may be packaged into sperm or eggs and passed on to the F1 generation and could affect gene expression in the F1. Red star indicates marks placed on chromatin by the detection mechanisms.

passed on to the next generation, where the marked or potentially silenced allele may cause a phenotype, perhaps by behaving similarly to a null allele or by being abnormally regulated. Taking into account the intergenerational effects of the *spr*-5 mutation (NOTTKE *et al.* 2011), this phenomenon may only cause phenotypic differences in certain genetic backgrounds lacking the enzymes to remove the marks.

Our prediction has significant implications for disease association studies, which, thus far, have focused on associating a CNV deletion allele with a disease phenotype. However, given the above-mentioned phenomena, I believe that the ability of the genome to detect and respond to differences in CNVs must be considered when analyzing genetic data. For example, if one of the parental genotypes was heterozygous for a CNV and the wild-type chromatin was marked during meiosis, the phenotype may be associated with the wild-type allele and would be missed by traditional genetic mapping (Figure 22). A better understanding of this phenomenon may shed light onto the subtler role that CNVs and meiotic processes play in predisposing humans to disease.

Might homolog pairing afford a unique opportunity for the assessment of population diversity?

My consideration of structural heterozygosity (Chapters 2 and 3) has also led me to question the function of pairing in meiosis, which is known primarily for its role in chromosome segregation and, therefore, the production of haploid gametes. Here, I would like to suggest a second, potentially equally important, function wherein the capacity of meiotic pairing to directly compare two parental genomes enables the meiotic cell to sense the level of genetic variation within the population and respond to that variation by decreasing, increasing, or maintaining variation. During such a comparison, CNVs would make an excellent measure of diversity, as they are larger than SNPs and, therefore, may be more easily detected.

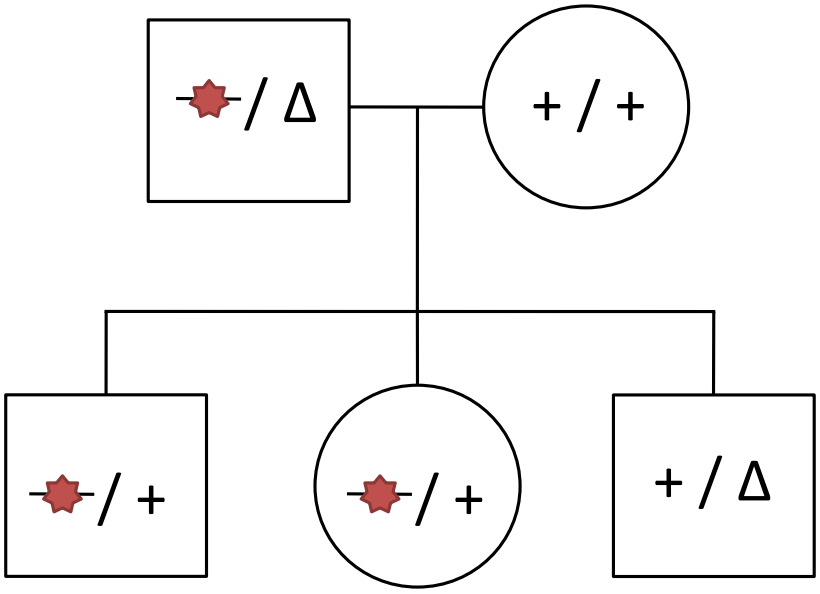


Figure 22. Pedigree Illustrating the Implications of Detection of Heterozygous Structural Variants in Humans. In this example, the male parent is heterozygous for a structural variant that is detected and marked by the meiotic machinery. Both the unmarked allele and the deletion alleles are passed on to the F1 generation. Disease phenotype may not depend on which allele is inherited, but rather the heterozygous state in the parental generation. Red star indicates marks placed on chromatin by the detection mechanisms.

Moreover, as individuals typically have hundreds of CNVs in a wide range of sizes distributed throughout the genome; these structural variants would allow for excellent coverage and comparison of the entire genome. Importantly, because CNVs can be produced by many different mechanisms, including errors in replication, DNA damage repair, or recombination, they would be able to serve as a common readout for many kinds of genomic stress or mutational process and, therefore, permit for sensing of heterology via a single, parsimonious mechanism.

