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"The BRCA/Fanconi recombination pathway in *C. elegans* meiosis"

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3. Results

Cells are always under attack from a plethora of deleterious agents to DNA, the keeper of genetic information. Unresolved DNA damage can lead to catastrophic consequences for the cell and for the organism: mutations or chromosomal aberrations that can alter the cellular growth or threaten the survival of the cell. Despite this permanent aggression, DNA remains an exceptionally stable carrier of genetic information. The safeguard of genomic integrity results from a highly regulated process of DNA replication and repair, cell cycle progression, chromosome segregation and programmed cell death. The accuracy with which these processes are performed determines how genetic information is transmitted from parent to daughter cell. Evolution has equipped living organisms with a complex system of surveillance/defence mechanisms, among which DNA repair plays a central role. These systems act as particular intra-molecular mechanisms, able to recognize and virtually eliminate possible lesions. Homologous Recombination (HR) is one of the main mechanisms of DNA repair, conserved during evolution, guaranteeing the faithful transmission of genetic information, included in a network of mechanisms responsible for the progression of the cell cycle.

In eukaryotes with a sexual cycle, many genes involved in DNA damage repair also have functions in meiotic recombination. Many of these genes are conserved during evolution, such as the genes implicated in Homologous Recombination. The double strand break (DSB) on DNA, in fact, represents an early meiotic event through which homologous chromosomes can have an exchange of genetic information, the right orientation of chromosomes at the meiotic spindle, and hence their correct segregation. Therefore, in nature, those same DSBs, which could lead to catastrophic consequences for the cellular life, represent an exceptional system for the maintaining of the species. While in low eukaryotes, such as yeast, the switch from the mitotic to the meiotic program is an induced response to the absence of nutriments, in metazoans and plants, germ line cells are distinct from somatic cells and specialized to perform meiosis. Thus, meiotic recombination can be interpreted as a mechanism to increase genetic diversity, and to decrease the risk of the extinction of the species. The conservation of the genes involved in both processes, meiotic recombination and DNA repair, during evolution, allows us to use simple organisms as model systems that contribute to a better understanding of the recombination machinery. Homologous Recombination has been studied in great detail in fungi. Through experiments on yeast, several mechanisms of homologous recombination have been elucidated, such as gene conversion, sister chromatid repair, and crossing over. All the main genes and gene networks involved in these mechanisms have been discovered in this system that still remains an

essential tool for this kind of analysis. The unicellular nature of the yeast model system represents, however, a limitation for the study of those molecular mechanisms that involve the cellular interactions which normally occur in higher organisms and the interactions between DNA repair and apoptosis. A metazoan model system such as *Caenorhabditis elegans*, offers the advantage of studying the interplay between different cells as well as the cross-talking between damage checkpoints, repair and apoptosis. It also allows the observation of the effects of a mutation in a repair gene in the context of an entire organism and during development. As a metazoan, *C. elegans* is relatively simple, characterised by a transparent body consisting principally of its reproductive system. The unique organization of meiotic nuclei in the gonads permits a rapid cytological assessment of defects in DNA repair, crossover formation and apoptosis. A collection of mutants in conserved and newly identified genes involved in homologous recombination and check-points/apoptosis, together with the rapid and powerful reverse-genetic techniques (RNA interference, germline co-suppression), makes *C. elegans* a suitable metazoan system to study the homologous recombination mechanism and the cross-talking between damage checkpoint, repair and apoptosis.

1. INTRODUCTION

1.1 DSB

The most deleterious DNA lesions are those that affect both strands of the DNA double helix, such as double strand breaks (DSB) and inter-strand cross-links. DSBs have been shown to be at the origin of deletion mutagenesis, chromosomal breakages, rearrangements, translocations and inversions (for a review, van Gent et al., 2001). DSBs can be accidentally produced either by the physiological DNA metabolism during the cellular cycle or by exogenous DNA damaging agents. A spontaneous DSB can arise from torsional stress of the DNA molecule or when a DNA replication fork encounters blocking structures. DSBs can arise during the repair processing of DNA damage, such as chemical modifications and mismatches, by free radical action or by products of oxidative metabolism. Accidental DSBs are also produced by a variety of DNA-damaging agents, including ionising radiation, radiomimetic chemicals, and a number of anti-cancer drugs (e.g. bleomycin, camptotecyn, cisplatin, etc.). Most of these chemical treatments lead to the cross-linking of the two complementary DNA strands (ICL, inter-strand cross-links). ICLs prevent replication, as well as transcription, precluding the use of information encoded by the complementary strand for repair. ICLs can occur during replication in the S-phase, where they lead to the collapse of replication forks and DSBs. The first step in the repair of this DNA structure is its identification, when the replication or transcription machinery encounters it and becomes blocked. It has been demonstrated in yeast that the repair system can eliminate this structure with the formation of a DSB and the replacement of the right sequence by Homologous Recombination Repair.

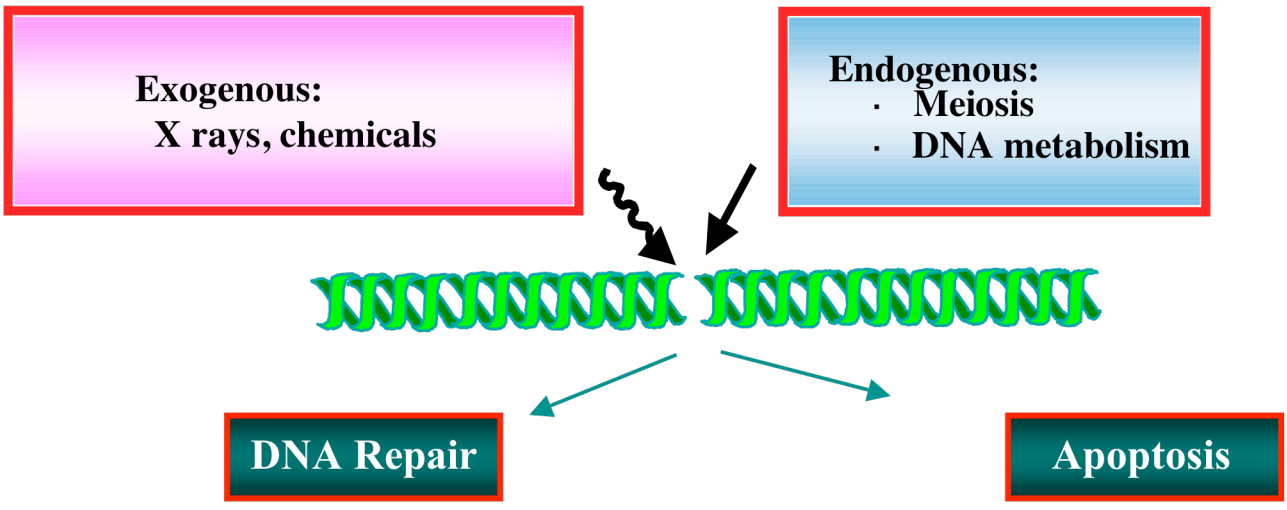
DSBs can also be programmed. Indeed, specific DSBs are intentionally produced during meiotic recombination allowing the creation of new combinations of genetic material in the gametes by crossing over. DSBs are also primary events during: (i) the process of integration of retroviruses, (ii) the process of mobile element transposition, and (iii) site specific V(D)J recombination and somatic hyper-mutation of immunoglobulin genes, two different mechanisms which create an extraordinary diversity of antigen receptors in vertebrates (for a review, Jackson and Jeggo, 1995; Papavasiliou and Schatz, 2000).

1.2 DSB repair pathways

Eukaryote somatic cells repair DSBs using mechanisms that appear to be highly conserved during evolution. Two major pathways have maintained the role of assuring the genome integrity: the DNA End-Joining pathway (also named Non-Homologous End Joining, NHEJ), and the Homologous Recombination Repair (HRR) (Figures 1.1 and 1.2). The relative contribution of each of these pathways varies during development and depends also on the stage of the cell cycle. In the G2-phase of the cell cycle, a higher contribution of HR in DSB repair is observed, because the intact sister chromatid is available as a donor of homologous sequences to the region containing the broken DNA. The DNA End-Joining instead operates during G0, G1, and early S phase. The balance between these two pathways determines the genome integrity. DSBs are mainly produced during DNA replication and only rarely in G0/G1 by accidental damage and therefore Homologous Recombination Repair represents the repair system that is principally activated during the cell cycle.

The cell has an additional possibility to respond to the DNA damage. When damage checkpoints are activated, the progression of the cell cycle is arrested, allowing the proteins associated directly or indirectly with the damaged DNA to recruit and modulate the different repair machinery. In the case of unresolved damage, the cell has the capacity to activate a specific apoptotic program that shifts the destiny of the cell towards death. Apoptosis is present in metazoans, both as a developmental programme and as a damage response. A specific protein, p53, has the capacity to transduce the DNA damage stimuli into the apoptotic programme (for a review, Rich et al., 2000).

Figure 1.1



1.2.1 Non Homologous End-Joining

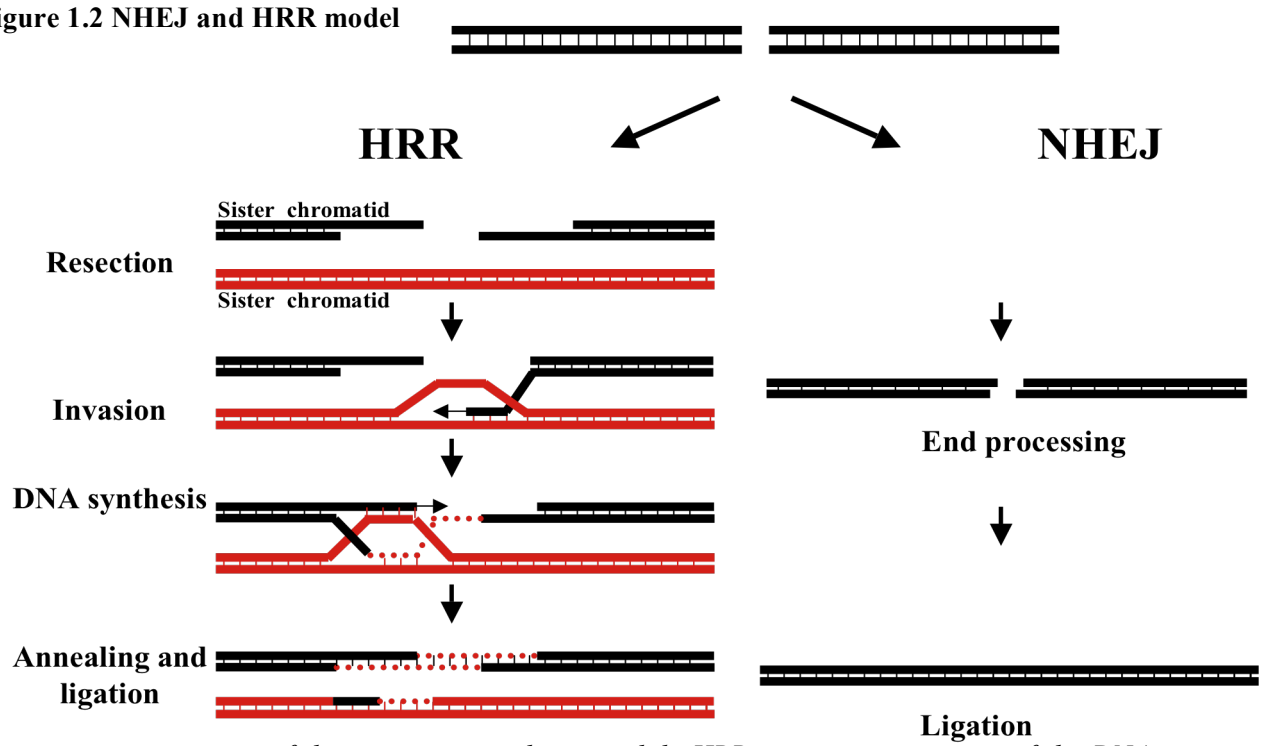
The term NHEJ was used for the first time in 1996 when Moore and Haber coined it in their work on yeast to describe an alternative DSB repair in the absence of a homologous donor. The yeast model was and still remains one of the best tools to understand the functions of repair systems such as NHEJ. Experiments in *S. cerevisiae* with plasmid transformation assay have contributed to an understanding of the way in which the NHEJ components are involved in this repair process, measuring the relative efficiency and accuracy of the DNA restoration (for a review, Daley et al., 2005). In the last few years, a general model of the repair process has been delineated, and its validity has been confirmed in all eukaryotes (Figure 1.2). NHEJ does not require any homology at the ends of the strands that have to be rejoined. A core of conserved proteins is recruited to the damage site in order to protect, process, and rejoin directly the DNA broken ends (for a review, Shrivastav et al., 2008). However, the direct joining of the broken DNA ends can, in theory, be precise but it often is error prone, due to the loss of those bases that may be removed for an efficient ligation. Moreover, since NHEJ does not require an extensive homologous repair sequence, it has the possibility to join DNA ends that were not originally contiguous. Indeed, inappropriate use of NHEJ could be one of the major causes of DNA rearrangements and translocations in cells (Weinstock, et al., 2006). It is clear that these errors are less dangerous in adult differentiated cells that express a limited number of genes and are unable to divide.

1.2.2 Homologous Recombination Repair

In the S/G2 phase of the cell cycle, after the DNA replication, Homologous Recombination Repair (HRR) is the main mechanism to repair DSBs. The molecular basis and genetic requirements of HR were initially defined by studies in bacteria and yeast cells. Early studies in cell killing by ionizing radiations gave rise to the notion that HRR is the dominant mechanism of DSB repair in yeast. DSB repair via HR requires the presence of long homologous sequences (several hundred bp homology) used as an intact donor molecule (sister chromatid, homologous chromosome, exogenous homologous DNA elements). The sequence of events, which leads from initial DNA broken ends to DSB repair via HR is conserved from *Escherichia coli* to humans. There are the following fundamental stages: (i) the first step, initiation, represents resection of the DNA DSB in the 5' to 3' direction. The generated 3' single-stranded DNA tails are then bound by RecA (*E.coli*) or Rad51 protein

(eukaryotes), which form long nucleoprotein filaments. (ii) The second step involves the strand exchange, which occurs between two homologous dsDNA molecules. In fact, the Rad51 nucleoprotein filament interacts with an undamaged DNA molecule and, when a homologous region has been located, Rad51 catalyses strand exchange events in which the damaged DNA molecule invades the homologous dsDNA, to produce a D-loop structure. (iii) During the next step, the 3' terminus of the damaged DNA is extended by a DNA polymerase that copies information from the undamaged partner used as a template, and finally the ends are joined. (iv) After branch migration, the created Holliday junctions are resolved by cleavage and religation to produce two intact DNA molecules. The model of HR, called "strand invasion", was proposed by Resnick (1976) and by Szostak (1983) (Figure 1.2). The model, based on the original Holliday model (Holliday, R. 1964), has since been modified, based on the analysis of molecular events associated with HR in yeast *S.cerevisiae* (for a review, Aylon and Kupiec, 2004) and has been confirmed for metazoans and plants. The search for an extensive homology, which is required between the region containing a DSB and a donor template from which the repair is directed, is essential for an efficient and accurate DSB HRR. The homology search process, however, may inappropriately choose, as homologous partners, repetitive regions from any of the chromosomes, which will lead to chromosomal rearrangements and chromosomal translocations.

Figure 1.2 NHEJ and HRR model



#Schematic representation of the two repair pathways: while HRR requires a resection of the DNA ends and a homologous template for repair, NHEJ does not require any homologous sequences and joins the DNA ends restoring the integrity of the chromosome.

1.3 Meiosis and Recombination

In those organisms with a sexual cycle, genes involved in somatic Homologous Recombination Repair have also functions in meiotic recombination. Given that "DNA damage" is a term that indicates an accidental event inducing a break in the DNA strand, when DSBs are caused during a restricted and controlled physiological process, such as meiosis, the term "cut" could be more appropriate. During meiosis, sexually reproducing organisms reduce diploid cells through two consecutive rounds of cell division (meiosis I and meiosis II) to generate haploid cells. The diploid state is reconstituted by fertilization in the subsequent generation assuring the continuity of the species. While meiosis II is quite similar to the mitotic process, unique molecular events are present during meiosis I. After one round of DNA replication (for which the genome is 4N), germ cells enter meiosis I. Their chromosomes undergo several modifications, which are characteristic features of prophase I, which allow us to distinguish its five sub-phases, called leptotene, zygotene, pachytene, diplotene and diakinesis. In the leptotene phase, proteinaceous cores form along the chromosomes and the homologs align with each other. In the zygotene phase, the homologs begin to be strictly connected along their entire length (synapsis) by the proteinaceous scaffold, the synaptonemal complex (SC). In the pachytene phase, the synapsis is completed, while, in the diplotene phase, the SC moves out. The disassembly of the SC reveals the homologs still linked together by points of attachment. These connections are visible at the diakinesis phase as cytological structures, called chiasmata that are the cytological consequence of the occurred crossing-over (exchange between homologous chromosomes via meiotic recombination). The correct completion of meiotic recombination is functional for the bi-orientation of homologous chromosomes in the spindle and for the proper chromosomal segregation through the tensional strength exercised by the chiasmata.

1.4 Meiosis of Nematode worm as a model system for DNA repair

Given that many of the genes in the HR pathway have been conserved during metazoan evolution, their roles can be investigated in model systems particularly suitable for the study of DSB repair in meiosis. Several features make *Caenorhabditis elegans* (*C. elegans*) an ideal organism for the study of meiosis and the recombination events during prophase I: the transparent body of the worm makes it simple to see the gonad organization,

permitting an easy cytological identification of the different phases of Meiosis I. The germ cell line of the worm is the only tissue in this organism where the cells are continuously dividing. Their state is controlled by damage checkpoints inducing cell cycle arrest, repair mechanisms and cellular programmed death. For this reason, the gonad of *C. elegans* represents one of the easier metazoan systems for a study of the cross-talking between the "surveillance" systems, composed of checkpoints, repair mechanisms and apoptosis. Moreover, *C. elegans* is a simple metazoan organism, in which many of its genes have human homologs (from WormBook). The entire *C. elegans* genome has been sequenced. Therefore, biological information from *C. elegans* may be directly applicable to more complex organisms, such as humans.

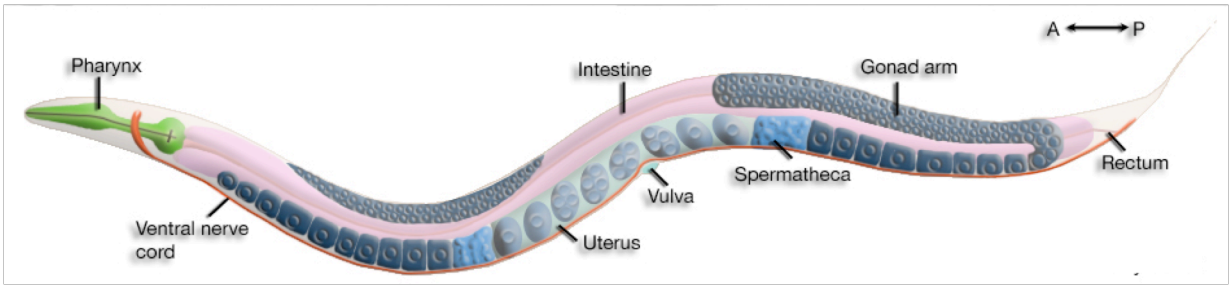
1.4.1 Features of *C. elegans*

Caenorhabditis elegans is a member of the Nematode phylum, belonging to the Rhabditidae family. It is a small animal (about 1 mm long when adult) (Figure 1.3). Its normal habitat is in soil, surviving on bacteria and fungi. *C. elegans* exists either as hermaphrodite or as male, characterized by clear different structures when adult. The number of sexual chromosomes determines the worm's sex. In *C. elegans*, six chromosomes are present: five pairs of autosomes (chromosomes I, II, III, IV, and V) and one pair of sex chromosomes, X (letter X). Hermaphrodites have two X chromosomes (designated XX), while males have one X chromosome (designated XO), the hemizygous state. Spontaneous males appear in the wild type population with a very low frequency (0.2%), due to the loss of one sexual chromosome for non-disjunction. Males can cross-fertilize hermaphrodites and give a mixed progeny of males and hermaphrodites. The phenotype in which the frequency of males is higher than the wild type is named Him phenotype (high incidence of males) indicating segregation defects of sexual chromosomes (Hodgkin et al., 1979).

