Università degli Studi di Napoli
"FEDERICO II"

DOTTORATO DI RICERCA IN GENETICA MOLECOLARE
XXII° CICLO

"Functional characterization of the Caenorhabditis elegans gene fcd-2 during meiosis and in maintenance of genomic stability"

Autore

Nicola Silva

Docente Guida
Prof.ssa Girolama La Mantia

Coordinatore:
Prof. Roberto Di Lauro

Tutor:
Dr. Adriana La Volpe

ANNO ACCADEMICO 2009/2010

- *Index* –

1. INTRODUCTION

1.1. Genomic Stability and Caenorhabditis elegans as model system

Living organisms spend their existence in environments that can be sometimes a source of deleterious stimuli, as their cells and tissues can be exposed to the action of a plethora of dangerous agents that can eventually compromise the integrity of the genetic information enveloped within the chromosomes. In order to preserve the integrity of their genetic heritage, all the living organisms have developed, throughout evolution, biological systems in charge to keep safe their genetic information and in order to correctly transmit it to the following generations: unresolved DNA damages in fact, can lead to catastrophic consequences for the cells, the organism and the species. Despite this permanent aggression, DNA remains a stable carrier of genetic information. Many processes exert their action in order to preserve genomic integrity, which comes out from a finely and orchestrated interaction among DNA replication and repair mechanisms, cell cycle progression, chromosomes segregation and cell death. Among these DNA repair mechanisms, Homologous Recombination (HR) is the most conservative pathway of DNA repair, it is conserved during evolution, guaranteeing the faithful transmission of genetic information, and it is responsible for the generation of genetic variability.

Some genes that are involved in DNA damage repair in eukaryotes with a sexual cycle, also function in meiotic recombination. Many of these genes are conserved during evolution, such as the genes implicated in the Double Strand Breaks (DSBs) repair via Homologous Recombination. While double strand breaks represent the result of an insult onto DNA and for this reason must be necessarily repaired, they also are the key for the triggering of meiotic recombination events. 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. 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. Metazoa and plants have evolved the formation of specific tissues in order to perform meiosis, switching by a situation in which meiosis is an induced response to the absence of nutriments (as in yeast), to a specialized process achieved by specialized cells (germ cells). A metazoan model system such as *Caenorhabditis elegans*, offers the advantage of studying the cross-talking between damage checkpoints, DNA repair and apoptosis and allows the observation of the effects of mutations in a number of genes in various combinations, in the context of an entire organism, and during the development. The unique organization of meiotic nuclei in the gonads permits a rapid cytological assessment of defects in chromosome pairing, DNA repair, crossovers formation and apoptosis.

1.2. Double Strand Breaks

Double Strand Breaks (DSB) on DNA are one of the worst kinds of damages that can be caused to DNA. DSBs have been shown to be at the origin of chromosome breakages, deletions, rearrangements, translocations and inversions (for review, van Gent et al., 2001). DSBs can be generated either during physiological processes such as meiosis and DNA replication, or they can be caused by exogenous insults to DNA. Among the several DNA-damaging agents responsible of DSBs generation, ionising radiation, radiomimetic chemicals, and a number of anti-cancer drugs (e.g. bleomycin, camptotecyn, cisplatin, etc.) can be included. Some of these chemical treatments lead to the cross-linking of the two complementary DNA strands (ICL, interstrand cross-links). ICLs prevent replication as well as

transcription, precluding the use of information encoded by the complementary strand for repair. ICLs when occurring during replication in S-phase, lead to collapse of replication forks. These DNA perturbations must be recognized and resolved: 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.
However, DSBs can also be the result of a physiological program: specific DSBs are endogenically produced during meiotic recombination, in order to generate crossovers that will confer, through chiasmata formation, the required physical connection among homologs and allow the correct segregation of the chromosomes at anaphase I.Furthermore, DSBs are also generated in the context of other biological phenomena, such as at stalled replication forks, integration of retroviruses, mobile element transposition and others.

1.3. Double Strand Breaks repair mechanisms

Two major pathways have maintained the role of assuring the genome integrity: the *Non-Homologous End Joining, NHEJ* (also named *DNA End-Joining pathway*), and the *Homologous Recombination Repair* (*HRR*) (Figure 1.1). These two pathways are not stochastically used within cells, as their activation varies during the development and it also depends on the stage of the cell cycle. After DNA replication, HR is preferentially used as a DSB repair mechanism, since the intact sister chromatid is available as a donor of homologous sequences to the region containing the broken DNA. The NHEJ instead operates during the pre-replicative stages of cell cycle (G0, G1), in which, under physiological conditions, the number of DSBs is extremely rare. 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; therefore Homologous

Recombination Repair represents the repair system that is principally activated during the cell cycle.