Figure 23 illustrates a model in which meiotic pairing acts to achieve or maintain an advantageous level of variation within the population. In the first scenario, two homologs, with an "advantageous" level of variation, pair during meiosis, and detecting no need to increase or decrease variation fail to trigger any down stream mechanisms to alter the level of structural variation (Figure 23B).

In a second scenario, the level of variation is below a certain threshold. Thus, mechanisms work to increase heterogeneity, possibly by increasing the rate at which mutations accumulate. Such processes would include: raising the rate of break-induced replication, decreasing DNA damage repair rates, activating transposons, increasing the level of double-strand breaks, decreasing the fidelity of replication, and altering levels of DNA methylation (Figure 23C) (HASTINGS *et al.* 2009a; HASTINGS *et al.* 2009b; VAN DER HEIJDEN and BORTVIN 2009).

In the third scenario, the variation is too great resulting in one or more of several outcomes: an increase in DNA repair, an increase in the fidelity of this repair, or lastly, germ cell apoptosis (Figure 23A). The latter option would result in a type of meiotic drive, skewing the meiotic outcome away from gametes with a level of diversity deemed too high. Taken together, the different responses to levels of variation could function at a population level to maintain a consistent and advantageous level of genetic difference among the members of the population.

Interestingly, there is evidence for mechanisms capable of detecting unpaired

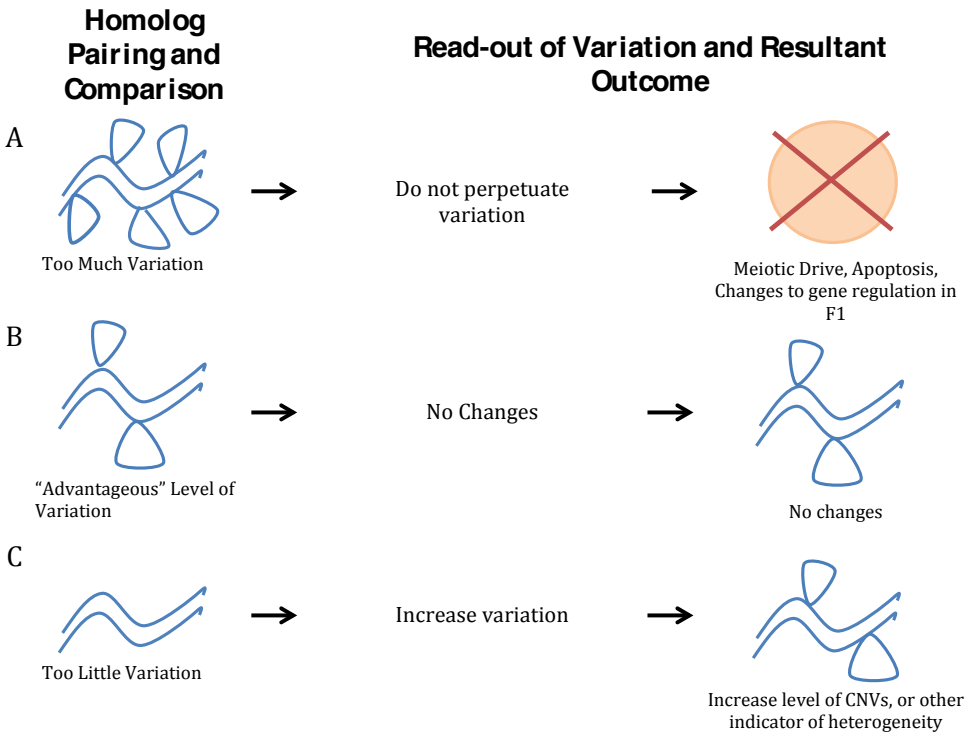


Figure 23. Homolog Pairing During Meiosis May Allow for Comparison of Genomic Variation Within the Population.
A) Detection mechanisms find that genomic variation is above a certain threshold. Variation is not passed on to the F1 generation.
B) Detection mechanisms find that variation is within an " advantageous" range and meiosis progresses normally.
C) Variation is found to be below the "advantageous" level and mechanisms to increase variation are activated.

regions, which we call Pairing-facilitated Assessment of Structural Heterology (PFASH). Meiotic silencing phenomena, including MSUD and MSUC, would be the most well characterized types of PFASH. There may also be evidence for PFASH in humans at micro- and mini-satellites, which are short series of DNA repeats ranging in size from 1-6bp and 10-60bp, respectively. When there is a large difference in

repeat number between the two satellites alleles, mutation rates increase in a process called heterozygote instability (AMOS 2010; AMOS *et al.* 2008; AMOS *et al.* 1996). These observations are in line with our ideas for PFASH. Further evidence for PFASH may also have been found in mice where Scavetta and Tautz have shown that during intersubspecific crosses, CNV copy number changes occur on all chromosomes (2010).