Therefore, the predominant sexual form is the hermaphrodite: this animal produces sperms and eggs. Most of the worm's body consists in the reproductive system. The gonad of the hermaphrodite adult worm is composed of two arms in which the physical location of the germ cells corresponds to their level of maturation during meiosis I. *C. elegans* hermaphrodite development has a developmental switch: at first sperms are produced and stored in the spermatheca during the L4 larval stage and then oocytes are produced for the whole adult life. The germ cells mature in a syncytium; later, just before fertilization, the nucleus and cytoplasm are completely surrounded by a plasma membrane. The oocytes pass into the

spermatheca and are fertilized. *C. elegans* fertilized eggs are laid a few hours afterwards: the embryos develop in the uterus until the state of about 40 cells (from WormBook, Sulston et al., 1983). The eggs hatch about 12 hours later and the animals proceed through 4 larval stages, each of which finishes in a moult, until they arrive at the adult form. In only three days, the worm goes through the complete reproductive life cycle.

Figure 1.3 Structure of an adult *C. elegans* hermaphrodite



#Picture from http://www.wormatlas.org/handbook/

1.4.2 Progression of meiosis I: programmed DSBs

In meiosis, the key to chromosomal segregation is a DNA DSB dependent mechanism. Programmed DNA cuts, in the leptotene phase, are necessary to induce the meiotic recombination. The strict regulation of the activity of the enzyme, SPO-11, leads to multiple cuts in the DNA during the first phase of prophase I. SPO-11 is a homolog of an archeal (TOPO VI) A subunit (Bergerat, et al., 1997). Type II topoisomerases generally provide for the topological disengagement of DNA, making one DNA molecule pass through another by generating a transient break in one of the two DNA molecules. Although SPO-11 is similar to type II topoisomerase, during evolution their functions have diverged: while type II topoisomerase normally cuts and rejoins the broken ends of DNA, SPO-11 has lost the ability to rejoin the DNA termini, introducing only double strand cuts (Bergerat et al., 1997; Keeney et al., 1997; Dernburg, et al., 1998). The SPO-11 cuts determine the initiation of recombination events. In many eukaryotes (such as yeasts, plants and mammals), the SPO11 activity is also essential for the proper formation of the synaptonemal complex. Indeed, the lack of meiotic cuts blocks not only the initiation of recombination, but also the synapsis between the homologous chromosomes (Storlazzi, et al.,2003; Grelon, et al., 2001; Baudat et al., 2000). Also in *Caenorhabditis elegans*, the absence of cuts induced by SPO-11 prevents

the initiation of recombination (crossovers are not formed). The direct consequence is the failure of a correct chromosomal segregation (aneuploidy), leading to high levels of embryonic lethality in the next generation (Dernburg, et al., 1998). However, in *Caenorhabditis elegans* as well as in *Drosophila melanogaster,* homologous synapsis occurs in the absence of SPO-11. An alternative system previously promotes the polymerization of the SC, bypassing a requirement for recombination intermediates to stabilize the pairing between homologous chromosomes (Smolikov et al., 2008). This could be a possible base for an explanation of the dependence of the synaptonemal complex formation on DSBs in the other systems. The induction of DSBs in the *spo-11* mutant through exposure to γ-irradiation gives a partial rescue of the lethal phenotype (Dernburg, et al., 1998). This result suggests how cuts of SPO-11 in *C. elegans* are only necessary for DSB induction. The autonomy of the meiotic progression from DSB induction makes the meiosis of *C. elegans* an extraordinary model in which molecular events such as the repair of SPO-11-dependent cuts, and structural modifications of chromosomes can be independently studied.

Together with the SPO-11 protein, in *Caenorhabditis elegans*, as well as in *Saccharomices cerevisiae,* MRE-11 is required for DSB formation (Chin and Villeneuve, 2001; Borde et al., 2004). All organisms, where the effects of the MRE11 mutation have been examined, show defects in meiotic recombination. *C. elegans mre-11* mutants show intact chromosomes at the diakinesis phase, without chiasmata, suggesting that either meiotic DSBs do not occur (as in *spo-11*) or that DSBs are repaired without crossing over (Chin and Villeneuve, 2001). The irradiation of the *C. elegans mre-11* mutants during meiotic prophase I, however, does not lead to a rescue (as in *spo-11* mutants), but causes chromosomal fragmentation and a high level of embryonic lethality (Chin and Villeneuve, 2001). The inability to repair IR induced DSBs and the presence of intact chromosomes at the diakinesis phase is consistent with a dual role of MRE-11 in both generating and repairing meiotic DSBs.

1.4.3 Processing and strand invasion

Similar to the role of MRE11 in yeast meiotic recombination, the worm MRE-11 is required in the 5’ to 3’ resection of DSBs to generate a substrate for a subsequent strand invasion step (Chin and Villeneuve, 2001; for a review, Borde, 2007). This resection of DNA ends forms a functional substrate 3’ssDNA for the binding with the strand exchange protein RAD-51. RAD-51 is one of the main proteins that have a recruitment in homologous

recombination during mitosis and meiosis. Its central role is emphasized by the fact that Rad51 has conserved the recombination function during evolution. Rad51 yeast mutants lead to un-repaired cuts, reducing the chromosomal pairing, and compromising the synaptonemal complex formation compared to wt (Rockmill et al., 1995). In mice, the loss of Rad51 causes embryonic lethality (Tsuzuki et al., 1996). This makes it complicated to study the recombination consequences of such a mutation. In *C. elegans*, the inability to repair the SPO-11 dependent cuts due to the *rad-51* depletion leads to the activation of DNA repair checkpoints, a strong increase of apoptotic levels, diffused chromosomes at the diakinesis phase, the defective segregation of chromosomes and finally an embryonic lethality of the offspring of the worm (Rinaldo et al., 1998; Gartner et al., 2000; Rinaldo et al., 2002).

In the last few years, several works have elucidated the mechanism that regulates the Rad51 sequestering on ssDNA. This phase of homologous recombination sees the involvement of the homologue gene BRCA2, a DNA repair gene that when mutated in humans causes an increased predisposition to breast and ovarian cancer (for a review, Narod and Foulkes, 2004). Important evidence about the role of BRCA2 has recently been obtained through the *C. elegans* model system. The *C. elegans* ortholog of BRCA2, BRC-2, interacts with RAD-51 *in vitro* and *in vivo*: yeast two-hybrids and pull down assays have shown how BRC-2 interacts directly with RAD-51 (Martin et al., 2005). Like *rad-51* mutants, *brc-2* mutants have defects in the repair of meiotic DSBs caused by a failure of RAD-51 loading onto DNA breaks; consequently, the *brc-2* mutant is characterized by embryonic lethality due to extensive chromosomal fragmentation (Martin et al., 2005). Based on these observations, it has been thought that BRC-2 sequesters RAD-51 to the site of the damage, promoting its nucleation on ssDNA (Martin et al., 2005), a model confirmed also in other eukaryote systems (for a review, Boulton, 2006).

The cytological localization of RAD-51 along the germ line reflects the processing and the resolution of DSBs induced by SPO-11. In *C. elegans,* as well as in other metazoans, RAD-51 forms multiple foci in the early phase of meiosis I (Ashley et al., 1995; Moens et al., 1997; Colaiacovo et al., 2003; Oliver-Bonet et al., 2005). In particular, the RAD-51 foci in the wild type gonad of *C. elegans* appear in the leptotene/zygotene phase, peaking in abundance in the early pachytene phase, and disappearing in the late pachytene phase (Colaiacovo et al., 2003). The decrease of RAD-51 foci reflects the progression of the repair events to the resolution of the cuts. Therefore, immunostaining against the RAD-51 protein can become an optimal tool in scientific applications for monitoring the progression of meiosis and checking the "health state" of the repair system.

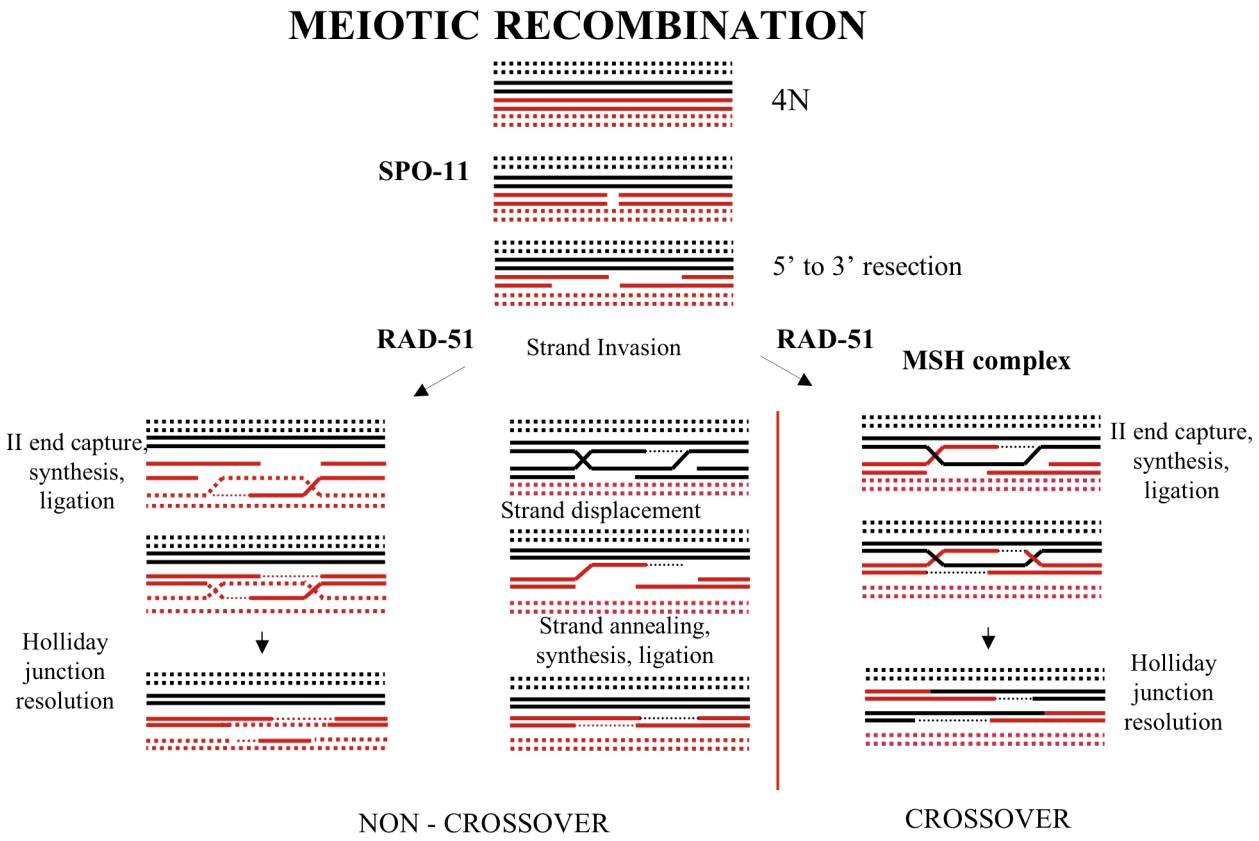
1.4.4 Crossover or non-crossover? That is the question

Homologous recombination, which in the meiotic context is the preferred repair pathway, may repair the DSBs either as a crossover, in which there is an exchange of chromosome arms between homologous chromosomes, or as a non-crossover, where the repair is either performed on the homologous chromosomes, involving only a local alteration of DNA (gene conversion) without an exchange of further material, or is entirely completed using the sister chromatid as a template. In higher eukaryotes, only a small fraction of DSBs are repaired as crossovers (for a review, Bishop and Zickler, 2004). In *C. elegans*, only one DSB for each pair of homologous chromosomes is selected for repair through the crossover recombination, which ultimately produces chiasmata between homologous chromosomes. The number of SPO-11 dependent cuts distributed along the entire length of the chromosomes and detected by immunostaining with RAD-51, is however higher than the number of repair events that are resolved in crossovers. Except for a single DSB repaired as a crossover for each pair of chromosomes, all other DSBs must be repaired through a repair pathway that gives non-crossovers. This evidence suggests that some proteins have roles either as barriers against the recombination events or as stabilizing factors for the Holliday junctions to give crossovers. The MSH complex acts as a pro-crossover factor. The MSH proteins belong to the Mut-S DNA mismatch repair family. MutS homologs (MSH) have been identified in all eukaryote organisms examined. Among the MutS members, MSH4 and MSH5 are expressed only in meiosis and form a heterodimeric complex. They do not have apparent functions in mismatch repair, but play an essential role in the meiotic recombination machinery. Previous studies in *S. cerevisae* and *C. elegans* had already suggested a function of these conserved proteins in the promotion of the crossover products (Ross-Macdonald and Roeder, 1994; Zalevsky et al., 1999; Kelly et al., 2000; Colaiacovo et al., 2003). In *C. elegans msh* mutants, alignment, pairing and synapsis between homologous chromosomes are efficient, but the diakinesis oocytes have twelve chromosomes (univalents) compared to the six bivalents of the wild type worm, due to the absence of chiasmata (Zalevsky et al., 1999; Kelly et al., 2000). In *him-14/MSH4* and *msh-5 C. elegans* mutants, the RAD-51 immunostaining shows an altered pattern characterized by the persistence of the RAD-51 foci along the gonad until the late pachytene phase suggesting that the SPO-11 protein cuts DNA, but the resolution of DSBs is delayed. The induction of additional damage, by γ-radiation, gives the same phenotype at the diakinesis phase as the untreated *msh* mutant, i.e. twelve proper univalents (Kelly et al., 2000). These mutants are, thus, competent for the repair of exogenous and endogenous DSBs

in meiosis suggesting that only the crossover repair pathway is impaired. The depletion of RAD-51 in the *msh-5* mutant shows nuclei at the diakinesis phase with a partial aggregation of bodies and chromosomal fragmentation (Rinaldo et al., 2002) indicating that in *msh* worms, the meiotic DSBs are resolved with a RAD-51 dependent non-crossover pathway. All these data together suggest a role of the MSH complex as a repair driving factor for the resolution of one DSB as crossover, while all other DSBs are repaired as non-crossover products on sister chromatids and/or inter-homologue chromosomes (gene conversion) (Figure 1.4) (Zalevsky et al., 1999; Kelly et al., 2000; Rinaldo et al., 2002; Colaiacovo et al., 2003). A recent work has shown how the human MSH4/MSH5 complex binds *in vitro* the Holliday junctions and stabilizes them favouring the crossover solution (Snowden et al., 2004). However, the contribution in meiosis of other alternative repair pathways, such as NHEJ, when homologous recombination is compromised, has not really been defined. In *C. elegans*, the homologue of DNA ligase IV (*lig-4*) has been identified. It is an essential enzyme for the last step of the joining of the broken ends of DNA. The *lig-4* mutant in *C. elegans* is viable, fertile and competent for crossover formation (Martin et al., 2005). To understand the possible contribution of NHEJ in meiosis when the crossover pathway is abrogated, the *lig-4* mutant has been crossed with the *him-14/MSH4* mutant (Adamo et al., 2008). Unlike the *msh-5;rad-51*^{RNAi} genetic background, where nuclei at the diakinesis phase show chromosomal fragmentation and a partial aggregation of bodies, the *lig-4;him-14/MSH-4* double mutant shows predominantly diakinesis nuclei with 12 proper univalents, like in the *him-14/MSH-4* single mutant. These data reveal that the SPO-11 dependent cuts are only repaired through homologous repair, which remains the main pathway in meiosis, while NHEJ has little or no role in meiotic DSB repair in *C. elegans*.

In *C. elegans*, the fact that there is only one crossover per bivalent suggests that the presence of a crossover suppresses the possible formation of a second crossover event along the entire length of the chromosome (Hillers and Villeneuve, 2003). Moreover, the distribution of crossovers seems to indicate that there are preferred “hot-spots” along the chromosomes. Each of the five autosomal chromosomes has a central cluster of tightly linked genes flanked by the chromosomal arms in which the genes are more widely spaced on the genetic map. The frequency of recombination in the chromosomal arms seems to be fivefold higher than the frequency of recombination in the central region (Brenner, 1974; Barnes et al., 1995; Hillers and Villeneuve, 2003).

Figure 1.4 Meiotic recombination



 # In meiosis, only one DSB is repaired as a crossover, all other DSBs are processed through alternative non-crossover pathways. The MSH complex, as well as the synaptonemal complex, is essential for the crossover formation.