DNA damages can also trigger the activation of *checkpoints*, which temporary block the cell cycle progression in order to allow the DNA repair machinery to resolve the damage. Once the damage has been resolved, the cell cycle can proceed again; if, instead, the damage cannot be repaired a death signal is activated, leading the cell to die by apoptosis. Apoptosis is present in metazoans and it works both during the development and in damage response in the adult tissues. A specific protein, p53, has the capacity to transduce the DNA damage stimuli into the apoptotic programme (for review, Rich et al., 2000).

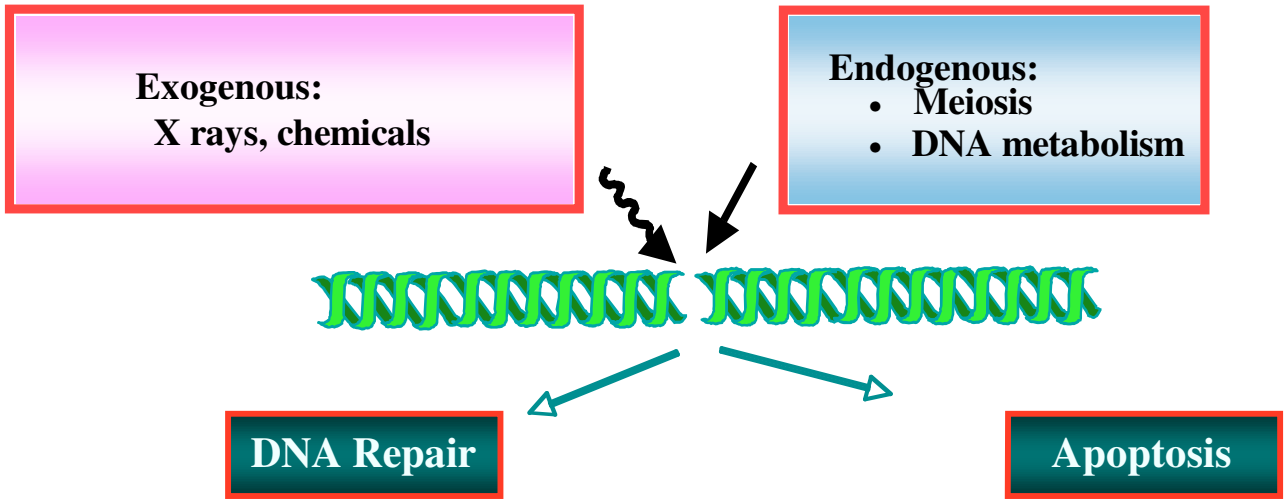


Figure 1.1- Schematic representation of DNA damages sources and pathways activated by damages.

1.3.1 Non Homologous End-Joining

The term Non-Homologous End Joining (*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. Experiments in *Saccharomyces cerevisiae* with plasmid transformation assay have contributed to the 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 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, right). NHEJ does not require any homology at the ends of the strands that have to be re-joined. A core of conserved proteins is recruited to the damage site in order to protect, process, and rejoin directly the DNA broken ends (for review, Shrivastav et al., 2008): a DNA-dependent protein kinase called DNA-PK, two proteins that form a heterodimer called Ku70/80 and the XRCC4/DNA ligase IV heterodimer (Pastwa et al.; 2003), It is assumed that Ku heterodimer could be the first sensor of DSBs repair during the NHEJ pathway, binding the DNA extremities flanking the DSBs, stabylizing and protecting them from degradation (Cary *et al.,* 1997; Pang *et al.,* 1997; Yaneva *et al.,* 1997; Feldmann *et al.;*2000). Once located onto DNA, Ku recruits also DNA-PK promoting its phosphorylation. This protein works either as a scaffold for the placement of the downstream proteins (such as XRCC4/Ligase IV) or promoting the relocation of XRCC4/Ligase IV to the DNA extremities in order to allow the ligation step occurring after the removal of Ku heterodimer *via* autophosphorylaton (Chan *et al.,* 1996; Calsou *et al.,* 1999; Chan *et al.;* 1999; Nick McElhinny *et al.,* 2000). 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 homologous template, 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.(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, but they can lead to serious developmental defects or lethality during development.

1.3.2 Homologous Recombination Repair

During S/G2 transition, after the DNA replication, Homologous Recombination Repair (HRR) is the elected mechanism to repair DSBs. DSBs repair via HR requires the presence of long homologous sequences (several hundred bp homology) used as an intact donor molecule (usually the sister chromatid, but also the homologous chromosome, or exogenous homologous DNA elements). The succession of the events through which HR is achieved, is conserved from Escherichia coli to humans and proceeds by an initiation stage, in which DSB is resected in the 5' to 3' direction (Figure 1.2, left). The 3' single-stranded DNA originated by this processing, are loaded by RecA (E.coli) or Rad51 protein (eukaryotes), which forms long nucleoprotein filaments. The successive 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 homologous dsDNA, producing a D-loop structure.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. 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", has been proposed by Resnick (1976) and by Szostak (1983) (Figure 1.2). The model, based on the original Holliday model (Holliday, R. 1964), has been then modified based on the analysis of molecular events associated with HR in yeast S.cerevisiae (for review, Khanna et al., 2001) 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