Moreover, mutation rates increase near insertions/deletions (indels) in many eukaryotes, possibly due to meiotic pairing disruptions targeting the indel region for additional mutational processes (TIAN *et al.* 2008). The implications of increasing the mutation rate could be very important. First, if the indel occurred as the result of a transposon insertion, mutating the area surrounding the insertion could serve to disable the transposition and prevent future transpositions. Second, if the indel is an aberrant insertion of genomic material, this process would disable the extra copies of genes present within this region. This, in turn, could hasten selection and evolutionary pressures at these loci. Third, mutations would continue to accumulate on the chromosomal regions associated with a deletion, potentially resulting in a loss of fitness, provided that this region is subjected to additional rounds of PFASH.

Finally, we raise the possibility that comparison followed by directed changes in structural variation may also hasten natural selection by promoting haploid advantage (WU and DUNLAP 2002). Here, the induction of loss-of-function variation,

such as deletions or null mutations, could accelerate the fixation of advantageous mutations or the purging of deleterious mutations from the population.

Ultimately, our model suggests that homolog pairing and the phenomena in which it plays a role may be important processes for controlling gene regulation and genomic variation within the population through interactions with chromosomal structural variants. For this reason, it will be important for future studies to compare parental genotypes when establishing an association between a particular structural variant, such as a CNV, to a disease phenotype. Given that current analyses do not take this approach, it is possible that many disease associations were passed over during prior studies. Lastly, our model suggests that meiosis may have an additional universal function in maintaining genome integrity, emphasizing the possibility that many currently understood genetic mechanisms may also have less obvious, though equally important consequences for the evolution of the genome.

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Appendix B

In chapter 2, statistical analysis comparing the mean number of RAD-51 foci in heterozygous deficiency strains to N2 and the average of all control strains, respectively, were shown. In this appendix, Mann-Whitney U-tests comparing the heterozygous deficiency strains to each of the five control strains independently are shown.

				RAD-51 FOCI				
			Min. ComplSize (kb)	Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N		Mean	Mean	Mean	Mean	Mean
N2	n/a	4	n/a	0.7	3.3	3.5	1.2	0.5
BW983	n/a	4	n/a	1.3	5.0	3.4	0.3	0.1
MT1655	n/a	4	n/a	0.9	5.1	2.0	0.2	0.1
CB364	n/a	4	n/a	0.6	2.5	1.2	0.1	0.0
CB195	n/a	4	n/a	1.4	4.9	2.2	0.8	0.2
CB1095	n/a	4	n/a	1.1	4.3	4.3	1.0	1.0
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJ803	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	344	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2

Table B1. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to control strain BW983. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or by molecular mapping. BW983 is highlighted in dark purple and other control strains in light purple. Mean number of RAD-51 foci is shown for each strain. Cells are highlighted in red if the mean number of RAD-51 foci is significantly higher than BW983 and highlighted in blue if the RAD-51 level is significantly lower than BW983 ($P<0.05$, Mann-Whitney U-Test). Based on this comparison, BW983 appears to have an overall higher level of RAD-51 foci in zones 4 through 6 compared to other control strains. As a result, the four other control strains displayed significantly lower numbers of RAD-51 foci than BW983 in one to three zones, respectively. Similarly, many heterozygous deficiency strains showed significantly lower numbers of RAD-51 foci in zones 4 through 6. This effect was somewhat diminished in strains with heterozygous deficiencies greater than 500kb, where strains show more zones with significantly higher numbers of RAD-51 foci than BW983.