1.4.5 Synaptonemal Complex

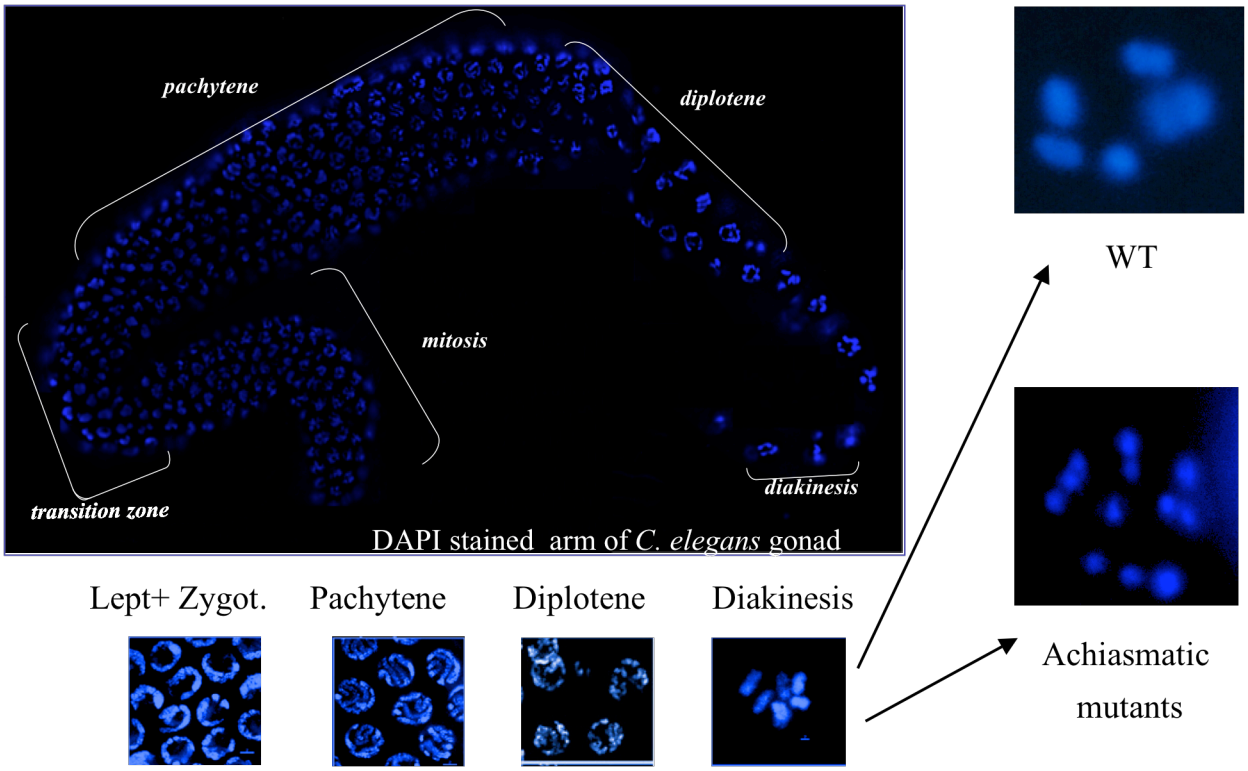
In *C. elegans*, during the progression of meiosis, chromosomes undergo several modifications, which reflect the level of maturation of the oocytes during meiosis I (Figure 1.5). As the mitotic cells move from the distal zone (the tip of the gonad), the nuclei undergo pre-meiotic DNA replication; they enter prophase I with the fully replicated genome. In the transition zone (Leptotene/Zygotene phases), the DNA encounters its first change. The structure of the chromosomes begins to change from a diffuse to a concentrated form. After the DNA duplication, the sister chromatids are tightly linked along their entire length through the interaction of the meiotic specific conserved cohesin proteins. REC-8, SCC-3, SMC-1 and SMC-3 constitute the *C. elegans* cohesin complex (for a review, Colaiacovo, 2006). The cohesin complex remains linked until the anaphase of the second meiotic division. If the cohesion between sister chromatids is impaired, as occurs in *rec-8* mutants, the formation of the synaptonemal complex is compromised, leading to a defective segregation of

chromosomes, a consequent aneuploidy and embryonic lethality (Pasierbek et al., 2001; Colaiacovo et al., 2003). In other words, the presence of the cohesin complex is essential to permit a correct disjunction of the homologous chromosomes and the correct progression of meiosis. REC-8 is required for the "recruitment" of the structural components of the synaptonemal complex (Pasierbek et al. 2001; MacQueen et al., 2002; Colaiacovo et al., 2003). This structure is ubiquitously present from yeast to mammals, as revealed by electron microscopy (EM) and fluorescent immunocytological studies, but its functions are not yet fully understood (for a review Roeder, 1997; Zickler D, Kleckner N., 1999; Colaiacovo, 2006). The synaptonemal complex is a tripartite structure composed of a pair of lateral elements, consisting of proteins assembled along the axes of each homologous chromosome, and a central region, consisting of transverse filament proteins interconnecting the lateral axes (zipper like structure). In *C. elegans*, the lateral elements are HORMA domain proteins, HIM-3, HTP-3, HTP-1 and HTP-2. The proper execution of pairing, synapsis, and crossing over depends on the activity of the meiosis-specific axis component (Zetka et al., 1999; Couteau et al., 2004; Martinez-Perez and Villeneuve, 2005; Couteau and Zetka, 2005). SYP-1, SYP-2, and SYP-3 are central region components of the SC (MacQueen et al., 2002; Colaiacovo et al., 2003; Smolikov et al., 2007). They are observed forming a single focus per nucleus at the entrance of meiosis I, followed by multiple foci and short stretches along the chromosomes. In the pachytene phase, they localize at the interface between the entire lengths of the homologous chromosomes. SYP-1, SYP-2, and SYP-3 are interdependent for their localization: the absence of one affects negatively the localization of the others. In *syp* mutants, the normal cytological morphology of DNA undergoes several changes. The absent polymerization of the central proteins affects the formation of the SC, which is no longer visible by transmission electron microscopy (TEM). Moreover, the chromosomes fail to re-disperse upon entrance into the pachytene phase, maintaining instead the polarized configuration of the leptotene/zygotene phase until the late pachytene phase (MacQueen et al., 2002; Colaiacovo et al., 2003; Smolikov et al., 2007). A second strong phenotype of these mutants is the high embryonic lethality. The absence of the SC between the homologous chromosomes represents a physical barrier that does not permit the repair of DSBs as crossovers, showing at the diakinesis phase nuclei with 12 intact univalents. The stochastic segregation of chromosomes and the consequent aneuploidy causes in the next generation a high level of non-viable eggs. Although the SC is not essential for the initiation of recombination, the completion of recombination events depends on the SC. Through the monitoring of the progression of meiosis I with α-RAD-51 antibody for the detection of

recombination intermediates, the SPO-11-dependent RAD-51 foci persist in *syp-2* worms until the late pachytene phase (Colaiacovo et al., 2003). In these mutants, the DSBs are created at the right time, indicating that the DSB meiotic formation does not depend on the SC formation. The depletion of RAD-51 leads to unresolved DSBs and at the diakinesis phase the nuclei show diffusions and unstructured chromosomes, compared to the single mutant *syp-2* (Colaiacovo et al., 2003). These data indicate that in the *syp* mutant, the DSBs are repaired through a RAD-51 dependent non-crossover pathway, giving 12 proper univalents at the diakinesis phase. Given the absence of the SC and the final resolution of DSBs, in the *syp* mutant the DNA repair system can use mainly the sister chromatids as default repair templates.

At the diplotene phase, the synaptonemal complex is dissolved and at the diakinesis phase each couple of chromosomes forms a bivalent linked together by a chiasma, a cytological structure that is evidence that crossover has occurred. This physical connection between the homologs gives the right chromosomal orientation at metaphase 1 and the tensional strength for a regular segregation in the meiotic spindle.

Figure 1.5 An arm of adult *C. elegans* gonad.



 # Representative image of a wild-type germline stained with DAPI (blue) (DAPI, 4,6-diamidino-2-phenylindole. During Meiosis I, chromosomes undergo characteristic modifications, which allow us to distinguish five sub-phases of prophase I, called leptotene, zygotene, pachytene, diplotene and diakinesis. In wt worms, diakinesis nuclei show 6 DAPI stained bodies (6 bivalents joined by chiasmata), while in achiasmatic mutants diakinesis nuclei show 12 DAPI stained bodies (12 univalents).

1.4.6 Physiological apoptosis in *C. elegans*

During the development of the hermaphrodite worm, 131 cells of the 1,090 generated, undergo programmed cell death in a highly reproducible way. Extensive genetic analyses have identified the evolutionary conserved apoptotic pathway in *C. elegans*. Four genes were originally identified for the regulation of the apoptotic pathway: *ced-3*, *ced-4*, *ced-9*, and *egl-1*. Loss of function of *ced-3*, *ced-4* and *egl-1* determines the survival of these 131 somatic cells that normally die (Ellis and Horvitz, 1986). In contrast, *ced-9* has an anti-apoptotic function: a gain of function mutation leads to a block of apoptosis, while a loss of function mutation determines a precocious death during early development, due to the activation of the unregulated apoptotic pathway (Hengartner et al., 1992). Epistasis studies have permitted us to understand that the progression of the death program in *C. elegans* consists in a change of state from inactive to active of CED-3, a protein belonging to the caspase family. For its activation, CED-3 has to associate with a CED-4 tetramer, a homologue to mammalian Apaf-1. CED-4 functions as a positive regulator of CED-3 and they together form the worm version of apoptosome, which in mammals is composed of three proteins: caspase 9, Apaf-1 and cytochrome-c (discovered years later) (Li et al., 1997). In those cells that survive, CED-4 is sequestered by the interaction with the positive competitor CED-9. This protein is homologous to Bcl-2, which plays a protective function in mammalian cells. The sequestration of CED-4 by CED-9 maintains the state of CED-3 inactive (for a review, Lettre and Hengartner, 2006). The fate of the cells, to die or not to die, depends on the level of the expression of *egl-1*. Like *ced-9*, *egl-1* also belongs to the Blc-2 family, but, differently from CED-9, which has an anti-apoptotic function, EGL-1 is pro-apoptotic. The binding between EGL-1 and CED-9 determines a conformational change of CED-9 that leads to the releasing of the CED-4 protein. Therefore, free CED-4 can form the CED-3/CED-4 complex, activating the apoptosis. The identification of these key genes regulating the cell death program in *C. elegans,* genes that are conserved in humans, has represented a significant advance in knowledge about apoptosis. The importance of this scientific contribution was confirmed when Sydney Brenner, H. Robert Horvitz and John E. Sulston, who are the main authors of this *C. elegans* apoptotic and development model, were awarded the Nobel prize in Physiology or Medicine in 2002.

1.4.7 Germline apoptosis and apoptosis induced by damage

In addition to the developmental apoptosis occurring during the first steps of embryogenesis and in the L2 larval phase, the adult hermaphrodite has a physiological apoptosis in the germ cell line. The gonad of an adult hermaphrodite is the only organ where the apoptosis is always activated (Gumienny et al., 1999). In this process, almost half of germ line cells during the entire life of the worm are physiologically condemned to die. The system of selection of germ cells that are designated to die, however, still remains unclear. However, the apoptotic levels in the germ line can vary: the presence of genotoxic stresses, for example, can activate the damage checkpoint, and consequently the damage apoptosis is added to the physiological apoptosis.

As in all organisms, the nematode cells have a system to monitor and signal the DNA damage. However, this pathway is only active in the germline, the only tissue actively proliferating in the adult organism. *hus-1*, *mrt-2* and *rad-5* are rad mutants, defective for the radiation-induced apoptosis (Hodgkin et al., 1979; Hartman and Herman, 1982; Ahmed et al., 2001). All three mutations abrogate the cell cycle arrest and apoptosis induced by DNA damage. *hus-1* and *mrt-2* encode proteins homologous to *S. pombe* Rad1 and Hus1 checkpoint proteins (Ahmed and Hodgkin, 2000; Hofmann et al., 2002). Hofmann's work has shown how HUS1::GFP associates with chromatin under normal conditions, while, after induction of DNA damage, HUS-1 relocates to particular sites, signalling probably un-repaired damage. For a correct localization on the nucleus, HUS-1 has to interact with MRT-2 and the Rad9 homolog HPR-9, forming a complex, a marker of DNA damage (Hofmann et al., 2002). Differently from *mrt-2*, *rad-5* is dispensable for the localization of *hus-1*. Moreover, the *rad-5/clk-2* mutant shows a more serious phenotype compared to the *mrt-2* and *hus-1* mutants: the complete elimination of the *rad-5* gene function leads to developmental arrest and embryonic lethality (Ahmed et al., 2001). However, all these proteins, when deficient, are unable to induce the cell cycle arrest and the damage-dependent apoptosis, indicating their roles in damage signalling. Even if it is still not clear how, these three *C. elegans* checkpoint proteins regulate the apoptotic machinery through the CEP-1 protein. CEP-1 is the only one member of the p53 family codified in the *C. elegans* genome. The depletion of CEP-1 by iRNA does not affect the physiological apoptosis during the worm development and in the germ cell line. In contrast, like p53, CEP-1 is a regulatory factor for responding to genotoxic stresses on DNA. It is required for the activation of DNA damage apoptosis, but not for the cell cycle arrest (Derry et al., 2001; Schumacher et al., 2001). Studies indicate how the damage

apoptosis in the germ cell line is transcriptionally regulated: mRNA levels of *egl-1* are up-regulated following DNA damage, consistent with the role of *egl-1* previously described for the induction of apoptosis (for a review, Stergiou and Hengartner, 2004). The proposed model sees the CEP-1 protein as a transactivator between the damage sensor proteins and their substrates. However, how CEP-1 is activated following DNA damage and whether it binds directly the transcriptional regulatory region of *egl-1* is still unclear.

1.5 BRCA pathway

In mammals, some of the genes involved in both somatic DSB repair and meiotic recombination, have been discovered for the serious pathologies linked to their mutations: in heterozygosis, BRCA1 and BRCA2 are both involved in the predisposition to breast and ovarian cancer. Inheritance of one mutant allele in either gene, BRCA1 or BRCA2, confers on women an 80% lifetime risk of developing breast and/or ovarian cancer (for a review, Narod and Foulkes, 2004). Studies on DT40 cells from chicken and on tumour derived human cell lines indicate how both genes are functional for the maintaining of genome stability. Their mutations, in fact, lead to chromosomal translocation, duplication, and aberrant fusions between non-homologous chromosomes (Venkitaraman, 2002). The role of BRCA2 has been well elucidated in the last few years through the *C. elegans* model system also (Martin et al., 2005; for review, Boulton, 2006) as previously described. BRCA2 participates in the early steps of homologous repair: BRCA2 mediates the recruitment and nucleation of RAD51 on the damage site. A description of the cellular BRCA1 function instead seems to be difficult given that BRCA1 plays different roles in several cellular processes. Sequence conservation of BRCA1 is weak in mammalian species, except for two particular domains present in the C- and N-terminal regions of the protein, a Ring and two tandem BRCT domains respectively. In BRCA1, a Ring motif is responsible for the heterodimerization with a structurally related protein, BARD1 (BRCA1 associated RING domain 1). The BARD1/BRCA1 interaction is supported by their co-localization at repair foci. Moreover, BARD1 and BRCA1 cell lines and knockout mice exhibit the same phenotype, indicating a common function (for a review, Boulton, 2006). The RING motif is not only responsible for the interaction between BRCA1 and BARD1, but also functions as an E3 ubiquitin ligase in the final step of the ubiquitylation process (Hashizume et al., 2001; Ruffner et al., 2001). Such a modification could alter the cellular protein localization or the enzymatic activity or regulate protein-protein interaction.

At the moment, no effective targets of the BRCA1/BARD1 ubiquitylation are known. However, recent work in human cells has shown that the BRCA1/BARD1 ubiquitylation activity occurs at the DNA repair sites. Through immunofluorescence with a specific antibody to conjugated ubiquitin, these events can be detected in stalled forks after hydroxyurea treatment in the S-phase and at DSB repair sites following the exposure to ionizing radiation (Morris and Solomon, 2004). In addition to BARD1, in S/G2-phase cells or following exposure to DNA damaging agents, BRCA1 forms foci at the sites of DNA damage, co-localizing with other repair proteins such as BRCA2 and RAD51 (Scully et al., 1997a; Scully et al., 1997b; Sharan et al., 1997). This co-localization reinforces the idea of a BRCA1 role in the homologous recombination repair of endogenous or exogenous damage. The absence of BRCA1, moreover, reduces the efficiency of the HR repair in mouse cells (Moynahan, et al.,1999). Consistent with these data, BRCA1 interacts with other different homologous repair proteins. A lot of evidence supports a connection of BRCA proteins with the FA pathway in the resolution of DSB damage, explaining thus the name "BRCA/Fanconi pathway". Fanconi Anaemia is a syndrome characterized by aplastic anaemia, cancer predisposition, genetic instability and cellular hypersensitivity to ICL agents (the cellular hypersensitivity is used as a diagnostic tool). FANC proteins (13 FA proteins) seem to have an important role in a network of cross-talking proteins involved in the DNA surveillance and repair. The FA complex, in response to stalled forks or to DNA damage induced by IR or ICL agents, monoubiquitylates FANC-D2 and FANC-I. The FANC-D2 monoubiquitylation is associated with its ability to assemble in nuclear repair foci. In these foci FANC-D2 co-localizes with other proteins involved in DNA repair such as BRCA1, RAD51, MRN complex and BRCA2, which has a biallelic mutation associated with a rare D1 complementation group of Fanconi anaemia. The absence of BRCA1 determines the failure of FANC-D2 translocation on RAD51-containing foci (Garcia-Higuera et al., 2001). These findings are interpreted as evidence that BRCA1, BRCA2, and RAD51 cooperate with FA proteins in a common biological response to DNA damage (for a review, Wang, 2007).

An additional role for BRCA1 in meiosis has been suggested from studies of knockout mice. BRCA1 knockout mice have a premature death before 10 days of embryonic development, characterized by high levels of apoptosis (Gowen et al., 1996). The abrogation of p53-dependent damage response leads to an extension of the embryonic life, assisting the analysis of the BRCA1 role in meiosis. BRCA-/-;p53-/- double mutants show defects in spermatogenesis: the testes are smaller than those of wt, the spermatogonia appear normal, while spermatids and spermatozoa are not observed. Monitoring the progression of meiosis

reveals how in these mutants, spermatocytes are arrested in the late pachytene phase and fail to enter the diplotene phase (Cressman et al., 1999; Xu et al., 2003). In wild type mice, immunolocalization against the BRCA1 protein reveals that BRCA1 localizes along chromosomal structures discernible as unsynapsed axial elements (Scully et al., 1997b). Turner, recently, has evidenced how BRCA1 staining on the X and Y-chromosomes depends on their unsynapsed state (Turner et al., 2004). The sex chromosomes are maintained in a transcriptionally silent state, obtained through the phosphorilation of ATR on the H2AX histone. BRCA1 determines the right localization of ATR on the sex chromosomes. In BRCA1 mutant mice, ATR is mislocated, H2AX phosphorilation is abolished, and the level of transcription of X and Y genes are upregulated compared to the controls, suggesting a failure of meiotic sex chromosome inactivation (MSCI) (Turner et al., 2004). It is evident that BRCA1 functions in mitotic and meiotic cells, but its contribution still remains unclear.

1.5.1 BRCA1 ortholog in *C.elegans*

In 2004, Boulton's laboratory first identified the BARD1 ortholog (BRD-1) through sequence research of the *C. elegans* genome: the *brd-1* gene encodes a protein, characterized by an N-terminal RING finger and two C-terminal BRCT repeat domains, with 23% of identity and 41% of similarity with the BARD1 human protein. Given that BARD1 exists as a heterodimer of BRCA1, BRCA1 ortholog (BRC-1) has been found through the yeast two-hybrid system screening. BRC-1 contains an N-terminal RING finger, a nuclear signal localization and two C-terminal BRCT repeat domains. Pull down assays and the yeast two-hybrid matrix confirm the interaction between these two proteins through the RING finger, suggesting a possible role of the BRD-1/BRC-1 complex in the DNA damage repair pathway, like in mammals (Boulton et al., 2004). The depletion of the *brd-1* gene or *brc-1*, through the RNA-mediated interference (RNAi), gives rise to different phenotypes: an increased level of chromosomal non-disjunctions, that is manifested as a modest increase of males in self progeny (HIM phenotype), and an increased level of apoptosis in the germ line (Boulton et al., 2004). The exposure of depleted worms at the L4 stage to γ-radiation determines a dramatic increase of apoptosis in the germ line, and compromises dramatically the morphology of chromosomes in the transition and pachytene phases. Moreover, after exposure, the hatching rate of the progeny is decreased, compared to controls, suggesting that both *brc-1* and *brd-1* depleted worms are radiation sensitive. The high level of apoptosis after

γ-radiation is suppressed when mutations of *cep-1/p53* or *rad-5* are present in either *brd-1* or *brc-1* depleted worms. The level of apoptosis is dependent on the *cep-1/p53* and *rad-5* checkpoint proteins (Boulton et al., 2004). All these data indicate how the BRC1/BRD1 complex could be required for DNA repair. To verify this possible role, experiments of biochemistry have characterized the proteins associated with BRD-1 from soluble and chromatin bound fractions under normal conditions or after exposure to radiation. BRC-1 and BRD-1 are present in both soluble and chromatin bound fractions before and after ionizing radiation (IR). In addition to the BRC1/BRD1 complex, RAD-51 is one of the proteins associated with chromatin bound fractions after exposure to IR (Polanowska et al., 2006). Similarly to other models, BRD-1 co-localizes with RAD51 foci at sites of DNA damage. After radiation, the absence of *brc-1* affects negatively BRD-1 localization on DNA: there is no detectable BRD-1 staining. Unlike *brc-1*, after IR treatment in the *mre-11* mutant the majority of the BRD-1 protein is defuse into nuclei, indicating a failure of recruitment of BRD-1 to DNA repair sites. Therefore, the capacity to form the BRC-1/BRD-1 complex on repair sites is dependent on the *C. elegans* MRN complex (Polanowska et al., 2006). These data suggest strongly that these proteins may have a function in DSB repair via homologous recombination.

Another protein associated with the BRC1/BRD1 complex is Ubc-5 (E2 conjugating enzyme). The association between the BRC1/BRD1 heterodimer and Ubc-5 forms a stable complex, detectable specifically through E3 Ub-ligase activity. Ubiquitylation events induced by the BRC-1/BRD-1/Ubc-5 complex can be visualized directly by immunostaining against the conjugated ubiquitin on the worm germ line. Under normal growth conditions, the ubiquitylation activity is present in the later stage of prophase I, but it is rapidly abolished, after radiation treatment. The exposure to IR determines multiple conjugated-Ub foci, visible at DNA damage sites in mitotic nuclei, where the replication forks are probably stressed. All these data reveal that the BRC-1/BRD-1 heterodimer participates in a conserved response to DNA damage. However, the pathway of action in homologous DNA repair has not been identified.