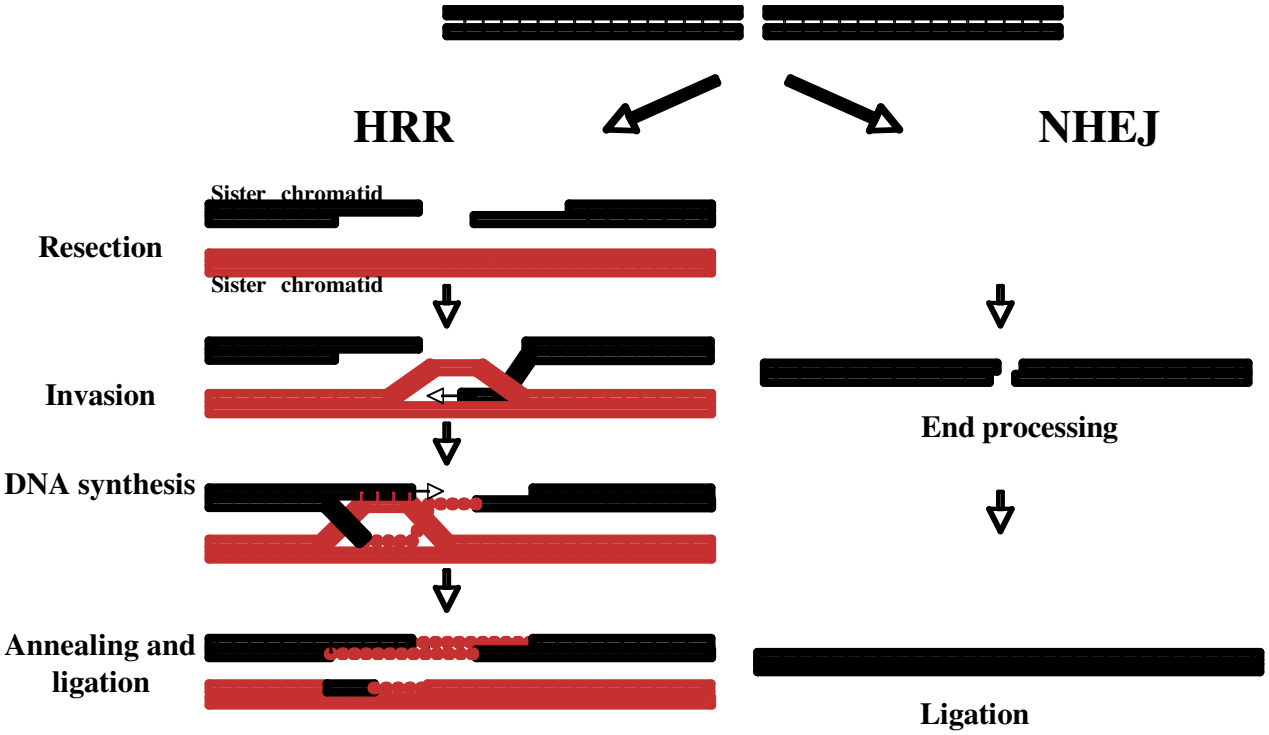


Figura 1.2- *Homologous Recombination Repair* and *Non-Homologous End Joining* pathways

1.4. Meiosis and Meiotic Recombination

Generation of functional gametes in species with a sexual reproduction, involves a specific process called *meiosis*. By meiosis, haploid cells are produced through two consecutive rounds of cell division called *meiosis I*, an equational division in which homologs are redistributed to daughter cells, and *meiosis II*, a reductional division, in which sister chromatids segregate generating four haploid gametes. After fertilization, cells reconstitute the diploid state, ensuring the continuity of the species.

Unique molecular events arise during meiosis I that is preceeded by single step of DNA replication leading to a genomic content of 4N. During the prophase of meiosis I, chromosomes display dramatic modifications in their

shape as a result of molecular processes that are occurring. Due to the chromosomal appearance, we can distinguish five sub-phases (*leptonema*, *zygonema*, *pachynema*, *dyplonema* and *diakinesis*) each one of them can be considered as a specific scenario in which meiotic process exert its features: in *C. elegans* during **leptonema-zygonema** stages (also called *transition zone*), chromosomes appear localized toward one side of the nucleus, in a characteristic "crescent" shape and during this phase, homologous chromosomes start to seek for each other in a process called *pairing*. By the end of the transition zone, a proteinaceous tri-partite structure called *Synaptonemal Complex (SC),* starts to be formed between each couple of homologues, working as a proteic zip by which chromosomes are tightly positioned and stabilized in a fully aligned architecture (*synapsis*). In the **pachynema** stage, synapsis is completed and the recombination events proceed through crossovers formation while, in **dyplonema**, the synaptonemal complex disassembles. The disappearing of the SC reveals the homologs are 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. Since many of the genes involved in the HR pathway are conserved during metazoan evolution, their roles and genetic interactions can be investigated in model systems particularly suitable for the study of DSB repair (in mitosis and meiosis) and DNA damage control. *C. elegans* very well fits several characteristics, that make it an ideal organism to clarify mechanisms that drive meiosis and the recombination events during prophase I: the germ cells, show a totally peculiar organization in the gonad, as they are polarized in a spatial/temporal fashion, so that all the meiocytes are synchronized in