				RAD-51 FOCI				
			Min. ComplSize (kb)	Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N		Mean	Mean	Mean	Mean	Mean
N2	n/a	4	n/a	0.7	3.3	3.5	1.2	0.5
BW983	n/a	4	n/a	1.3	5.0	3.4	0.3	0.1
MT1655	n/a	4	n/a	0.9	5.1	2.0	0.2	0.1
CB364	n/a	4	n/a	0.6	2.5	1.2	0.1	0.0
CB195	n/a	4	n/a	1.4	4.9	2.2	0.8	0.2
CB1095	n/a	4	n/a	1.1	4.3	4.3	1.0	1.0
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJB03	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	344	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2

Table B2. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to control strain MT1655. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or by molecular mapping. MT1655 is highlighted in dark purple and other control strains in light purple. Mean number of RAD-51 foci is shown for each strain. Cells are highlighted in red if the mean number of RAD-51 foci is significantly higher than MT1655 and highlighted in blue if the RAD-51 level is significantly lower than MT1655 ($P<0.05$, Mann-Whitney U-Test). Control strains BW983, CB195, and CB1095 showed 2 to 3 zones with significantly higher levels of RAD-51 foci than MT1655, while CB364 showed 1 zone with significantly lower levels of RAD-51. Heterozygous deficiency strains with deficiencies greater than 19kb showed an overall increased level of RAD-51 foci in zones 4, 6, and 7. This response is especially apparent in strains with deficiencies greater than 500kb. Many strains displayed significantly lower levels of RAD-51 compared to MT1655 in zone 5, likely because MT1655 had a higher level of RAD-51 foci in that zone.

				RAD-51 FOCI				
			Min. ComplSize (kb)	Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N		Mean	Mean	Mean	Mean	Mean
N2	n/a	4	n/a	0.7	3.3	3.5	1.2	0.5
BW983	n/a	4	n/a	1.3	5.0	3.4	0.3	0.1
MT1655	n/a	4	n/a	0.9	5.1	2.0	0.2	0.1
CB364	n/a	4	n/a	0.6	2.5	1.2	0.1	0.0
CB195	n/a	4	n/a	1.4	4.9	2.2	0.8	0.2
CB1095	n/a	4	n/a	1.1	4.3	4.3	1.0	1.0
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJ803	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	344	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2

Table B3. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to control strain CB364. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or by molecular mapping. CB364 is highlighted in dark purple and other control strains in light purple. Mean number of RAD-51 foci is shown for each strain. Cells are highlighted in red if the mean number of RAD-51 foci is significantly higher than CB364 and highlighted in blue if the RAD-51 level is significantly lower than CB364 ($P<0.05$, Mann-Whitney U-Test). All of the four additional control strains showed significantly increased levels of RAD-51 compared to CB364 in 2 to 5 zones. This indicates that CB364 has lower average levels of RAD-51 foci overall than the other control strains. Similarly, all but one heterozygous deficiency strain showed significantly elevated RAD-51 foci in at least one zone when compared to CB364.

				RAD-51 FOCI				
			Min. ComplSize (kb)	Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N		Mean	Mean	Mean	Mean	Mean
N2	n/a	4	n/a	0.7	3.3	3.5	1.2	0.5
BW983	n/a	4	n/a	1.3	5.0	3.4	0.3	0.1
MT1655	n/a	4	n/a	0.9	5.1	2.0	0.2	0.1
CB364	n/a	4	n/a	0.6	2.5	1.2	0.1	0.0
CB195	n/a	4	n/a	1.4	4.9	2.2	0.8	0.2
CB1095	n/a	4	n/a	1.1	4.3	4.3	1.0	1.0
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJ803	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	344	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2

Table B4. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to control strain CB195. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or by molecular mapping. CB195 is highlighted in dark purple and other control strains in light purple. Mean number of RAD-51 foci is shown for each strain. Cells are highlighted in red if the mean number of RAD-51 foci is significantly higher than CB195 and highlighted in blue if the RAD-51 level is significantly lower than CB195 ($P<0.05$, Mann-Whitney U-Test). When the four additional control strains were compared to CB195, 2 strains, BW983 and CB1095, showed significantly elevated levels of RAD-51 foci in at least one zone. Three of the control strains, MT1655, CB364, and CB1095, had significantly decreased levels of RAD-51 in at least one zone when compared to CB195. Heterozygous deficiency strains with deficiencies over 19kb typically had significantly increased or decreased levels of RAD-51 in two or more zones, with a greater response in zones 4 through 7.