Rationale and Aim

Genomic stability is constantly threatened by a plethora of exogenous and endogenous stresses that can lead to a loss or alteration of genetic information. The integrity of the genome is maintained by an efficient cross-talk between DNA repair and checkpoint pathways, efficiently coordinated with the developmental cell cycle progression of the organism. In order to highlight the role of the surveillance/response network for genomic preservation, I chose to use *Caenorhabditis elegans* as my model system. Unicellular model organisms such as bacteria or yeast, have an obvious limitation in the study of surveillance systems. On the other hand, development in mammals is complex and still largely unknown and so it is difficult to study the effect of mutations in an entire organism. Therefore, most studies in mammals are not done in the context of a complete organism, but, *ex vivo*, in cell culture derived from tissue. This is an expedient that provides only a partial and possibly artificial picture of what really happens in the organism. *C. elegans*, however, is a simple pluricellular organism that permits an easy analysis of the interplay between different cells. Its body is principally composed of the reproductive system, which is immediately apparent upon viewing under the microscope, allowing a trouble-free monitoring of the progression of oogenesis. Before the entrance in meiosis, the mitotic cells are continuously dividing. Their state is controlled by damage checkpoints inducing cell cycle arrest and repair mechanisms so they represent a suitable model to study the cross-talking between the "surveillance" systems in proliferating tissues. During meiosis, through different steps, the meiocytes develop from a 4N asset to mature haploid germ cells. For a successful segregation of chromosomes, the DNA undergoes endogenous double strand breaks (DSBs) induced by the topoisomerase-like protein SPO-11. One of the DSBs is responsible for inducing and is sufficient to ensure recombination crossover for each pair of homologs, essential to give the right tensional strength for chromosomal segregation. In eukaryotes, many genes involved in meiotic recombination also have functions in DNA damage repair. Many of these genes are conserved during evolution. The meiosis of *C. elegans*, therefore, becomes an appropriate model system, suitable for a study of the role of genes and possible protein interactions in the DNA repair mechanism.

Moreover, the study of the nematode apoptotic mechanism has been relevant for the comprehension of human apoptosis since the key components of the cell death pathway are conserved from *C. elegans* to humans. During nematode meiosis, basal apoptosis condemns

half of the germ line cells to death. In response to genotoxic stresses, apoptotic levels increase throughout the germ cell line by activation of specific checkpoints. Nematode meiosis once more represents a powerful model that allows an easy investigation of the cross talk between the repair mechanism and apoptosis.

Among the metazoan-specific DNA repair genes in *C. elegans*, there is the homologue of the breast cancer susceptibility BRCA1 gene. In mammals, the loss of BRCA1 causes defects in the resolution of DSBs by homologous recombination repair. However, the exact role of BRCA1 in the DNA repair process still remains elusive. The aim of this thesis is to exploit the *C. elegans* oogenesis model to clarify the role of the BRCA1 ortholog, BRC-1, during the DSB repair mechanism.

2. Materials and Methods

2.1 Strains

The worms were grown on Nematode Growth Medium (NGM) plates containing the bacterial strain known as OP50, a uracil-requirent mutant of *Escherichia coli*. An OP50 strain of *E. coli* was used to prevent overgrowth of the bacterial layer. The medium has limited uracil, and the bacteria cannot grow into a thick layer, which could obscure the worms. The worms were grown at 20°C. The maintaining of the worms was carried out as described by Sulston and Hodgkin, 1988. The following strains used in this work were kindly provided by the *Caenorhabditis Genetics Centre*:

- N2, wild-type strain Bristol;
- AV106, *spo-11(ok79)IV/nT1[unc-?(n754)let-?]IV;V*;
- AV308, *him-14 (it21)/mnCI*;
- AV276, *syp-2 (ok307)V/nT1[unc-?(n754)let-?(qls50)] (IV;V)*;
- DR102, *dpy-5(e61) unc-29(e403)I*;
- BC3217, *unc-60(m35) dpy-11(e224)V; sDp30 (V;X)*;
- VC172, *cep-1(gk138)*;
- MT2405, *ced-3(n717) unc-26(e205)IV*.
- DW102*, brc-1 (tm1145)* strain was generated and kindly provided by Shoehi Mitani of the National Bio-resource Project for the *nematode*, Japan.

Three of these strains (AV106, AV308, AV276) carry recessive mutations and cannot be kept as homozygotes. They are stably maintained as heterozygotes through genetic constructs or chromosomal rearrangements, called genetic balancers. These rearrangements abrogate the crossing over between the homologs in the region where alleles are mutated. The genetic balancers have visible markers that allow a distinction between the genotypes of worms and the maintenance of the strains for generations. The maintenance of the heterozygous genotype from one to the next generation required selection of heterozygous individuals. AV106 has an Unc (Uncoordinated) genetic marker: *unc* worms are characterized by locomotion problems due to defects of the muscle system. AV308 has a balancer that determines a *rod* phenotype, i.e. sterile worms with a reduced body size and an inability to

move starting from early larva phases. Finally, AV276 has a balancer carrying different genetic markers, an *unc* gene, a GFP marker, and a let (lethality) gene, which in homozygosis leads to unviable eggs.

NGM plates were prepared with NaCl 0.3%, Peptone 0.25%, and Agar 2%. After sterilization, cholesterol (5μg/ml), $CaCl_2$ (1mM), $MgSO_4$ (1mM), and $NaKPO_4$ (25mM pH6) were added.

OP50 bacteria were grown in an LB solution (NaCl 1%, Yeast Extract 0.5%, Bacto tryptone 1%) at room temperature over night and stored at 4°C.

2.2 Genetic strategy

2.2.1 Primers and PCR

Some deletion mutants were monitored during genetic crosses using PCR primers flanking the deletions:

brc-1(tm1145)

- 5'TGTCGCATCGTCGGCATTAA3' and 5'AATATAGGCACCGGCGGGGA3'

cep-1(gk138)

- 5'TAAAATGGGATGTCTAGTGC3' or 5'TAAAATGGGATGTCTAGTGC3' and 5'GAATGTCTTGGGAATTAGAG3'

The PCR products were amplified using a genomic DNA prepared according to the following protocol: single animals were picked up with a platinum wire and each placed in a 3 μl of lysis buffer (20000U/ml proteinase K in 10 mM Tris [pH 8.2], 50 mM KCl, 2.5 mM MgC12, 0.45% Tween 20 and 0.05% gelatin) in a tube suitable for PCR. The tubes were frozen at -70°C for 15 min, and afterwards heated for 1hr at 65°C and for 15 min at 95°C. Genomic DNA was stored at -20°C.

Symmetric PCR (polymerase chain reaction) was performed to monitor the *brc-1* mutation with a PCR mix with 0.5 μl DNA, 1 μl *brc-1* primers 10μM, 2 μl dNTP 2.5mM, 2.5 μl 10x buffer 17.5mM $MgCl_2$, 0.4 μl Ampli Taq Gold [Roche], 17.6 μl H_2Odd. The reactions were heated at 94°C for 8min and cycled 35 times: 30s at 94°C, 1min at 59°C and 1min at

72°C, following by 10min at 72°C for a final extension. For the *cep-1* mutation, two parallel PCRs were performed using, in one a couple of primers external to the deletion, and in the other internal and external primers. The same PCR program as that used for the *brc-1* mutation was used for *cep-1*, but with an annealing temperature of 60°C.The PCR products with the internal and external primers gave the wild type pattern in an electrophoretic run on gel of agarose 1%.

2.2.2 Genetic crosses

To generate *brc-1;spo-11, the spo-11(ok79)*IV homozygous hermaphrodite worms, were crossed with *brc-1* males. *spo-11(ok79)*IV hermaphrodites were crossed with 3-4 *brc-1* males to ensure fecundation. The parents were moved each 12 hours onto new Petri plates with fresh OP50 bacteria for five times in total. From the F1 hermaphrodites with the wild type phenotype, the F2 were cloned and screened for the *spo-11* embryonic lethal phenotype. Adult hermaphrodites that laid only unviable eggs were dissected, one of the two arms of the gonad was analyzed by PCR for the *brc-1* deletion and the other was immuno-stained with α-RAD-51.

To generate *brc-1;him-14/MSH4* worms, *him-14 (it21)/mnC1* hermaphrodites were crossed with *brc-1(tm1145)III* males. Almost 50% of the F1 progeny had to be male for the mating to be considered successful. Therefore, the heterozygous hermaphrodites (F1) were cloned and the lines that showed the inherited balancer in F2 were not considered. The F2 were cloned and screened for the *him-14/MSH4* embryonic lethal phenotype. Adult hermaphrodites that laid a majority of unviable eggs were analyzed by PCR for the *brc-1* deletion and the double mutant progeny were DAPI stained and their diakinesis nuclei were analyzed. Similar strategies were used to generate *brc-1;syp-2*. Double mutant progenies were DAPI stained and their diakinesis nuclei were analyzed.

brc-1;cep-1 double mutants were isolated by PCR and screened for embryonic lethality and diakinesis aberrations. To generate *brc-1;ced-3* worms, *ced-3(n717) unc-26(e205)* hermaphrodites were crossed with *brc-1(tm1145)III* males and the F2 were screened for the *unc-26* phenotype. Adult Unc hermaphrodites were analyzed by PCR for the *brc-1* deletion. The double mutants obtained were checked for the absence of apoptotic nuclei and the progeny were screened for embryonic lethality.

To study recombination frequency in *brc-1* mutant, males of genotype *brc-1(tm1145)III* were crossed with *unc-60(m35)dpy-11(e224)V* hermaphrodites. The F2 were

screened for the *unc-60 and dpy-11* phenotypes. Adult Unc Dpy hermaphrodites were analyzed by PCR for the *brc-1* deletion. The same method was performed for analysis of recombination frequency on the I chromosome through the genetic cross between *brc-1(tm1145)III* and *dpy-5(e61)unc-29(e403)I.*

2.3 Screening of laying worms

Each single and double mutant worm was cloned during the L4 larval state on single Petri plates and kept at 20°C, producing and laying eggs for 4 days. Every 12 hours the laying worms were transferred onto fresh plates until the deposition of non-fertilized oocytes. Each plate was monitored for 24/72 hours to analyse two different parameters: embryonic lethality and the presence of males among the progeny. These two phenotypes can indicate defects of the meiosis mechanism like non-disjunction of the chromosomes. The non-disjunction of autosomic chromosomes can lead to aneuploidy, causing embryonic lethality in the next progeny. If a defect of segregation affects the sexual chromosomes, the effect will be the Him phenotype (high incidence of males) in the next generation. The value of embryonic lethality was calculated as the ratio of unviable eggs to laid eggs, while the percentage of males was calculated as the ratio of males to the viable progeny.

2.4 Quantitative analysis of DAPI-staining bodies in diakinesis nuclei

After their laying of eggs, adult hermaphrodites were picked out for quantitative analysis of DAPI-staining bodies in diakinesis nuclei. The worms were transferred and suspended in 15μl M9 solution (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml of 1mM $MgSO_4$ in 1liter) on glass slides. The samples were permeabilized and fixed through 15μl of absolute ethanol. Once the samples had been dried, ethanol was added again. To visualize the DNA in the fixed animals, 15μl of the 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI) (2ng/μl) diluted in M9 were added. The observed nuclei were from both arms of each gonad, and about 20 worms in different genetic backgrounds (wt, *brc-1*, *him-14/MSH-4*, *syp-2, brc-1;him-14/MSH-4*, *brc-1;syp-2*) were sacrificed. The quantitative analysis was performed on *z* series of images acquired using a Leica DM6000 fluorescence microscope, Leica DC 350 FX camera under the control of Leica LAS AF 6000 software. Optical sections were collected at 0.50 μm increments.

2.5 Affinity Purification of RAD-51 antibody

Rabbit α-RAD-51 antibody was generated using a His-tag fusion protein expressed from plasmid pET28a containing the entire RAD-51S coding sequence (Colaiacovo et al., 2003; Rinaldo et al., 1998). Affinity purification of α-RAD-51 was performed as in Koelle and Horvitz (Koelle and Horvitz, 1996): 2ml of antiserum were purified by binding to a nitrocellulose filter strip carrying 1mg of RAD-51 fusion protein and the specifically bound antibodies were eluted with 100mM glycine-HCl (pH 2.5). In particular, the protein, run on 6% gel acrilamide, was blotted onto nitrocellulose by standard electrophoretic transfer. The protein was visualized by Panceau S staining and the corresponding band was cut out from the membrane. The filter strip was transferred into a 15 ml conical tube and those proteins poorly bound were removed with 100mM glycine-HCl (pH 2.5) for 5min. After two washings, each of 2 min, in TBS (20mM Tris pH 7.4, 500mM NaCl, 0.05% Tween-20), the filter strip was blocked with blocking buffer (5% dry milk powder, 50mM Tris pH 7.5, 150mM NaCl) for 1hr at room temperature on a rocker. Two further washings in blocking solution (in this case without milk) were carried out each for 2min. 2ml of serum were diluted with 8ml of TBS and added to the filter strip for 2-3hours at room temperature on a rocker. The filter strip was washed for 5min with washing buffer (100mM Tris pH 7.5) and subsequently twice with 1xPBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0,024% KH_2PO_4,). To elute the specifically bound antibodies, 1ml of 100mM glycine-HCl (pH 2.5) was added to the filter strip and this was then incubated for 10min. The elution step was repeated twice .The elution was stored at -80°C.

2.6 Immunostaining of meiotic nuclei

Gravid hermaphrodites were dissected in 15µl M9 solution (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml of 1mM $MgSO_4$ in 1liter) on poly-lisine slides with. Coverslips, treated previously with sigmacote (a special silicone solution), were added and freeze crack using on dry ice. The samples were permeabilized and fixed through three steps at -20°C in methanol, methanol/acetone (1:1), and acetone respectively. The preparations were washed three times for 5 min in 1x PBS and blocked with 0,3% BSA in 1x PBS under a coverslip for 30 min at 37°C in a humid chamber. The coverslips were rinsed off and the specimens were incubated with the primary antibodies diluted in Ab buffer (0,1% BSA, 0.04% Tween-20,

0.05% sodium azide in 1x PBS). Dilutions were 1:50 anti-REC-8 (Pasierbek et al., 2001), 1:200 anti-RAD-51. Slides were incubated with the primary Ab for 90 min at room temperature in a humid chamber. The coverslips were then rinsed off and three washings were carried out in 1x PBS, each one for 5 min. Incubation with the secondary antibodies, anti-rat for REC-8 (1:200) and anti-rabbit for RAD-51 (1:200) in Ab buffer, was carried out for 55 min at room temperature. Finally, the slides were washed and mounted in anti-fading medium (5mg phenylenediamine, 500μl PBS, 4.5ml glycerol, 20μl NaOH for a final pH 6-9) containing DAPI (1ng/μl). Quantitative analysis of RAD-51 foci was performed on *z* series of images acquired using a Leica DM6000 fluorescence microscope, Leica DC 350 FX camera under the control of Leica LAS AF 6000 software. Optical sections were collected at 0.25 μm increments. The quantitative analyses of RAD-51 foci were performed by dividing the germ line into 6 zones (tip, mitotic zone, transition zone, early pachytene, middle pachytene, late pachytene), in accordance with their cytological features.

2.7 Recombination Frequency

The genetic distance separating two genes (or any two points on a chromosome) is determined by the frequency of meiotic recombination that takes place between them. The nearer the two genes are to each other, the less likely that a recombination event will occur in that span. Most *C. elegans* chromosomes are on average about 50 map units long. We used two different intervals to estimate the distribution and the frequency of crossovers. Two marker mutations of worm were used as tools for standard genetic mapping: Dumpy mutation that leads to a short and fat phenotype, and Uncoordinated mutation, a worm with strong locomotion problems.

Males of genotype *brc-1(tm1145)III* were crossed with *brc-1(tm1145)III; unc-60(m35)dpy-11(e224)V* hermaphrodites. Cross-progeny hermaphrodites were picked out and placed on single plates and transferred daily for 4 days, and complete broods were scored for Unc Dpy, wild-type, and Unc non-Dpy, and Dpy non-Unc recombinant progeny. The genotype of F2 was derived from the F3 phenotypes, (obviously the F2 males have not been considered in the general estimation). *brc-1(tm1145)III; dpy-5(e61)unc-29(e403)I* were crossed with *brc-1(tm1145)*III and similarly screened. Therefore, the estimation of frequency of recombination was calculated through the ratio between the recombinant alleles and all screened alleles from F2 progeny.

2.8 Apoptosis assay

We performed and set the apoptosis assay with the syto-12 staining. We chose Syto-12, a vital dye, that directly permits the recognizing of cells that undergo apoptosis; it stains DNA or RNA, but in apoptotic cells, the refraction power increases because of the more compact DNA conformation (Gumienny et al., 1999). To obtain an estimation of the relative numbers of apoptotic corpses in different genetic backgrounds (wt, *brc-1*, *cep-1*, *ced-3*, *brc-1;ced-3*, *brc-1;cep-1*, *spo-11*, *brc-1;spo-11*), adult animals (24-hours from L4) were suspended in M9 solution and stained by incubating with 33 μM of syto-12 for 2 hours at room temperature in the dark. The worms were then transferred to seeded plates to allow stained bacteria to be purged from the gut. After 45 minutes, the animals were mounted on 2% agarose pads and immersed in levamisole 2mM. The quantitative analysis was performed using a Leica DM6000 fluorescence microscope, Leica DC 350 FX camera under the control of Leica LAS AF 6000 software. The estimation of apoptotic levels was calculated as the average number of apoptotic nuclei per arm screened for each genotype.

2.9 Statistical tools

Statistical analyses of DAPI stained bodies in diakinetic nuclei, apoptosis levels and RAD-51 foci patterns were computed through t-Student test for independent samples using the VassarStats software (http://faculty.vassar.edu/lowry/VassarStats.html). All DAPI stained bodies from single and double mutants represented two pools from which the relative two tails P value was estimated. Nuclei with a mis-shapen, unstructured chromatin were assumed to contain more than 17 fragments and were pooled in one category to which a value of 18 bodies was arbitrarily assigned for statistical analysis.

The level comparison of embryonic lethality of the different genotypes (*brc-1*, *ced-3*, *cep-1*, *brc-1;ced-3*, *brc-1;cep-1*) was obtained by GraphPad software on www.graphpad.com website. The unviable eggs and laid eggs from an equal number of single and double mutant hermaphrodites were summarized in a contingency table 2x2 and the P-value was computed by chi-square.