subsequent stages of prophase I; several cytogenetics techniques can be applied on this system, allowing, among other things, rapid analysis of chromosomal aberrations. The germ 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 to study the cross-talking between the "surveillance" systems, composed of checkpoints, repair mechanisms and apoptosis, moreover, the entire *C. elegans* genome has been sequenced, therefore, biological information from *C. elegans* may be extrapolated and applicable to more complex organisms, such as humans.

1.5. Use of Caenorhabditis elegans meiosis as model system to clarify processes involved in DSBs metabolism

Since many of the genes involved in the HR pathway are conserved during metazoan evolution, their roles and genetic interactions can be investigated in model systems particularly suitable for the study of DSB repair (in mitosis and meiosis) and DNA damage control . *C. elegans* very well fits several characteristics, that make it an ideal organism to clarify mechanisms that drive meiosis and the recombination events during prophase I: the germ cells, show a totally peculiar organization in the gonad, as they are polarized in a spatial/temporal fashion, so that all the meiocytes are synchronized in subsequent stages of prophase I; several cytogenetics techniques can be applied on this system, allowing, among other things, rapid analysis of chromosomal aberrations. The germ 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 to study the cross-talking

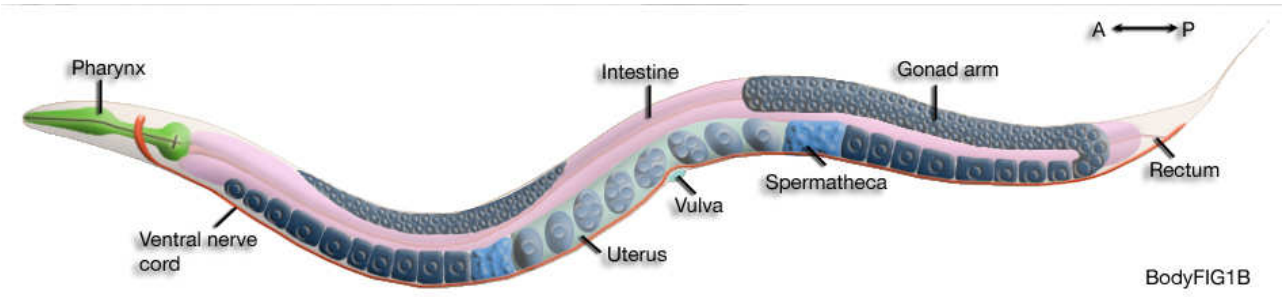
between the “surveillance” systems, composed of checkpoints, repair mechanisms and apoptosis, moreover, the entire *C. elegans* genome has been sequenced, therefore, biological information from *C. elegans* may be extrapolated and applicable to more complex organisms, such as humans.

1.5.1. C. elegans scenario

Caenorhabditis elegans is a small nematode (about 1 mm long when adult) (Figure 1.3) normally spending its existence in soil, surviving on bacteria. There is a sexual dimorphism in this organism, as the individuals of this specie are divided into hermaphrodites and males, characterized by clear different structures when adult. Sex is determined by the number of sexual chromosomes: there are five pairs of autosomes (chromosomes I, II, III, IV, V) and one pair of sex chromosomes, X (letter X), so hermaphrodites show a complement of two X chromosomes (designated XX), while males have only one X chromosome (designated XO). Males spontaneously arise in the wild-type population due to X-chromosome non-disjunction, although this phenomenon is very rare (0.2%). Males can cross-fertilize hermaphrodites and give a mixed progeny of males and hermaphrodites. Phenotype in which the frequency of males is higher than wild type is named Him phenotype (high incidence of males) indicating a segregation defects of sexual chromosomes (Hodgkin et al., 1979).

The hermaphrodites hence are the most abundant and are basically in charge of perpetuation of the specie producing both sperms and eggs and being able to self-fertilize. The reproductive system occupies most of the worm’s body, and it is formed by the gonad, consisting of two arms (sharing a single uterus) in which the physical location of the germ cells corresponds to their level of maturation during meiosis I. *C. elegans* is able to produce eggs for the whole duration of its life cycle, while sperms are generated only during the L4 larval stage and are therefore available for fertilization just in a limited number. The

germ cells achieve their maturation in the gonad, which shows a syncytial organization: only in diakinesis before fertilization they become individual cells in which nucleus and cytoplasm are completely surrounded by a plasma membrane. The oocytes are fertilized passing through the spermatheca and are laid a few hours later: 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 it arrives at the adult form. In only three days, the worm goes through the complete reproductive life cycle.