				RAD-51 FOCI				
			Min.	Zone 4	Zone 5	Zone 6	Zone 7	Zone 8
Strain	Chr	N	ComplSize (kb)	Mean	Mean	Mean	Mean	Mean
Avg. Cntr	n/a	n/a	n/a	1.0	4.2	2.8	0.6	0.3
N2	n/a	4	n/a	0.7	3.3	3.5	1.2	0.5
BW983	n/a	4	n/a	1.3	5.0	3.4	0.3	0.1
MT1655	n/a	4	n/a	0.9	5.1	2.0	0.2	0.1
CB364	n/a	4	n/a	0.6	2.5	1.2	0.1	0.0
CB195	n/a	4	n/a	1.4	4.9	2.2	0.8	0.2
CB1095	n/a	4	n/a	1.1	4.3	4.3	1.0	1.0
SP705	II	3	1.1	0.9	4.0	1.5	0.3	0.1
GR1311	X	3	7.5	0.9	5.0	2.3	0.2	0.2
PJB03	II	4	19	0.4	2.3	5.2	0.2	0.1
BC199	I	4	34	0.8	2.2	3.7	2.8	1.3
BC694	I	4	34	0.4	2.9	5.2	3.2	0.8
VC100	X	3	44	1.1	6.1	4.4	2.0	0.0
DM3006	X	4	185	0.7	1.5	0.8	0.2	0.0
DM3007	X	3	187	1.0	3.9	0.8	0.0	0.0
GE2180	III	4	197	3.2	4.4	3.9	0.7	0.2
ML610	II	4	267	0.6	3.6	2.8	0.3	0.1
BW219	V	4	279	0.6	4.5	2.7	0.1	0.1
GE2204	III	3	344	1.5	2.3	1.1	0.1	0.0
RW1333	IV	4	359	0.0	3.0	2.3	0.4	0.1
EJ275	I	4	461	1.1	3.7	1.2	0.3	0.0
JK1726	I	4	503	3.8	7.1	4.3	3.0	0.8
GE2172	III	3	549	2.6	6.2	1.8	1.0	0.6
MT2180	I	4	675	0.3	2.2	3.7	2.1	0.8
BW1535	III	3	924	2.5	5.5	1.7	0.0	0.0
VT454	II	4	1350	1.9	3.8	3.7	0.8	0.2
SP1540	I	4	1552	2.4	5.9	3.0	1.2	0.2
MT1978	III	4	1624	2.4	5.6	4.7	2.4	1.4
SP719	II	4	1902	2.3	5.1	3.0	1.0	0.2
DA768	IV	4	2083	0.8	6.9	2.2	0.0	0.0
GE2167	III	4	2351	3.4	7.7	5.4	3.7	1.1
SP755	II	3	2643	2.7	6.1	3.4	0.4	0.2

Table B5. Quantitation of RAD-51 foci in heterozygous deficiency strains compared to control strain CB1095. Heterozygous deficiencies are ordered by minimum deficiency size determined by complementation or by molecular mapping. CB1095 is highlighted in dark purple and other control strains in light purple. Mean number of RAD-51 foci is shown for each strain. Cells are highlighted in red if the mean number of RAD-51 foci is significantly higher than CB1095 and highlighted in blue if the RAD-51 level is significantly lower than CB1095 ($P<0.05$, Mann-Whitney U-Test). Two control strains, BW983 and CB195, showed one zone with significantly higher RAD-51 levels than CB1095. No other zones were significantly different when CB1095 was compared to the other control strains, indicating that CB1095 has overall similar RAD-51 levels to the other control strains throughout meiotic prophase I. When CB1095 was compared to heterozygous deficiency strains, strains with deficiencies greater than 500kb tended to display significantly increased levels of RAD-51 in zones 4,5, and 7. A response was also seen in zone 8, however, three strains showed significantly decreased levels of RAD-51 in this zone. Interestingly, the only strain with significantly different levels of RAD-51 in zone 6 was a 34kb deficiency strain.

When the control strains are compared to each other, it is evident that there is a considerable level of overall variation among each of the strains. CB364 has lower levels of RAD-51 foci across zones 4 to 8 when compared to the heterozygous deficiency strains, whereas BW983 and CB195 have higher levels of RAD-51 compared to many of the deficiency strains. CB1095 has similar levels of RAD-51 foci to strains with deficiencies less than 500kb and an increase in RAD-51 response can be seen in strains with larger deficiencies. Taken together, the variation among the five control strains highlights the importance of using an average of several different strains to accurately draw conclusions about the levels of RAD-51 in the strains with heterozygous deficiencies.