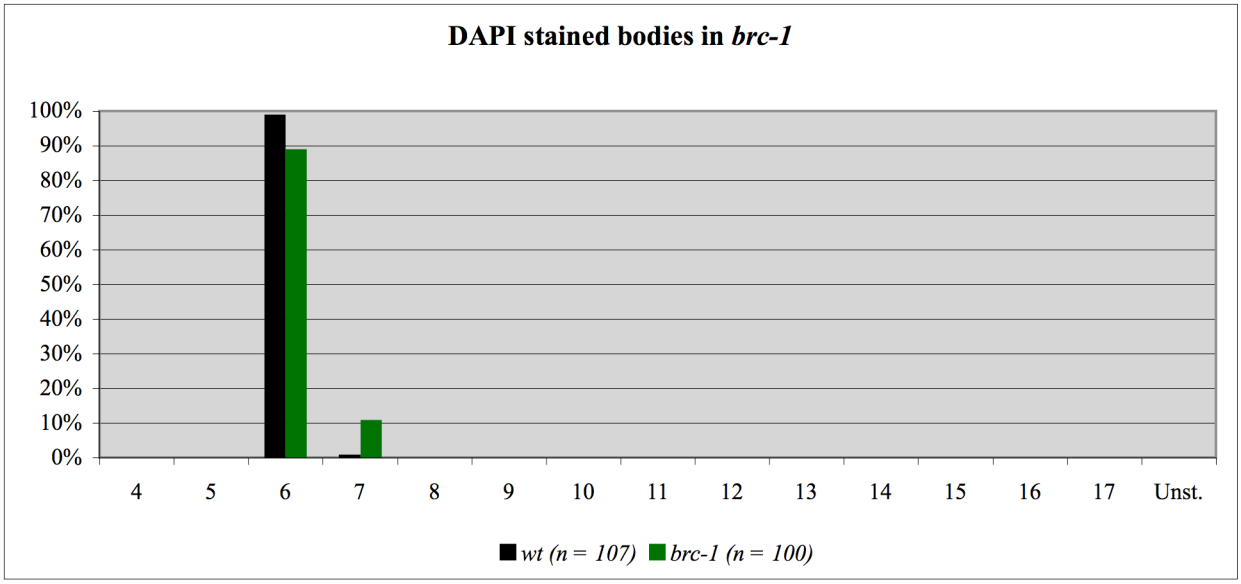
3. RESULTS

3.1 *brc-1* mutants

The structure of *C. elegans* is suitable for the study of the DSB Homologous Recombination Repair (HRR) mechanism, a molecular pathway that is strongly conserved in worms and mammals. Throughout the worm gonad, which is the main part of the worm's body, the programmed DSBs are processed at the same time as the chromosomes undergo structural changes: alignment, pairing, and synapsis. The staining of the gonad with 4, 6-diamidino-2-phenilindole (DAPI) allows the observation of meiotic nuclei in a spatial-temporal organization permitting a rapid cytological assessment of defects in subsequent steps of recombination repair and crossover formation.

In our assay**,** DAPI staining revealed no particular defects of the chromosomal organization along meiotic prophase I in *brc-1* mutants: alignment, pairing and synapsis between homologous chromosomes appeared normal, as reported in literature (Boulton et al., 2004). At the diakinesis phase, the *brc-1* mutants showed prevalently six normal DAPI stained bodies, representing bivalents joined together by chiasmata. However, 11% of oocytes at the diakinesis phase had seven DAPI-stained bodies, revealing a significant difference compared to wild type diakinesis (P-value < 0.0059) (Figure 3.1). We considered that this particular diakinesis pattern might be a consequence of defective chromosomal segregation. The Him phenotype (high incidence of males) is a typical meiotic phenotype in *C. elegans*, that reflects a defect during the segregation of the sexual chromosomes. A higher incidence of males (> 2%) appeared in *brc-1* mutant progeny than in wild type (0.1%), confirming our prediction from diakinesis data and data reported in literature (Boulton et al., 2004). If the defective segregation had affected also the autosomic chromosomes, the following aneuploidy would have led to increased levels of embryonic lethality in the next generation. In *brc-1*, the levels of embryonic lethality were not significantly increased (0.38%), suggesting that the defect of non-disjunction did not seem to affect the autosomic chromosomes during the meiosis. These data indicate that BRC-1 is dispensable for the crossover formation in meiosis in autosomes.

Figure 3.1 DAPI-stained bodies at diakinesis in *brc-1 mutant*



Statistical analysis of DAPI stained bodies in diakinesis nuclei	
brc-1 = wt	P = 0.0059

#Histogram representing quantification of the DAPI-stained bodies. The number (n) of observed nuclei is indicated next to each genotype. The y axis represents the percentage of nuclei in each class and the x axis indicates the number of DAPI-stained bodies; Unst, nuclei with unstructured chromatin; wt, wild type. Statistical analysis of DAPI stained bodies in diakinesis nuclei obtained by T-student test for independent samples.

3.2 *brc-1* apoptosis

Although the *brc-1* worms are viable and fertile, it was observed in the relevant work of Boulton (2004) that, in normal growth conditions, the *brc-1*(RNAi) depleted worms have an increase of the apoptotic levels in germ cell lines. Normally, a precise number of cells during development in *C. elegans* undergo a programmed apoptosis. Almost half of the germ line oocytes undergo apoptosis throughout the entire life of the worm, and a steady-state level of zero to four apoptotic cells can be observed at any given time. While for developmental apoptosis the number of cell deaths is fixed, the number of germ cell deaths can vary in relation to the activation of the DNA damage checkpoint. The germ line damage apoptotic program is activated at the end of the pachytene zone and is the only "apoptotic barrier" to DNA damage present from the mitotic tip to the end of the pachytene phase. The increase of germ line apoptosis in *brc-1* mutants might indicate an accumulation of DNA unresolved damage. We performed and standardized the apoptosis assay in the *brc-1* deletion mutant through syto-12 staining (Gumienny et al., 1999). We confirmed that, in normal growth

conditions, the *brc-1* mutant, like the *brc-1*(RNAi) interfered worm, has a level of apoptosis twofold the wild type level with an average of 7.71 per gonad arm compared to 3.96 in the wild type (Table 3.1).

In brc-1 mutants, does the increase of apoptosis depend on DNA damage?

Unresolved DNA damage should activate the *cep-1* dependent checkpoint pathway regulating apoptosis. CEP-1 is the only p53 family member encoded in the *C. elegans* genome. The *cep-1/p53* is not involved in the physiological germ cell death, but, like p53, regulates apoptosis in response to genotoxic stress in the germ line (Derry et al., 2001). If the *brc-1* mutation causes unresolved damage activating the DNA damage checkpoint and apoptosis, absence of *cep-1/p53* should carry apoptosis to basal levels. To understand if the increase of *brc-1* apoptosis is *cep-1/p53* dependent, *brc-1* males were crossed with *cep-1/p53* hermaphrodites and the F2 double mutants isolated (see Materials and Methods). We quantified and compared the levels of apoptosis in the germ line of the single mutant *cep-1/p53* and the double mutant *brc-1;cep-1/p53*. The *brc-1;cep-1/p53* double mutant showed a number of apoptotic corpses with an average of 3.58 per gonad arm, similar to a physiological level of the single mutant *cep-1/p53*, with an average of 3.07 (Table 3.1). In the absence of BRC-1, some DNA defects are not repaired and are recognized by this checkpoint system inducing the CEP-1/p53 dependent apoptosis.

Does the apoptotic increase depend on mitotic or meiotic defects?

We did not know if the DNA damage that we observed in the *brc-1* mutants comes from a pre-meiotic phase during the cellular divisions or depends on meiotic defects. If the damage has a pre-meiotic origin, the *brc-1* mutation will affect the resolution of stalled forks during DNA replication in cellular divisions. On the other hand, in meiosis, SPO-11 dependent cuts may be improperly repaired. To understand which molecular processes *brc-1* affects, we analysed the impact of the *brc-1* mutation in the *spo-11* genetic background through genetic crosses. SPO-11 is a conserved topoisomerase II like protein, responsible for double strand cuts in the first step of prophase I. *spo-11* deletion results in intact chromosomes, but the absence of DSBs abrogates crossover events, with a visible pattern of 12 univalents at the diakinesis phase (Dernburg et al., 1998). The subsequent aneuploidy due to mis-segregation causes high levels of embryonic lethality and a severe Him phenotype in the next generation (Dernburg et al., 1998). We considered that if the double mutant *brc-1;spo-11* shows a basal level of apoptosis, as the single mutant *spo-11* does, the SPO-11 dependent DSBs are not repaired in the *brc-1* mutant, inducing apoptosis. On the other hand, if the apoptotic levels maintain the same values, the damage inducing apoptosis will have a

pre-meiotic origin. Therefore, the two mutations, *brc-1* and *spo-11,* were combined by genetic cross. The double mutant *brc-1;spo-11* showed a normal apoptotic level: the *spo-11* mutation suppressed the *brc-1* apoptotic phenotype, reverting the apoptotic level to a wild type value of 3.66 per gonad arm (Table 3.1).

Table 3.1 Germline apoptosis and embryonic lethality in the *brc-1* mutant

	Apoptosis			Lethality	
	# gonad arms analyzed	apoptotic nuclei	average apoptotic nuclei/arm	# scored embryos	%Embryonic lethality
wt	85	337	3.96	1,977	<0.05
*brc-1**	73	563	7.71	1,048	0.38
spo-11	82	267	3.26	ND	ND*
brc-1; spo-11	65	238	3.66	ND	ND*
ced-3	64	6	0.09	1,240	1.94
brc-1; ced-3	83	7	0.08	1,369	9.72
cep-1/p53	89	273	3.07	1,032	0.19
brc-1; cep-1/p53	94	337	3.58	1,339	0.22

Statistical analyses of apoptotic levels by T-student	P value
brc-1 = wt	P < 0.0001
brc-1 = brc-1; cep-1/p53	P < 0.0001
cep-1/p53=brc-1	P < 0.0001
cep-1/p53= brc-1; cep-1/p53	P = 0.172908
spo-11=brc-1	P < 0.0001
spo-11= brc-1; spo-11	P = 0.37
brc-1= brc-1; spo-11	P < 0.0001

*# *brc-1 is statistically different from wild type, brc-1;spo-11 and brc-1;cep-1/p53 (Student's t-test; P-value <0.0001). spo-11 and brc-1;spo-11 embryonic lethality has not been scored as it is more than 99% due to progeny aneuploidy. ND, not determined. Statistical analyses of apoptotic levels are computed through t-Student test for independent samples.*

The increase of apoptosis in the *brc-1* mutant is directly correlated with the SPO-11 activity during prophase I. These data suggest an involvement of BRC-1 in the processing of SPO-11 dependent DSB repair during prophase I. Some of the SPO-11 cuts probably do not have a normal processing in the *brc-1* genetic background, and might represent damage incompatible with the survival of the next generation. In order to verify this hypothesis, we monitored the effects of the *brc-1* mutation, when apoptosis is totally abrogated. *ced-3* is a coding gene for a member of the caspase family and represents a key factor in programmed cell death: its activation promotes the cell disassembly process and nuclear DNA

fragmentation (WormBook). Through the genetic cross of the *brc-1* mutant with the *ced-3* null mutation worm (Yuan et al., 1993), we obtained the double mutant *brc-1;ced-3* where apoptosis was completely abrogated. The absence of apoptosis should lead to an accumulation of damage with the evident effects in the next progeny. As expected, the *brc-1;ced-3* double mutant shows a statistically significant fourfold increase in embryonic lethality (9.72%) compared to the single mutant *ced-3* (1.92%) (Table 3.1). The increase of embryonic lethality indicates that apoptosis, in *brc-1* mutants, eliminates the compromised oocytes that are incompatible with the production of viable offspring.

All these data indicate a possible role of BRC-1 after DSBs are induced by SPO-11 during early prophase I. Some DSBs, in the absence of BRC-1, cause deleterious consequences for the DNA that the CEP-1/p53 system recognizes as incompatible with the survival of offspring.

3.3 Increased RAD-51 foci in meiosis I in the *brc-1* mutant

Given that the increase of SPO-11 dependent apoptosis is due to the DNA damage checkpoint activation, BRC-1 would seem to perform a role, after the induction of SPO-11 dependent cuts, in the DSB meiotic repair mechanism. This hypothesis is strengthened by different works in which the participation of BRC-1 has been described in DNA repair after the induction of genotoxic stress (Polanowska et al., 2006).

Is Homologous Recombination perturbed in the brc-1 mutant?

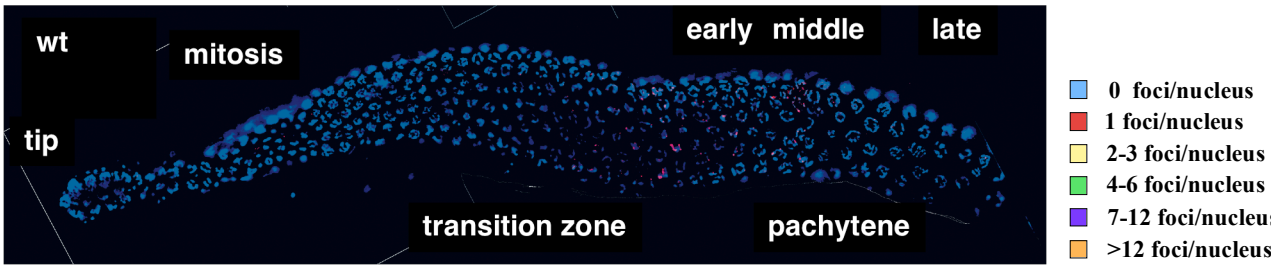
To monitor the meiotic progression of DSBs induced by SPO-11 and to better understand the possible role of *brc-1* in DSB repair through homologous repair, dissected gonads of *brc-1* hermaphrodites were stained with an antibody against RAD-51. RAD-51 is a member of the RecA-strand exchange protein family and catalyzes the invasion of DNA single strand overhangs into a recipient double strand of a homologous template. In the wild-type *C. elegans*, RAD-51 foci represent nascent meiotic homologous repair events; they arise during the zygotene stage, peak in abundance in early pachytene, and diminish in number during mid/late pachytene as meiotic DSB repair progresses (Figure 3.2 [A]) (Colaiacovo et al., 2003). The immunostaining of RAD-51, therefore, is used as a tool to monitor DSB repair kinetics. Several meiotic mutants show an abnormality in appearance and the persistence of RAD-51 foci: for instance, RAD-51 foci are missing or drastically reduced in *spo-11, mre-11* and *brc-2,* (Alpi et al., 2003; Martin et al., 2005), while they increase and persist nearly up to diplotene in *msh-5, syp-1* and *syp-2* (Colaiacovo et al., 2003). Thus, a defect in resolution of

double strand breaks in germ line cells is directly reflected in the pattern of RAD-51 immuno-staining. We quantified the RAD-51 foci along the worm germ line, dividing the germ line into 6 zones (tip, mitotic zone, transition zone, early pachytene, middle pachytene, and late pachytene), in accordance with their cytological features (Figure 3.2 [A]). Given that the values of apoptosis were increased, we expected an altered pattern of RAD-51 foci in the *brc-1* mutant in accordance with the unresolved damage.

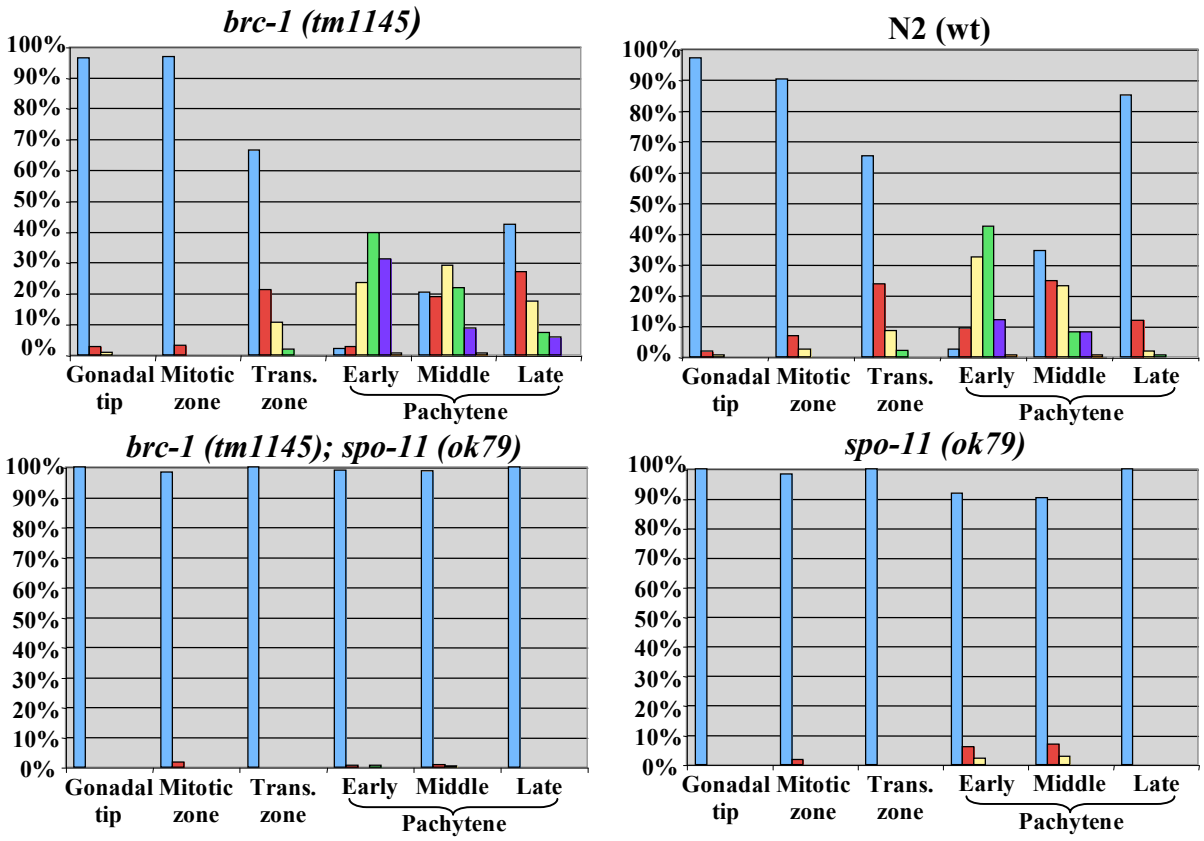
In figure 3.2 [B], the distribution and disappearance of RAD-51 foci on meiocyte nuclei along the germ cell line in the *brc-1* mutants is shown. The *brc-1* mutants did not exhibit elevated levels of spontaneous RAD-51 foci in the pre-meiotic zone, the only compartment in the adult animal where mitotic proliferation is active. In the early pachytene phase, instead, the *brc-1* mutants showed abnormally higher levels of RAD-51 foci compared to the wild type. The proportion of nuclei (30%) with 7-12 RAD-51 foci in the *brc-1* mutants was significantly different compared to the levels of the wild type. The differences in RAD-51 levels between the wild type worms and *brc-1* were statistically significant (P-value < 0.0001). It is not surprising that a similar increase of RAD-51 foci was also observed in the *brd-1* mutants (Adamo et al., 2008). BRD-1 is the heterodimeric partner of BRC-1, and co-localizes with BRC-1 and RAD-51 in DNA damage sites (Boulton et al., 2004; Polanowska et al., 2006). These data considered together strongly suggest that BRC-1/BRD-1 can have a function in DSB repair via homologous recombination during meiosis.