#Picture from http://www.wo rmatlas.org/handbook/

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

1.5.2. Meiosis initiation: physiological generation of DSBs

At the end of meiosis I, chromosomes, that underwent several molecular processes, must be redistributed in the correct number after the first cell division. In order to achieve this goal, chromosomes segregation relies on a mechanism dependent, in the first place, on the generation of physiological DSBs, which represent a *conditio sine qua non* for meiotic recombination induction. The protein responsible of the DSBs generation is SPO-11, that makes 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 topoisomerase generally provide for the topological disengagement of DNA,

making one DNA molecule pass through the other 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 breaks operated by SPO-11 activity, represent the initiation of recombination events. In many eukaryotes 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 (Baudat et al., 2000; Grelon, et al., 2001; Storlazzi, et al.,2003). 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. However, in *Caenorhabditis elegans* homologous synapsis occurs also in the absence of SPO-11 providing the CRA-1 protein is present (Smolikov et al.; 2008). An alternative system, in fact, promotes the polymerization of SC, bypassing a requirement for recombination intermediates to stabilize the pairing between homologous chromosomes (Smolikov et al., 2008). 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 *Saccharomyces cerevisiae,* MRE-11 is required for DSB formation(Usui et al.;

1998, Rinaldo et al.: 2002). *C. elegans mre-11* mutants show intact chromosomes at diakinesis, 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 *mre-11 C. elegans* 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) therefore this mutant is not able to repair DSBs. Furthermore, depletion of RAD-51 in a *mre-11* genetic background, result in intact 12 univalents. The inability to repair IR induced DSBs and the presence of intact chromosomes at diakinesis in absence of homologous repair, is consistent with a dual role of MRE-11 in both generating and repairing meiotic DSBs (Rinaldo et al., 2002).

1.5.3. Processing and strand invasion

Mre11 is required in the 5' to 3' resection of DSBs to generate a substrate for a subsequent strand invasion step (Borde et al., 2004). This resection of DNA ends forms a functional substrate 3'ssDNA for the binding with the strand exchange protein RAD51. RAD51 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 from bacteria to humans. 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 complicates the study the recombination consequences of such 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, diffusion of nuclear DNA that appears not be compacted in chromosomal bodies at diakinesis,

defective segregation of chromosomes and finally the 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 RAD-51 sequestering on ssDNA. This phase of homologous recombination sees the involvement of of the BRCA2 gene, a DNA repair gene that when mutated in humans causes an enhanced predisposition to breast and ovarian cancer (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 RAD51 (Martin et al., 2005)., *brc-2* mutants fail to load RAD-51 onto DNA breaks, consequently, have defects in the repair of meiotic DSBs like *rad-51* mutants and they are characterized by embryonic lethality due to extensive chromosomal fragmentation (Martin et al., 2005). These observations are consistent with BRC-2 being responsible of RAD51 loading to the site of the damage, promoting its nucleation on ssDNA (Martin et al., 2005), a model later confirmed also in other eukaryotic systems (for review, Boulton, 2006a).

The RAD-51 expression profile 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 leptotene/zygotene phase, peaking in the early pachytene, and quickly disappearing long before late pachytene (Colaiacovo et al., 2003). The decrease of RAD-51 foci reflects the progression of the DSBs resolution. Achiasmatic mutants that are not defective in DSBs formation display abnormal levels and distribution of RAD-51 foci (Colaiacovo et al 2003, Adamo et al 2008). Therefore, immunostaining against the RAD-51 protein

can become an optimal tool for monitoring the progression of meiosis and checking the “health state” of the repair system.

1.5.4. Meiotic DSBs Repair

During meiosis, all the double strand breaks that have been produced by the action of spo-11, are repaired by homologous recombination, that can lead to either crossovers products, that involve the physical exchange of DNA molecules between homologous chromosomes, or non-crossovers products, in which the homolog is still chosen as template for repair, but with no exchange of DNA molecules (gene conversion); also, the non-crossover repair can involve homologous repair on the sister chromatid, that is used as template for repair. It has been calculated that among all the double strand breaks that are made during meiosis, only very few of them are destined to generate a crossing-over (for review, Bishop and Zickler, 2004). In *C. elegans*, for each pair of homologs, just one crossover arises, that will ultimately produce chiasmata between homologous chromosomes. However, the *spo-11*-dependent DSBs, are more than the number of repair events that are resolved in crossovers. So, all those DSBs, that do not produce crossovers, must be repaired through a repair pathway not involving crossovers products. Among the proteins that are necessary for COs generation, there is the MSH complex. This is composed by two proteins that form a heterodimer, MSH-4 and MSH-5, and that belong to the Mut-S DNA mismatch repair family. MutS homologs (MSH) have been identified in all eukaryote organisms examined. Among the MutS members, the MSH4 and MSH5 do not have functions in mismatch repair, but play an essential role in the meiotic recombination machinery (Hollingsworth et al.,1995; Kelly et al., 2000; Winand et al., 1998; Zalevsky et al., 1999). 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). The *C. elegans him-14/MSH*4 and *msh-5* mutants show twelve DAPI-stained bodies (univalents) in the diakinesis oocytes compared to the six bivalents observed in the *wild type*, 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 late pachytene suggesting that the SPO-11 protein cuts DNA, but the resolution of DSBs is delayed (Colaiacovo et al.,2003). The induction of additional damage, by γ-radiation, gives the same phenotype at diakinesis as the untreated *him-14/MSH*4 and *msh-5* mutant, i.e. twelve proper univalents. These mutants are, thus, competent for the repair of exogenous and endogenous DSBs in meiosis (Kelly et al., 2000) suggesting that only the crossover repair pathway is impaired. The depletion of RAD-51 in the *msh-5* mutant shows nuclei at diakinesis with partial aggregation of bodies and chromosomal fragmentation (Rinaldo et al., 2002) indicating that in *him-14/MSH*4 and *msh-5* 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 the one DSB/chromosome 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). In *C. elegans*, during meiosis, homologous DNA repair may be the mechanism by which repair is achieved, while other DNA repair pathways such as NHEJ are silenced (Clejan et al., 2006). In *C. elegans*, the homologue of DNA ligase IV (*lig-4*) has been identified. It is the enzyme necessary for the last step of the joining of the broken ends in NHEJ. The *lig-4* mutant in *C. elegans* is viable, fertile and competent for crossover formation (Clejan et al., 2006). 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 diakinesis show chromosomal fragmentation and partial aggregation of bodies, the *lig-4;him-14/MSH-4* double mutant shows predominantly diakinesis nuclei with 12 proper univalents, like 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*.

It has been demonstrated that when a crossover occurs in a region, no other crossovers arise along the chromosome, suggesting 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), due to the well known phenomenon *cross-over interference* operating in most eukaryotes. Moreover, the distribution of crossovers seems to indicate that there are preferred “hot-spots” along the chromosomes. On the *C. elegans* genetic map each of the five autosomess has a central cluster of tightly linked genes flanked by the chromosomal arms in which the genes are more spaced. 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).