Figure 3.2 Increase of SPO-11 dependent RAD-51 foci in the *brc-1* mutant

A)



B)



Total number of nuclei scored per zone

C)

	Gonad tip	Mitotic zone	Trans. zone	Early pach.	Middle pach.	Late pach.
Wild-type	146	187	173	148	121	141
brc-1	106	126	113	141	137	137
spo-11	70	61	58	98	72	55
spo-11; brc-1	70	61	58	186	246	111

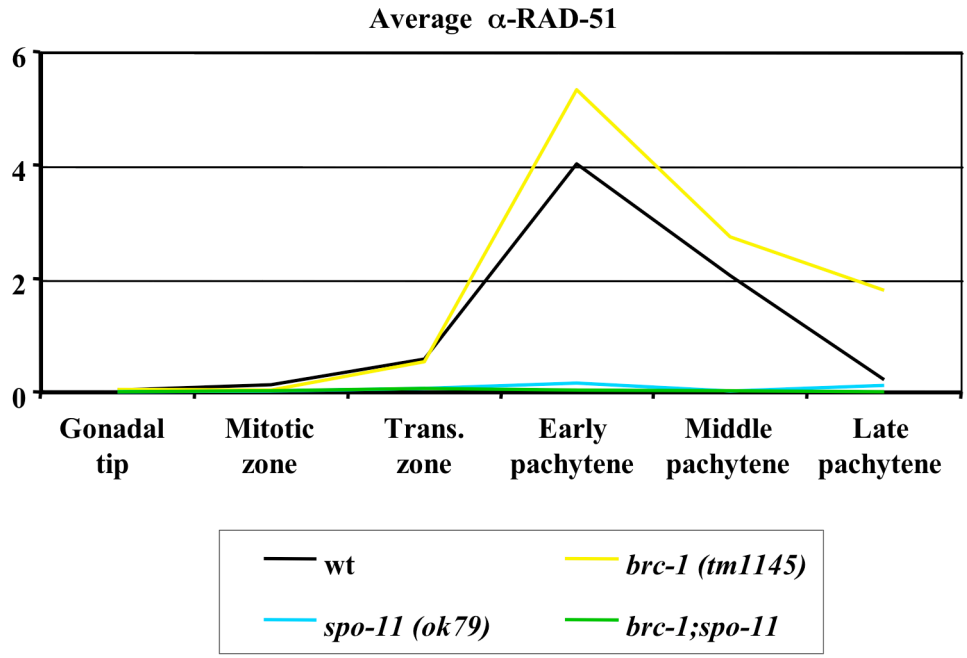
Student's test P value

	Gonad tip	Mitotic zone	Trans. zone	Early pach.	Middle pach.	Late pahy.	Pach (total)
N2 (wt)=*brc-1*	0.66	0.018	0.77	<0.0001	0.034	<0.0001	<0.0001
brc-1=brc-1;spo-11	1	0.31	1	0.024	0.028	1	0.09

#A)Representative image of a wild-type germline immunostained with anti-RAD-51 (red) and DNA counterstained with DAPI (blue). B)Histograms representing quantifications of RAD-51 foci in germlines of animals of the indicated genotypes. The y axis represents the percentage of nuclei with the number of foci indicated. The x axis represents the position (zone) along the germ-line. The tables indicate the number of nuclei scored per zone and statistical analysis using the Student's t-test. DAPI, 4,6-diamidino-2-phenylindole; wt, wild type.

If the averages of the number of RAD-51 foci per meiotic zone are plotted on a Cartesian graphic, it is possible to visualize better the kinetics of RAD-51 loading during the pachytene phase. In contrast to the abnormal increase of RAD-51 foci, the kinetics of RAD-51 loading at meiotic DSB sites appeared normal in the *brc-1* mutant compared to the wild type pattern (Figure 3.3). The *brc-1* mutation did not affect the loading or disassembly of RAD-51.

Figure 3.3 The kinetics of RAD-51 loading at meiotic DSB sites



#Plotting of the averages of RAD-51 foci per zone throughout germline of a wild-type, spo-11, brc-1 and the double mutant brc-1;spo-11. The y axis represents the average of RAD-51 foci per zone. The x axis represents the position (zone) along the germ-line.

To determine if ***the abundance of RAD-51 foci in the meiotic compartment of the brc-1 mutants is actually due to an additional amount of pre-meiotic damage added to the SPO-11 dependent cuts***, gonads of the double mutant *brc-1;spo-11* were immunostained with the RAD-51 antibody. The *spo-11* mutant does not have any cuts during meiosis (Dernburg et al., 1998) and consequently the homologous recombination machinery is not activated and the RAD-51 loading does not occur. In the absence of the SPO-11 cuts, the only damage, detected by RAD-51 immunostaining, may only be of pre-meiotic origin, suggesting that the altered RAD-51 pattern throughout the *brc-1* germ line may be the result of the sum of pre-meiotic and meiotic damage. As for the apoptosis, the abnormal pattern of RAD-51 foci in the *brc-1* mutant is SPO-11 dependent: the *brc-1;spo-11* double mutant showed no significantly different levels of RAD-51 foci compared to the levels of the *spo-11* single mutant (Figures

3.2 and 3.3). Thus, in *brc-1* mutants, the SPO-11 dependent DSBs are not normally processed, causing an increase of RAD-51 foci in the pachytene phase.

3.4 Frequency of recombination in the *brc-1* mutant

The abnormal increase of RAD-51 foci along the germ line for several meiotic mutants such as *him-14/MSH4*, *msh-5* and *syp-2* (Colaiacovo et al., 2003) directly reflects a defect in the resolution of double strand breaks on homologous chromosomes by the crossover pathway. The subsequent aneuploidy causes high levels of embryonic lethality in the next generation. In contrast to these mutants, the increase of RAD-51 foci in the *brc-1* germ line does not lead to an abnormal level of embryonic lethality. Given that *brc-1* is dispensable for the crossover formation, because at the diakinesis phase six normal bivalents occur, ***the next question was whether or not the RAD-51 pattern in the brc-1 mutant may reflect an up-regulation of the crossover pathway, increasing the frequency of crossover events.***

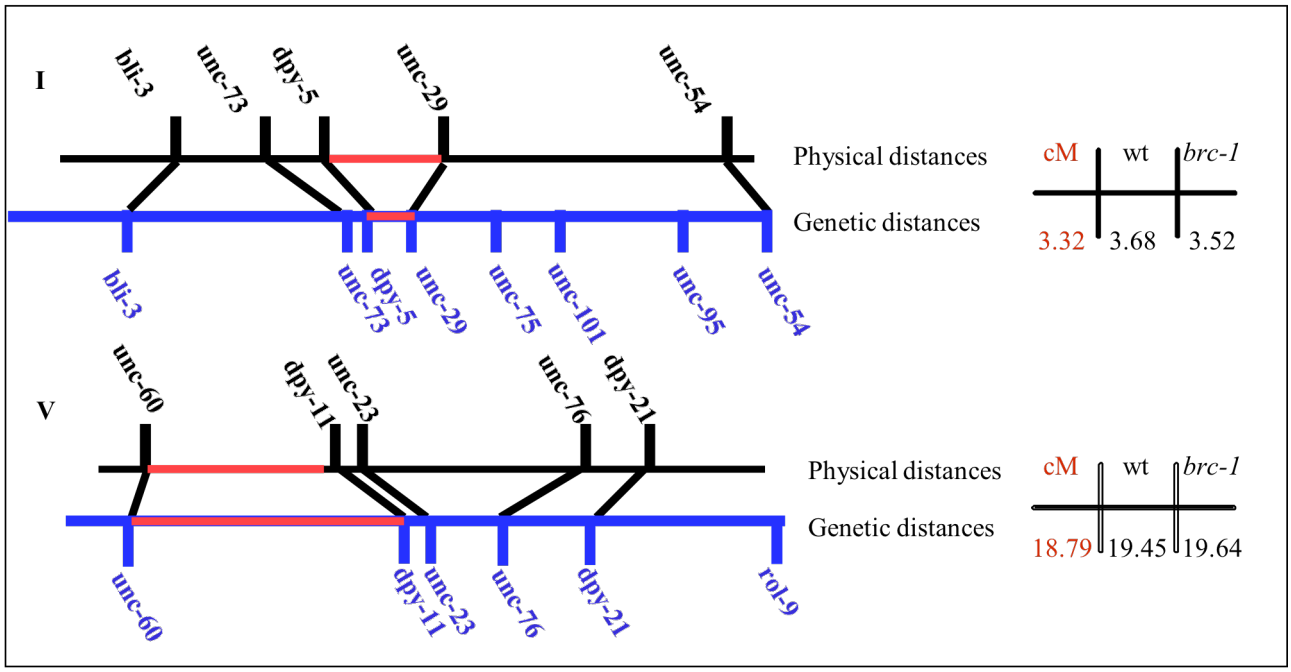
We answered this question by testing the frequency of recombination in the *brc-1* genetic background. In *C. elegans*, crossovers preferentially tend to accumulate in the chromosomal ends (Barnes et al., 1995; Brenner, 1974; Hillers and Villeneuve, 2003). We estimated the frequency of recombination on two different chromosomal intervals, one, *dpy-5 unc-29* in the centre of chromosome I, spanning a region with a low level of recombination, about 1cM/Mb, while the other, *unc-60 dpy-11* on chromosome V, where the frequency of recombination is higher, 3.7cM/Mb (Figure 3.4). We used these two different intervals to check whether the frequency and distribution of crossovers throughout the length of the chromosomes were altered (Figure 3.4). *dpy* (Dumpy) and *unc* (Uncoordinated) are two phenotypic markers, used as tools for standard genetic mapping: Dumpy is a short and fat phenotype while Uncoordinated, as the name indicates, is characterized by strong locomotion problems, a failure to move correctly.

From the genetic cross between *brc-1(tm1145)III; dpy-5(e61)unc-29(e403)I* and *brc-1(tm1145)III,* the F2 progeny was cloned and scored for Unc Dpy, wild-type, Unc non-Dpy, and Dpy non-Unc recombinant progeny. The genotype of F2 was derived from the F3 phenotypes. From a total of 936 chromosomes, we obtained 454 wild type and 449 Dpy Unc parental chromosomes, and 16 Dpy non-Unc and 17 Unc non-Dpy recombinant chromosomes. The same protocol was carried out for the second interval, starting from the

genetic cross between *brc-1(tm1145)III; unc-60(m35)dpy-11(e224)V* and *brc-1(tm1145)*III. In this case, from a total of 794 chromosomes analyzed, we obtained 311 wild type and 327 Unc Dpy parental chromosomes, and 81 Dpy non-Unc and 75 Unc non-Dpy recombinant chromosomes. In both intervals, the observed frequencies of recombination, 3.52% and 19.64% respectively (Figure 3.4), were not significantly distant from the frequencies observed in wild-type controls and from the map units reported previously in literature (Edgley & Riddle, 1993).

Therefore, the *brc-1* mutation does not influence either the frequency of recombination or the distribution of crossovers along the chromosomes. The increase of RAD-51 foci during the pachytene phase represents a problem in the DNA break repair mechanism. Given that BRC-1 is dispensable for the crossover formation, the difficult resolution of double strand breaks induced by SPO-11 may depend on a defective non-crossover pathway.

Figure 3.4 Recombination frequencies



 # Genetic and physical maps of C. elegans. The genetic map in brc-1 (tables on the right) is produced by scoring crossover frequencies in individuals heterozygous for pairs of cis linked markers on V and I chromosomes. The standard genetic map was obtained using a wild-type hermaphrodite and from literature data (Edgley & Riddle, 1993).

3.5 Role of *brc-1* in the absence of crossovers

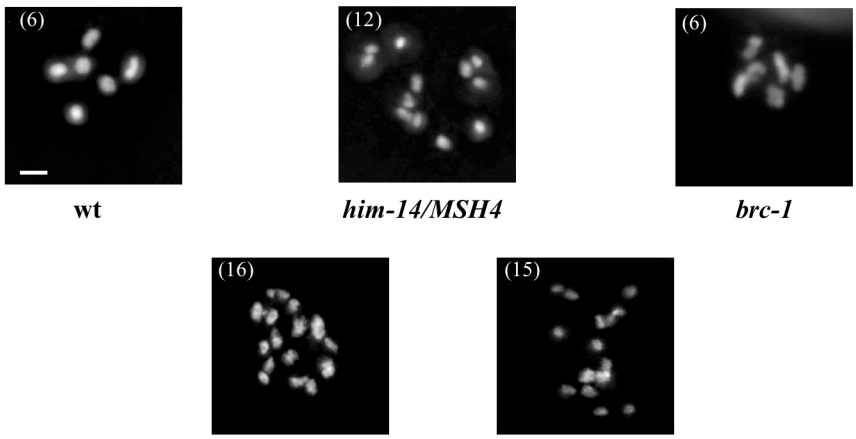
The recent work of Hayashi et al. (2007) has described a model in which "different repair mechanisms" are ordered in time and space throughout the worm germ line: the crossover repair should occur in the first steps of the pachytene phase, while the non-crossover pathway should be functional later on. Since only one of the SPO-11-dependent DSBs is repaired as a crossover (Barnes et al., 1995; Meneely et al., 2002; Hillers and Villeneuve, 2003), any additional DSBs must therefore be resolved by a non-crossover pathway, through repair on homologous chromosomes without chromosomal exchanges (gene-conversion) and/or repair on sister chromatids. Given that the *brc-1* mutation does not affect either the proper formation of chiasmata (showing six DAPI stained bodies in diakinesis nuclei), or the frequency of recombination, we wondered whether ***the increase of RAD-51 foci in the pachytene phase might depend on a defect in the non-crossover repair pathway***. To answer this question, the *brc-1* mutation was inserted in a genetic background where the crossover is abrogated in *him-14/MSH4* and in *syp-2* mutants. The double mutants should give two possible phenotypes at the diakinesis phase: 1) twelve proper chromosomes suggesting no role of *brc-1* in DNA repair pathways; 2) chromosomal defects such as chromosomal fragmentation highlighting an impaired non-crossover pathway.

3.5.1 *brc-1* combined with the *him-14/MSH4* mutation

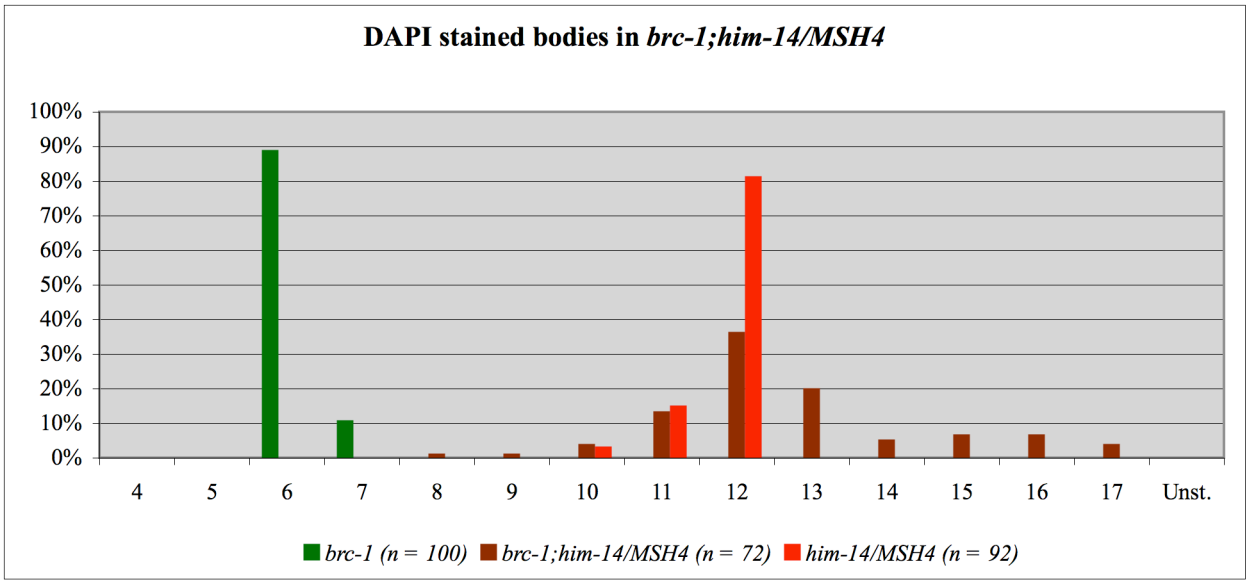
The preferential choice of homologous chromosomes, as templates for the repair of the SPO-11-dependent cuts, is coordinated by pro-crossover factors such as the heterodimer MSH-4/MSH-5 complex. This conserved complex is thought to be a key component of the recombination machinery, promoting crossover of the initial recombinant events. Mutants in *him-14/MSH-4* and *msh-5* genes exhibit a normal synaptonemal complex, but at the diakinesis phase show twelve DAPI stained achiasmatic chromosomes (Figure 3.5 [A]). However, the twelve DAPI stained bodies at the diakinesis phase correspond to twelve properly repaired univalents, since DSBs are processed in a RAD-51 dependent manner through a non-crossover pathway (Zalevsky et al. 1999, Rinaldo et al. 2002). To analyse the potential role of *brc-1* in non-crossover pathways, we examined the effect of the *brc-1* mutation in the *msh-4* genetic background.

Figure 3.5 Increase of DAPI-stained structures at the diakinesis phase in *brc-1;him-14/MSH4*

A)



B)



C) Statistical analysis of DAPI stained bodies in diakinesis nuclei

brc-1 = brc-1; him-14/MSH4	P < 0.0001
him-14/MSH4 = brc-1; him-14 /MSH4	P < 0.0001

 # A) Representative images of DAPI-stained oocyte nuclei at the diakinesis phase in the indicated double mutant genotypes. The number in parenthesis within each image indicates the number of DAPI-stained bodies that were detectable through the z stack of the nucleus. B) Histogram representing quantification of the DAPI-stained bodies. The number (n) of observed nuclei is indicated next to each genotype. The y axis represents the percentage of nuclei in each class and the x axis indicates the number of DAPI-stained bodies Scale bar, 2 μm. DAPI, 4,6-diamidino-2-phenylindole; Unst, nuclei with unstructured chromatin; wt, wild type. C) Statistical analysis of DAPI stained bodies in diakinesis nuclei obtained by T-student test for independent samples. The differences in DAPI-stained bodies between brc-1;him-14/MSH4 and him-14/MSH4, are statistically significant.

The *brc-1* males were crossed with the *him-14/MSH-4* hermaphrodites and the F2 double mutants were analyzed (see Materials and Methods). The DAPI staining of gonads did not reveal particular differences during the progression of meiosis between the *him-14/MSH-4* single mutant and the *brc-1;him-14/MSH-4* double mutant, except at the diakinesis phase. As shown in figure 3.5 [A, B], diakinesis nuclei in the *brc-1;him-14/MSH-4* double mutants had a significant increase of DAPI-stained bodies compared to *him-14/MSH-4* (P value < 0.0001). Almost 40% of diakinesis nuclei analyzed showed more than 12 DAPI stained bodies. The combination of the *brc-1* mutation with the *him-14/MSH-4* mutants might determine a chromosomal fragmentation, due to inefficient meiotic DSB repair in the absence of crossing-over.

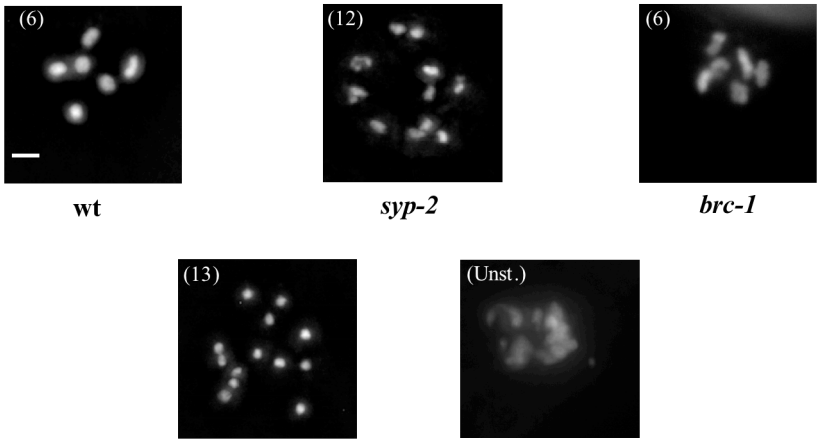
3.5.2 *brc-1* combined with the *syp-2* mutation

In contrast to the *him-14/MSH-4* or *msh-5* mutants, *syp-2* mutants are defective in SC formation. SYP-2 is a central component of the SC and is essential for the alignment of homologous chromosomes at the pachytene phase. However, chromosomes always undergo the action of SPO-11 inducing DSBs. These meiocytes exhibit persistent meiotic DSBs through the RAD-51 immunostaining, giving rise to twelve intact chromosomes lacking chiasmata at the diakinesis phase (Colaiacovo et al., 2003). The absence of the SC does not allow the repair of DSBs by inter-homologue recombination, and consequently the formation of crossover between homologous chromosomes is impaired. However, at the diakinesis phase, *syp-2* oocytes show 12 intact chromosomes (Figure 3.6 [A]), since they are repaired on the sister chromatid (Colaiacovo et al., 2003). We analyzed the effect of the *brc-1* mutation in the absence of the synaptonemal complex. We wondered whether the lack of BRC-1 may partially affect the *syp-2* diakinesis phenotype (i.e. the twelve intact univalents) and lead to defective repair on sister chromosomes, indicating a role of *brc-1* specifically in sister chromatid repair.