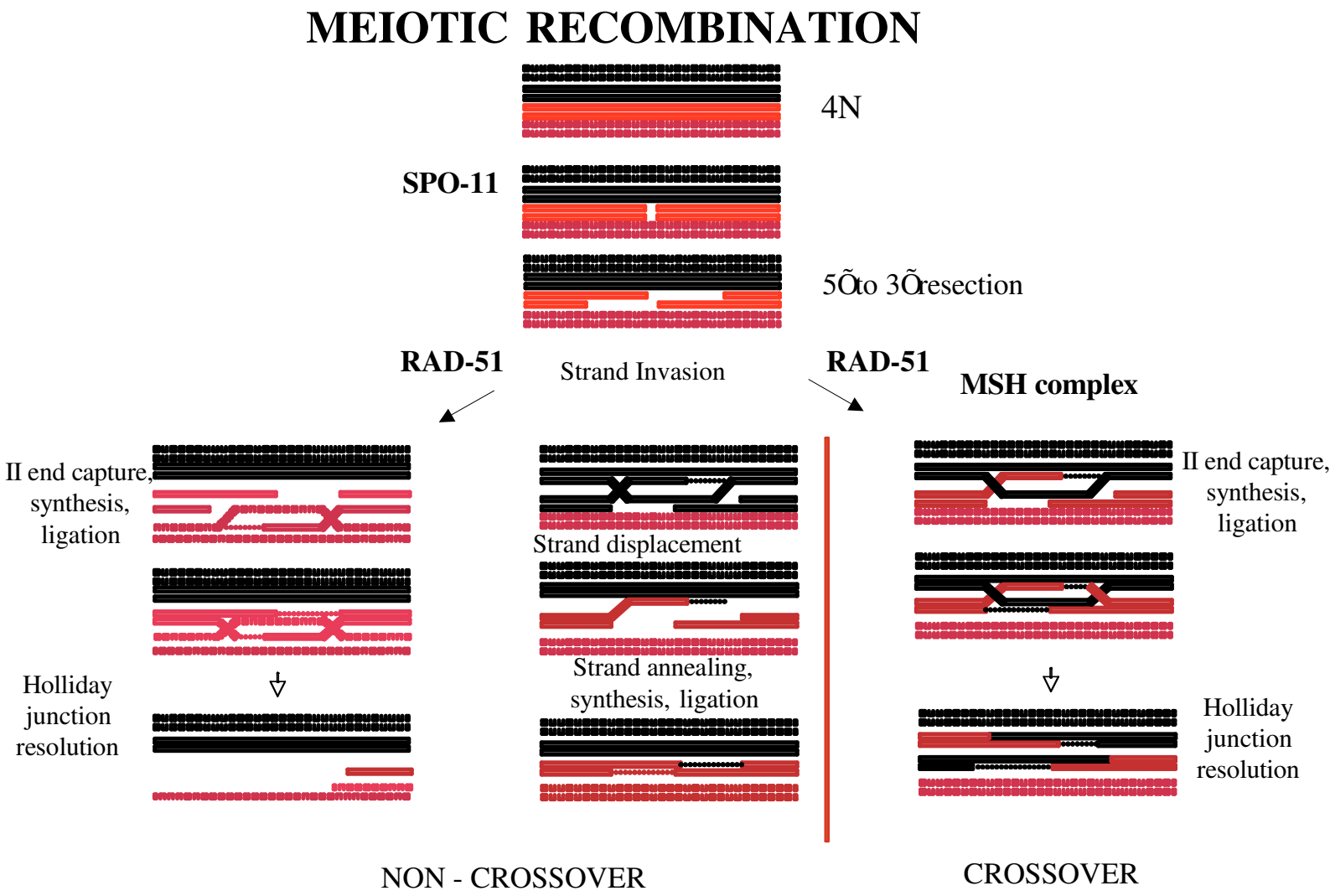


Figure 1.4- Meiotic recombination. In meiosis, only one DSB is repaired as crossover, all other DSBs are processed through alternative non-crossover pathways. MSH complex as well as the synaptonemal complex are essential for the crossover formation

.

1.5.5. The meiotic protein zip: Synaptonemal Complex (SC)

During the progression of meiosis, chromosomes undergo dramatic morphological changes, strictly coupled with the stage of meiosis in which oocytes are going through (Figure 1.5). In the distal part of the gonad respect to the uterus (mitotic tip), cells undergo mitotic divisions. 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 (for review, Colaiacovo, 2006). The cohesin complex remains linked until the anaphase of the second meiotic division. Immediately beyond this phase, cells enter the pre-meiotic phase, also called transition zone, composed by leptonema and zygonema stages. In the transition zone chromosomes start to change their shape: in fact DNA in transition nuclei appears to be localized toward one side of the nucleus, in a "*crescent*" shape, and the homologs start to seek for each other, in a process called *homologs pairing*. The presence of cohesins between sister chromatids is required for the synaptonemal complex formation: if cohesion is lost in fact, as it occurs in *rec-8* mutants, chromosomes do not segregate properly, causing a consequent aneuploidy and embryonic lethality (Pasierbek et al., 2001; Colaiacovo et al., 2003). In other words, the presence of the cohesin complex is also necessary to permit a correct disjunction of the homologous chromosomes and for the correct progression of meiosis. The Synaptonemal Complex (SC) is ubiquitously present from yeast to mammals, as revealed by electron microscopy (EM) and fluorescent immunocytological studies (for review Roeder, 1997; Zickler D, Kleckner N., 1999; Colaiacovo, 2006). It is constituted of proteins assembled along the axes of each homologous chromosome, and a central region, formed of transverse filament proteins interconnecting the lateral axes (zipper like structure). In *C. elegans*, the lateral elements are constituted by HIM-3 (Zetka et al., 1999), HTP-3 (Goodyer et al., 2008; Severson et al., 2009), HTP-1 and HTP-2 (Martinez-Perez et al., 2005; Martinez-Perez et al., 2008) proteins. The proper execution of the fundamental events occurring in meiosis, such as pairing, synapsis, and crossing over, strongly depends on the controlled activity of the meiosis-specific axis component (Zetka et al., 1999; Couteau et al., 2004; Martinez-Perez and Villeneuve, 2005; Couteau and Zetka, 2005). Central region of SC is built by SYP proteins: SYP-1 (MacQueen et al., 2002), SYP-2 (Colaiacovo et al., 2003) and SYP-3 (Smolikov et al., 2007). These proteins localize in discrete foci at the end of leptonema/zygonema stage, and by the