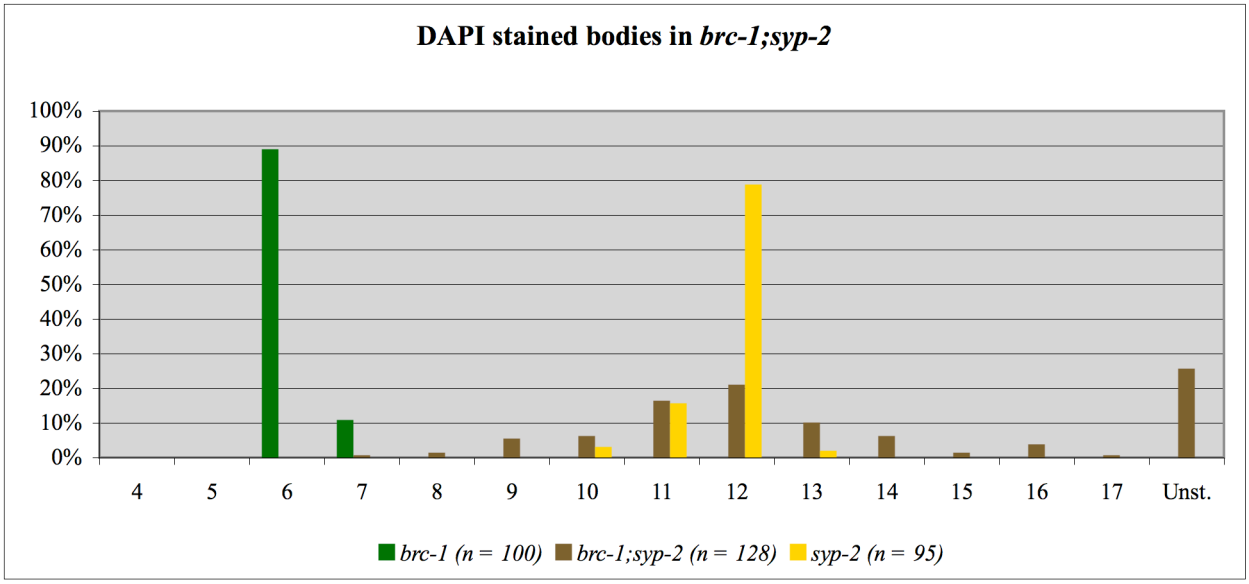
The *brc-1;syp-2* mutant revealed a significant increase in the number of DAPI-stained bodies at the diakinesis phase, compared to that observed in the *syp-2* single mutant (P value < 0.0001) (Figure 3.6 [A, B]). Almost 20% of diakinesis nuclei showed more than twelve spots. This phenotype is similar to that observed in the *brc-1;him-14/MSH-4* double mutants.

Figure 3.6 Increase of DAPI-stained structures at the diakinesis phase in *brc-1;syp-2*

A)



B)



C) **Statistical analysis of DAPI stained bodies in diakinesis nuclei**

brc-1 = brc-1; syp-2	P < 0.0001*
syp-2 = brc-1; syp-2	P < 0.0001*

 # A) Representative images of DAPI-stained oocyte nuclei at the diakinesis phase in the indicated double mutant genotypes. The number in parenthesis within each image indicates the number of DAPI-stained bodies that were detectable through the z stack of the nucleus. B) Histogram representing quantification of the DAPI-stained bodies. The number (n) of observed nuclei is indicated next to each genotype. The y axis represents the percentage of nuclei in each class and the x axis indicates the number of DAPI-stained bodies. Scale bar, 2 μm. DAPI, 4,6-diamidino-2-phenylindole; Unst, nuclei with unstructured chromatin; wt, wild type. C) *Statistical analysis of DAPI stained bodies in diakinesis nuclei obtained by T-student test for independent samples. *Nuclei with a misshapen, unstructured chromatin were assumed to contain more than 17 fragments and pooled in one category to which a value of 18 bodies was arbitrarily assigned for statistical analysis. The differences in DAPI-stained bodies between brc-1;syp-2 and syp-2, are statistically significant.*

Interestingly, in the *brc-1*;*syp-2* double mutants, but not in the *brc-1;him-14/MSH-4* double mutants, a large number of diakinesis nuclei (approximately one-quarter) showed a misshapen poorly condensed chromatin (Figure 3.6 [A, B]). This particular phenotype is similar to that observed in the *rad-51* or in the *brc-2* backgrounds in which the repair of all DSBs through the homologous recombination pathway is gravely compromised (Rinaldo et al., 2002; Martin et al., 2005). This does not occur in either *brc-1*;*him-14/msh-4* or *msh-5;rad-51*^{RNAi} backgrounds (Rinaldo et al., 2002). This observation suggests that the failure to repair DSBs leads to a major destabilization of chromosomes that is dependent on the HIM-14/MSH-4 - MSH-5 complex.

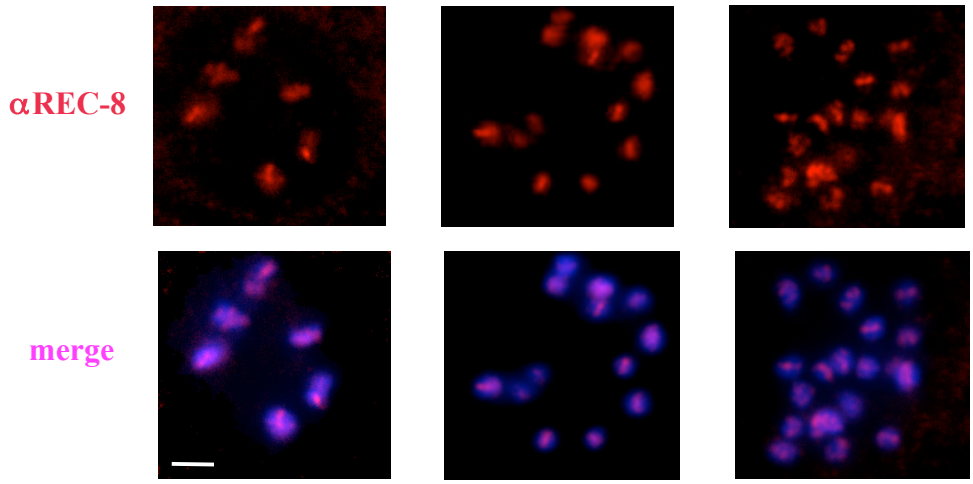
Since in the *syp-2* mutant the homologous repair uses the sister chromatids as default DNA templates and in both *brc-1;him-14/MSH4* and *brc-1;syp-2* double mutants the diakinesis nuclei show a similar increase of DAPI stained bodies, all these data taken together suggest that BRC-1 may be involved in homologous DSB repair using the sister chromatid as a template.

3.5.3 REC-8 immunostaining in *brc-1;him-14/msh-4* and *brc-1;syp-2* double mutants

One possible objection to this interpretation may be that this particular phenotype is caused by premature disassembling of the cohesin complex rather than by a defect of DNA DSB repair. After the DNA duplication, the sister chromatids are linked along their entire length through the interaction of the meiotic specific cohesin proteins, REC-8, SCC-3, SMC-1 and SMC-3. Their presence is essential to permit a correct disjunction of the homologous chromosomes in meiosis I and for the formation of the synaptonemal complex. The depletion of these proteins leads to aneuploidy, embryonic lethality and the Him phenotype (Pasierbek et al., 2001; Colaiacovo et al., 2003). In *rec-8* mutants, the meiotic cohesin complex is deficient and the sister chromatids are not held together, showing at the diakinesis phase 24 DAPI stained bodies, representing 24 individual sister chromatids (Pasierbek et al., 2001).

To distinguish a structural problem that may alter the cohesion between sister chromatids from a molecular defect in DSB repair, dissected gonads from both *brc-1;him-14/msh-4* and *brc-1;syp-2* double mutants were immunostained with an antibody against the REC-8 protein (Pasierbek et al., 2001). The absence of REC-8 from some diakinesis structures could explain the increased number of bodies in the double mutants.

Figure 3.7 REC-8 immunostaining in *brc-1; him-14/MSH4 and in brc-1; syp-2* at the diakinesis phase



 # Representative images of DAPI-stained (blue) brc-1;him-14/MSH4 and brc-1;syp-2 oocyte nuclei at the late diakinesis phase, immunostained with αREC-8 (red). Thirteen individual spots in brc-1; him-14/MSH4 and eighteen in brc-1; syp-2 can be individually distinguished, each staining with the meiosis specific cohesin. Scale bar, 2μm

In both double mutants, diakinetic nuclei showed the localization of the REC-8 protein on all DAPI stained bodies (Figure 3.7 where REC-8 is stained in red). The presence of REC-8 on all the stained bodies suggests that the cohesion between the sister chromatids is maintained.

4. Discussion

The meiosis I of *C. elegans* represents a good model to study the roles and the interactions of those proteins involved in the repair of DSBs. Recent evidence indicates that the meiotic DSBs are repaired through different DNA repair pathways depending on the temporal and space positions of the oocytes along the germ line of *C. elegans* (Hayashi et al., 2007). Only one DSB for each pair of homologous chromosomes is selected for repair through the crossover recombination, which ultimately produces chiasmata between homologous chromosomes. Given that the number of SPO-11 cuts exceeds the number of final crossovers, all extra cuts must be repaired using alternative pathways. It is assumed that in the presence of an intact synaptonemal complex and the HIM-14/MSH4-MSH5 complex, excess DSBs may be repaired as inter-homologue non-crossovers in the early prophase. Evidence suggests that the bias towards inter-homologue repair is released before the end of the pachytene phase to ensure that any remaining DSBs are repaired before the first meiotic division using the sister chromatid as a template. This is based on the observation that mutants, such as *syp-2,* where the synaptonemal complex is absent, show persistent meiotic DSBs that are ultimately repaired late in the meiotic prophase, giving rise to twelve intact univalents (Colaiacovo et al., 2003). The data presented in this thesis support a role for BRC-1 in meiotic DSB repair through inter-sister homologous recombination.

This is suggested by the fact that BRC-1 is dispensable for crossover formation, but *brc-1* mutants show abnormally high levels of apoptosis and meiotic RAD-51 foci, which are both SPO-11 dependent. In the absence of the synaptonemal complex (*syp-2* mutant) where inter-homologue recombination is eliminated, the repair of meiotic DSBs can only proceed by inter-sister recombination. The observation that *syp-2* mutants show 12 intact univalents, yet *brc-1;syp-2* double mutants show a large number of diakinetic nuclei either with fragmented chromosomes or with uncondensed, misshapen chromatins (similar to that previously observed in the HR-deficient mutants *rad-51* and *brc-2* [(Rinaldo et al., 2002; Martin et al., 2005), strongly suggests that BRC-1 is required for meiotic DSB repair by inter-sister recombination. Given the subtle meiotic phenotype of the *brc-1* single mutant, our data suggest that once the single obligate crossover per homologue pair is generated in *C. elegans*, most remaining meiotic DSBs are repaired by inter-homologue noncrossover recombination, rather than by inter-sister recombination. A specific role for BRC-1 in DSB repair using the sister chromatid as a template is consistent with the known function of BRCA1 in mitotic

cells, where the synaptonemal complex is absent and crossover-promoting genes are not expressed. Similar to its human counterpart, BRC-1 is recruited to sites of DSBs (Polanowska et al., 2006; Scully et al., 1997b), but how BRC-1/ BRCA1 promotes DSB repair in mitotic or meiotic cells remains unclear. A BRCA1 deficiency in both *C. elegans* and humans is associated with a DSB repair defect (Boulton et al., 2004; Moynahan, et al.,1999). However, one important difference is that BRC-1 is dispensable for RAD-51 recruitment to sites of DSBs in *C. elegans* (Polanowska et al., 2006). This difference, coupled with our present finding that BRC-1 is required for inter-sister recombination, raises the possibility that BRCA1 might have additional functions in DSB repair independent of its role in recruiting Rad51 to DSBs. As BRC-1/BRCA1 have an E3-ubiquitin ligase activity, which is stimulated by DSBs, identification of substrates for this activity might provide important insights into the control of DSB repair (Morris and Solomon, 2004; Polanowska et al., 2006). So far, CtIP and H2AX are the only definitive BRCA1 substrates identified in mitotic cells, whereas substrates in meiotic cells have yet to be found (Yu et al, 2006; Zhao et al, 2007). Recent studies have also revealed connections between BRCA1 and the Fanconi anaemia proteins during the DNA-damage response (for a review, Wang, 2007). It will be important to assess possible interactions between BRC-1 and CeFANCD2 during *C. elegans* meiosis (Collis et al., 2006). These studies might provide mechanistic insights into the function of BRCA1 and FANCD2 that could have important implications for the understanding of genome stability and tumorigenesis.

This work demonstrates, moreover, how a simple model system such as the nematode *Caenorhabditis elegans* can be usefully employed for the study of genes involved in cancer predisposition syndromes (such as breast cancer and Fanconi anaemia) to identify genetic interaction between repair genes and pathways involved in genome stability, and between repair genes and checkpoint/apoptosis genes (Adamo et al., 2008; Boulton, 2006; Collis et al., 2006; Polanowska et al., 2006). The availability of deletion mutants in all the relevant genes and the sophistication of techniques for the analysis of recombination intermediates, chromosome rearrangements, and apoptosis make the *C. elegans* gonad an ideal toolkit for such analyses.

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6. Published article

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BRC-1 acts in the inter-sister pathway of meiotic double-strand break repair

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The breast and ovarian cancer susceptibility protein BRCA1 is evolutionarily conserved and functions in DNA double-strand break (DSB) repair through homologous recombination, but its role in meiosis is poorly understood. By using genetic analysis, we investigated the role of the *Caenorhabditis elegans* BRCA1 orthologue (*brc-1*) during meiotic prophase. The null mutant in the *brc-1* gene is viable, fertile and shows the wild-type complement of six bivalents in most diakinetic nuclei, which is indicative of successful crossover recombination. However, *brc-1* mutants show an abnormal increase in apoptosis and RAD-51 foci at pachytene that are abolished by loss of *spo-11* function, suggesting a defect in meiosis rather than during premeiotic DNA replication. In genetic backgrounds in which chiasma formation is abrogated, such as *him-14/MSH4* and *syp-2*, loss of *brc-1* leads to chromosome fragmentation suggesting that *brc-1* is dispensable for crossing over but essential for DSB repair through inter-sister recombination.

Keywords: *Caenorhabditis elegans*; meiosis; DNA repair

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INTRODUCTION

DNA interruptions, including double-strand breaks (DSBs), are caused by endogenous (DNA synthesis, recombination) and exogenous (genotoxic agents) factors. In all organisms, imprecise repair of such discontinuities leads to an assortment of genetic alterations, such as point mutations, deletions and rearrangements. There are two main DSB repair pathways—homologous recombination (HR) and non-homologous end joining (NHEJ) (West, 2003; Wyman & Kanaar, 2006). Homologous recombination is an accurate repair pathway that uses an intact sister/homologue as a template, whereas NHEJ involves direct religation of the DSB and is prone to errors. Failure to repair DSBs accurately is associated with several cancer-predisposition syndromes, such as Fanconi anaemia (FANCD2), Bloom syndrome (BLM) or breast and ovarian cancer susceptibility (BRCA1, BRCA2/FANCD1), which are affected for DSB repair by homologous recombination (Venkitaraman, 2002; D'Andrea, 2003; Wu & Hickson, 2003; Mankouri & Hickson, 2004). As many of the genes in the homologous recombination pathway, including breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2/FANCD1* (Martin *et al*, 2005; Petalcorin *et al*, 2006), have been conserved during metazoan evolution, their roles and interactions can be investigated in model systems that are suited for the study of DSB repair and meiosis.

The U-shaped gonad of *Caenorhabditis elegans* contains germ cells, the physical location of which correlates with their stage in meiotic progression, which facilitates the study of the effect of gene depletions at various stages of meiotic prophase (for reviews, see Couteau *et al*, 2004; Colaiacovo, 2006; Garcia-Muse & Boulton, 2007). During meiotic prophase, DSBs are induced by the conserved meiotic protein SPO-11 (Keeney *et al*, 1997; Dernburg *et al*, 1998). The repair of at least one meiotic DSB to generate a crossover between each pair of homologous chromosomes is essential for accurate chromosome segregation at the first meiotic division (Brenner, 1974; Hawley, 1988; Hillers & Villeneuve, 2003). Failure to generate the obligate crossover results in chromosome non-disjunction and aneuploidy in the next generation. Mutants affecting homologous recombination repair of meiotic DSBs show increased embryonic lethality owing to aneuploidy, high incidence of males owing to X-chromosome non-disjunction, anomalies in the number and shape of chromosomes at diakinesis, and altered levels and distribution of RAD-51 foci at the pachytene stage.

A battery of mitotically silent genes, including *him-14/MSH4*, *msh-5*, *syp-1*, *syp-2* and *him-3*, have been described in *C. elegans* that are involved in directing recombination between homologous chromosomes during meiosis (Zalevsky *et al*, 1999; Zetka *et al*, 1999; Kelly *et al*, 2000; MacQueen *et al*, 2002; Colaiacovo *et al*,

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2003). Null mutants in these genes show 12 well-structured univalents at diakinesis instead of the 6 bivalents in wild-type (representing homologous chromosomes held together by chiasmata). In these mutants, meiotic DSBs are believed to be repaired using non-crossover pathways, but the factors that promote this type of homologous recombination repair are not known (Colaiacovo *et al*, 2003).

Studies in mammalian cells have established a role for BRCA1 and its heterodimeric partner BARD1 in DSB repair by homologous recombination (Scully *et al*, 1997; Moynahan *et al*, 1999). A role for BRCA1 in meiosis is also suggested from knockout mice that are infertile as a result of pachytene arrest (Xu *et al*, 2003). Recent studies have shown that BRCA1 has a specialized meiotic role in the XY body that is maintained in a transcriptionally silent state through meiotic sex chromosome inactivation (MSCI; Turner *et al*, 2004). BRCA1 is required for the localization of ATR and subsequent phosphorylation of H2AX at the XY body, which is important for the establishment of MSCI (Turner *et al*, 2004). It is clear that BRCA1 functions in both mitotic and meiotic cells, but its contribution to meiotic DSB repair is less well understood. Here, we investigate the contribution of BRCA1 of *C. elegans* (*brc-1*) during meiotic recombination by exploiting specific aspects of *C. elegans* meiosis: meiotic DSBs induced by SPO-11 are not a prerequisite for the assembly of the synaptonemal complex (Dernburg *et al*, 1998), and axis morphogenesis and synaptonemal complex formation are not required for loading RAD-51 onto DSBs (Colaiacovo *et al*, 2003). Our data indicate that BRC-1 contributes to meiotic DSB repair by a non-crossover pathway.

RESULTS

Apoptosis and RAD-51 foci increase in the *brc-1* mutant

Previous studies have shown that the *brc-1* mutant is viable and fertile, but shows a weak meiotic phenotype: the incidence of males (Him phenotype), reflecting the frequency of X-chromosome non-disjunction in *C. elegans* meiosis, is elevated in the *brc-1* mutant (~2% versus ~0.1% in wild type; Boulton *et al*, 2004). However, the levels of embryonic lethality (0.38%) are not significantly increased in the absence of *brc-1*, indicating that the segregation of autosomes during meiosis occurs as normal. Most of the diakinetic nuclei in *brc-1* mutants show the wild-type complement of six 4,6-diamidino-2-phenylindole (DAPI)-stained bivalents at diakinesis, representing homologous chromosomes joined by chiasmata (Fig 1). However, approximately 12% of oocyte nuclei show seven DAPI-stained bodies, which might account for the Him phenotype. Thus, *brc-1* is largely dispensable for crossover recombination on the autosomes.

A possible meiotic defect is also suggested by findings that *brc-1* mutants show elevated basal levels of apoptosis of pachytene nuclei (Boulton *et al*, 2004). To determine whether this apoptotic phenotype is dependent on meiotic DSB formation, we generated a *brc-1;spo-11* double mutant. Eliminating meiotic DSB formation with the *spo-11* mutation (Dernburg *et al*, 1998) suppressed the apoptotic phenotype of *brc-1* mutants (Table 1). To examine the consequence of eliminating apoptosis in the absence of *brc-1*, we combined *brc-1* with a mutation in *ced-3*, which is essential for apoptosis (Yuan *et al*, 1993). A fourfold elevation in embryonic lethality was observed in the *brc-1;ced-3* double mutant when compared with the single mutants alone (Table 1), suggesting that in the absence of BRC-1, apoptosis is required to eliminate compromised meiotic cells that are incompatible with producing viable offspring.

To analyse further a potential meiotic DSB repair defect in *brc-1* mutants, we examined RAD-51 foci, which mark homologous recombination events (Alpi *et al*, 2003; Colaiacovo *et al*, 2003). The loading of RAD-51 at meiotic DSBs in the early stages of meiotic prophase occurs as normal in *brc-1* mutants. However, *brc-1* mutants show abnormally high levels of RAD-51 foci in early pachytene nuclei, a phenotype that is suppressed by the *spo-11* mutation (Fig 2). A similar elevation in RAD-51 foci is also seen in the meiotic nuclei of *brd-1* mutants, which lack the heterodimeric partner protein of BRC-1 (Fig 2; Boulton *et al*, 2004; Polanowska *et al*, 2006). These data indicate a role for *brc-1* in the efficient repair of a subset of SPO-11-dependent meiotic DSBs.

BRC-1 acts in DSB repair in the absence of crossovers

In *C. elegans*, one crossover per homologue pair is necessary and sufficient to generate interhomologue connections irrespective of the length of the chromosome (Hawley, 1988; Edgley & Riddle, 1993; Hillers & Villeneuve, 2003), therefore, it follows that additional DSBs generated by SPO-11 must be repaired by non-crossover pathways. The bias towards the homologue as a homologous recombination template is regulated by pro-crossover factors such as the HIM-14/MSH4–MSH-5 complex and the synaptonemal complex. Mutants in either of these complexes are achiasmate and show 12 intact univalents at diakinesis (Zalevsky *et al*, 1999; MacQueen *et al*, 2002). To analyse the potential role of *brc-1* in non-crossover DSB repair pathways, we examined the effects of combining the *brc-1* mutant with (i) the *him-14/MSH4* mutant, in which the synaptonemal complex is present, but crossing over is abolished, and (ii) the *syp-2* mutant, in which both synaptonemal complex formation and crossing over are absent (Fig 1). Combining the *brc-1* mutation with either *him-14/MSH4* or *syp-2* mutants resulted in a statistically significant increase in DAPI-stained bodies at diakinesis, which is indicative of chromosome fragmentation (Fig 1; supplementary Table A online). Unlike the *brc-1;him-14/MSH4* double mutant, approximately 25% of the observed *brc-1;syp-2* nuclei at diakinesis resolve in misshapen, poorly condensed chromatin (Fig 1). This phenotype is similar to that observed in *brc-2*- and *rad-51*-deficient strains, which are compromised for repair of all meiotic DSBs through the homologous recombination pathway (Rinaldo *et al*, 2002). These data indicate that in the absence of crossing over, BRC-1 contributes to meiotic DSB repair by using the sister chromatid as a template.

It is possible that the fragmentation observed in *brc-1;him-14/msh-4* and *brc-1;syp-2* double mutants might result from premature loss of chromosome cohesion or from a DSB repair defect. To distinguish between these two possibilities, *brc-1;him-14/MSH4* and *brc-1;syp-2* diakinetic nuclei were immunostained for the meiosis-specific cohesin REC-8 (Pasierbek *et al*, 2001). In both double mutants, DAPI-stained bodies retained REC-8, indicating that chromosome fragmentation arises as a result of a repair defect rather than the premature loss of cohesion (supplementary Fig A online).

If a non-crossover pathway of homologous recombination is impaired in the absence of *brc-1*, this defect might be compensated by an elevation in the frequency of crossovers. Therefore, we assayed the frequency of recombination in the *brc-1* mutant in two different chromosomal intervals: *dpy-5 unc-29* on

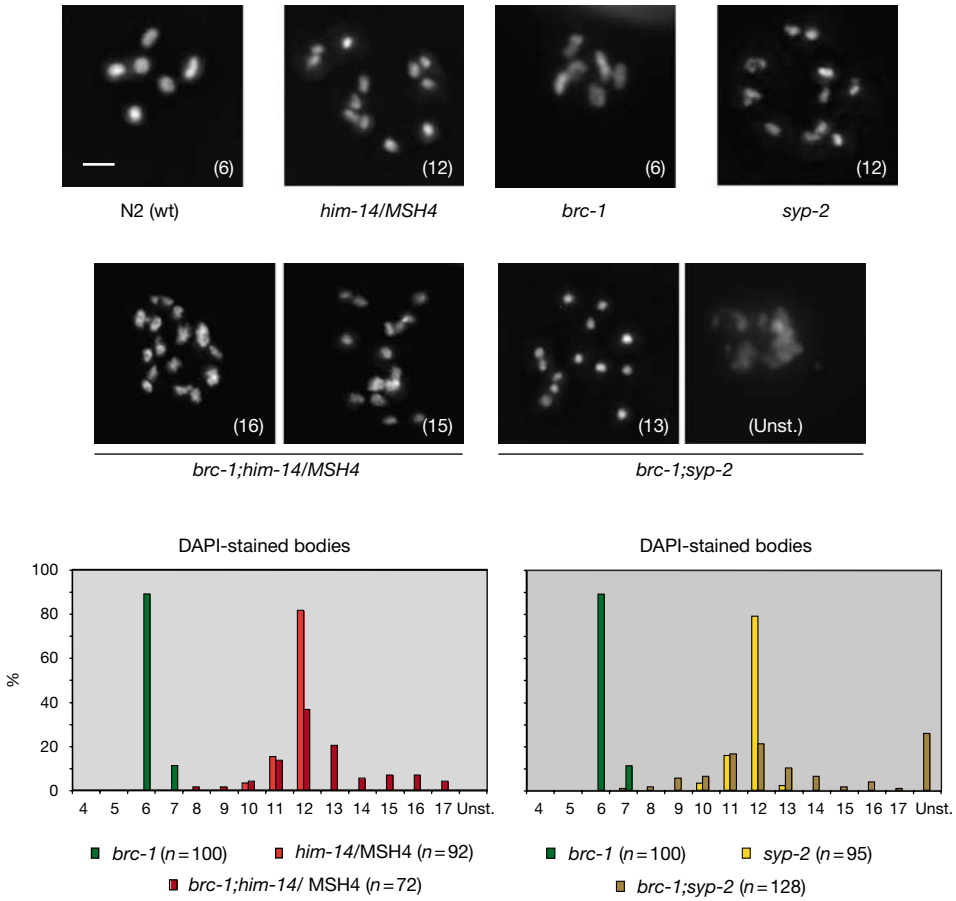


Fig 1 | Increased number of DAPI-stained structures at diakinesis in *brc-1*;*him-14/MSH4* and in *brc-1*;*syp-2* double mutants. Representative images of DAPI-stained oocyte nuclei at diakinesis in the indicated genotypes double mutants. The number in parenthesis within each image indicates the number of DAPI-stained bodies that were detectable through the z stack of the nucleus. Histograms represent quantification of the DAPI-stained bodies. The number (*n*) of observed nuclei is indicated next to each genotype. The *y* axis represents the percentage of nuclei in each class and the *x* axis indicates the number of DAPI-stained bodies. The difference in DAPI-stained bodies between *brc-1*;*him-14/MSH4* and *him-14/MSH4*, and between *brc-1*;*syp-2* and *syp-2* are statistically significant, (see supplementary Table A online). Scale bar, 2 μm. DAPI, 4,6-diamidino-2-phenylindole; Unst, nuclei with unstructured chromatin; wt, wild type.

chromosome I, which spans a region with a low level of recombination (about 1 cM/Mb), and *unc-60 dpy-11* on chromosome V, where the frequency of recombination is higher (3.7 cM/Mb). In both intervals tested, the frequency of recombination (3.52% and 19.64%, respectively) was comparable with that observed in the wild type and with the map units reported previously (Edgley & Riddle, 1993). Therefore, *brc-1* does not significantly influence either recombination frequency or crossover distribution.

DSBs can be repaired by homologous recombination or by NHEJ. NHEJ is generally believed to act in somatic cells before S phase, but might contribute to meiotic DSB repair when homologous recombination is compromised. To assess the latter, we compared the meiotic outcome of *him-14/MSH4*;*lig-4* (*C. elegans* DNA ligase-4; Martin *et al*, 2005) and *brc-1*;*him-14/MSH4* double mutants. In contrast to *brc-1*;*him-14/MSH4*, chromosome fragmentation was not observed in *him-14/MSH4*;*lig-4* double mutants. Rather, the diakinetic nuclei in *him-14/MSH4*;*lig-4* showed a pattern similar to that of *him-14/MSH4* single mutants (supplementary Fig B online). These data indicate that *brc-1* and *lig-4* function in different DSB repair pathways. Furthermore, NHEJ has little or no role in meiotic DSB repair in wild-type *C. elegans*.

Collectively, our results are consistent with a role for BRC-1 in a non-crossover homologous recombination pathway of meiotic DSB repair through inter-sister recombination.

DISCUSSION

Recent evidence indicates that germ cells switch between specific DSB repair pathways depending on their temporal and spatial position in the *C. elegans* germ line (Hayashi *et al*, 2007). In the case of meiotic DSB repair, a subset of DSBs per nucleus are selected for repair through crossover recombination, which ultimately produce chiasmata between homologous chromosomes. As the number of SPO-11-induced DSBs created at the onset of meiotic prophase exceed the number of final crossovers, these extra breaks must be repaired using alternative pathways. It is assumed that in the presence of an intact synaptonemal

Table 1 | Germline apoptosis and embryonic lethality in the *brc-1* mutant

	Apoptosis			Lethality	
	No. of gonad arms analysed	Apoptotic nuclei	Average apoptotic nuclei/arm	No. of embryos scored	Embryonic lethality (%)
Wild type	85	337	3.96	1,977	<0.05
brc-1[*]	73	563	7.71	1,048	0.38
spo-11	82	267	3.26	ND	ND[†]
brc-1;spo-11	65	238	3.66	ND	ND[†]
cep-1/p53	89	273	3.07	1,032	0.19
brc-1;cep-1/p53	94	337	3.58	1,339	0.22
ced-3	64	6	0.09	1,240	1.94
brc-1;ced-3	83	7	0.08	1,369	9.72

[*]*brc-1* is statistically different from wild type, *brc-1;spo-11* and *brc-1;cep-1/p53* (Student's *t*-test; *P*-value <0.0001). [†]*spo-11* and *brc-1;spo-11* embryonic lethality has not been scored as is more than 99% due to progeny aneuploidy. ND, not determined.

complex and the HIM-14/MSH4-MSH5 complex, excess DSBs are repaired as inter-homologue non-crossovers. Evidence suggests that the bias towards inter-homologue repair is released before the end of pachytene, to ensure that any remaining DSBs are repaired before the first meiotic division. This is based on the observation that mutants that lack the synaptonemal complex, for example, *syp-2* mutants, show persistent meiotic DSBs that are ultimately repaired late in meiotic prophase in a *rad-51*- and *rec-8*-dependent manner, giving rising to 12 intact univalents (Colaiacovo *et al*, 2003).

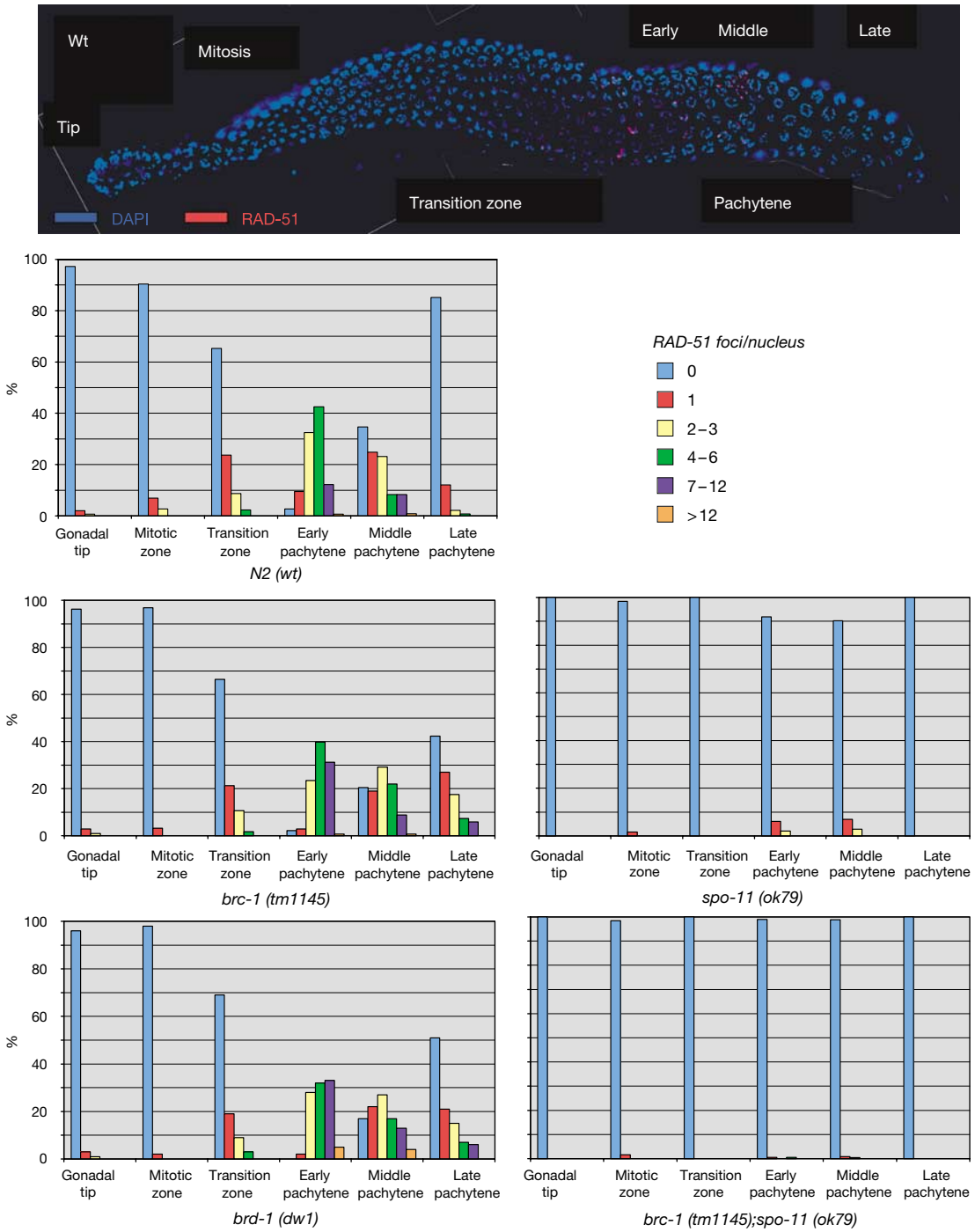
The data presented here support a role for BRC-1 in meiotic DSB repair through inter-sister homologous recombination. This is suggested by the fact that BRC-1 is dispensable for crossover formation, but *brc-1* mutants show abnormally high levels of apoptosis and meiotic RAD-51 foci, which are both SPO-11 dependent. In the absence of the synaptonemal complex (*syp-2* mutant) where inter-homologue recombination is eliminated, repair of meiotic DSBs can only proceed by inter-sister recombination. The observation that *syp-2* mutants show 12 intact univalents, yet *brc-1;syp-2* double mutants show a large number of diakinetic nuclei with uncondensed, misshapen chromatin, similar to that previously observed in the HR-deficient mutants *rad-51* and *brc-2* (Chin & Villeneuve, 2001; Rinaldo *et al*, 2002; Colaiacovo *et al*, 2003; Martin *et al*, 2005), strongly suggests that BRC-1 is required for meiotic DSB repair by inter-sister recombination. Given the subtle meiotic phenotype of the *brc-1* single mutant, our data suggest that once the single obligate crossover per homologue pair is generated in *C. elegans*, most remaining meiotic DSBs are repaired by inter-homologue non-crossover recombination, rather than by inter-sister recombination. A specific role for BRC-1 in DSB repair using the sister chromatid as a template is consistent with the known function of BRCA1 in mitotic cells, where the synaptonemal complex is absent and crossover-promoting genes are not expressed.

Similar to its human counterpart, BRC-1 is recruited to sites of DSBs (Scully *et al*, 1997; Polanowska *et al*, 2006), but how BRC-1/BRCA1 promotes DSB repair in mitotic or meiotic cells remains unclear. BRCA1 deficiency in both *C. elegans* and humans is associated with a DSB repair defect (Moynahan *et al*, 1999; Bhattacharyya *et al*, 2000; Boulton *et al*, 2004). However, one important difference is that BRC-1 is dispensable for RAD-51 recruitment to sites of DSBs (Polanowska *et al*, 2006). This difference, coupled with our present finding that BRC-1 is required for inter-sister recombination, raises the possibility that BRCA1 might have additional functions in DSB repair independent of its role in recruiting Rad51 to DSBs. As BRC-1/BRCA1 have E3-ubiquitin ligase activity, which is stimulated by DSBs, identification of substrates for this activity might provide important insights into the control of DSB repair (Morris & Solomon, 2004; Polanowska *et al*, 2006). So far, CtIP and H2AX are the only definitive BRCA1 substrates identified in mitotic cells, whereas substrates in meiotic cells have yet to be found (Yu *et al*, 2006; Zhao *et al*, 2007). Recent studies have also revealed connections between BRCA1 and the Fanconi anaemia proteins during the DNA-damage response (Wang, 2007). It will be important to assess possible interactions between BRC-1 and CeFANCD2 during *C. elegans* meiosis (Collis *et al*, 2006). These studies might provide mechanistic insight into the function of BRCA1 and FANCD2 that could have important implications for the understanding of genome stability and tumorigenesis.

METHODS

DAPI analysis and immunostaining. DAPI-staining, immunostaining and analysis of meiotic nuclei were carried out as described by Colaiacovo *et al* (2003). Quantitative analysis of RAD-51 foci was carried out on *z* series of images acquired using a Leica DM5000 fluorescence microscope, Leica DC 350 FX camera under the control of Leica LAS AF 6000 software. Optical sections were collected at 0.25 μm increments. Quantitative analysis of DAPI-staining bodies in diakinetic nuclei was carried out on *z* series of images (optical sections 0.50 μm increments). The numbers of nuclei scored for genotype are indicated in Fig 1 next to the coloured bar codes.

Apoptosis assay. Germline apoptosis was visualized with SYTO12 (Invitrogen, Molecular Probes, San Giuliano Milanese, Italy) as described by Gumienny *et al* (1999).



	Student's *t*-test *P*-value						
	Gonadal tip	Mitotic zone	Transition zone	Early pachytene	Middle pachytene	Late pachytene	Pachytene (total)
N2 (wt)=*brc-1*	0.66	0.018	0.77	<0.0001	0.034	<0.0001	<0.0001
brc-1;spo-11=spo-11	1	0.31	1	0.024	0.028	1	0.09

Fig 2 | *brc-1* mutants show *spo-11*-dependent elevation of RAD-51 foci. Representative image of a wild-type germline immunostained with anti-RAD-51 (red) and DNA counterstained with DAPI (blue). Histograms represent quantification of RAD-51 foci in germlines of animals of the indicated genotypes. The *y* axis represents the percentage of nuclei with the indicated number of foci. The *x* axis represents the position (zone) along the germline. Statistical analysis was carried out using the Student's *t*-test. DAPI, 4,6-diamidino-2-phenylindole; wt, wild type.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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