entrance in pachynema stage, these proteins acquire a linear shape that localizes 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 negatively affects the localization of the others. In *syp* mutants, the normal cytological morphology of DNA undergoes several changes. Without the SYP proteins, SC does not form anymore, as it is demonstrated by electron microscopy (TEM), furthermore the chromosomes fail to align since the protein "glue" represented by SC is absent, and for this reason they keep a decondensed configuration at pachynema (Colaiacovo et al., 2003; MacQueen et al., 2002; Smolikov et al., 2007). Without possibility to be juxtaposed, the homologs are physically not able to exchange DNA molecules between them, so they cannot undergo any crossover formation, and therefore no chiasmata are formed. As a consequence of it, diakinesis nuclei of *syp* mutants show 12 intact univalents, segregation of chromosomes at anaphase I is stochastic, and the consequent aneuploidy causes in the next generation a high level of embryonic lethality. Although the SC is not essential for the initiation of recombination, the completion of crossover events depends on SC. Through the monitoring of 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 late pachytene stage (Colaiacovo et al., 2003). In these mutants, the DSBs are created at the right time, indicating that the meiotic DSB formation does not depend on SC formation. The depletion of RAD-51 leads to unresolved DSBs and at diakinesis the nuclei show diffusions and unstructured chromosomes, that are absent in the single mutant *syp-2* (Colaiacovo et al., 2003). These data indicate that in *syp* mutant, the DSBs are repaired through a RAD-51 dependent non-crossover pathway, giving 12 proper univalents at diakinesis. In synthesis in the *syp* mutants meiotic DSBs are repaired by a RAD-51 dependent homologous repair using the only available template: the sister chromatid.

In wild type at diplotene phase, the synaptonemal complex is dissolved and at diakinesis 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 correct chromosomal orientation at metaphase I and the required tensional strength for a regular segregation in the meiotic spindle. All the morphological changes that occur during prophase I, are visible in the figure 1.5.

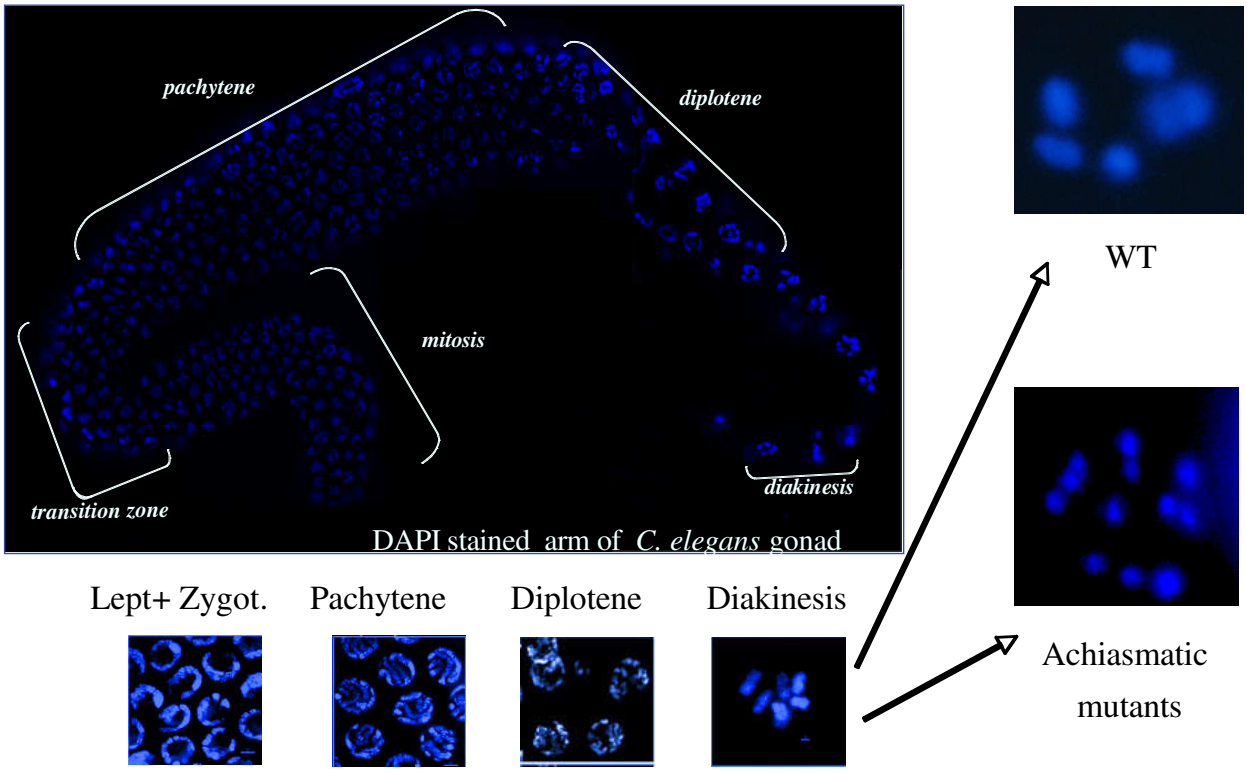


Figure 1.5- DAPI-staining of one arm of an adult C. elegans'gonad. ***Representative image of a wild-type germline stained with DAPI (blue) (DAPI, 4,6-diamidino-2-phenylindole). During Meiosis I, chromosomes undergo characteristic modifications, which allow us to distinguish five sub-phases of prophase I, called leptonema, zygonema, pachynema, dyplonema 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).***

1.5.6 Apoptotic Pathway in Caenorhabditis elegans

During the embryonic development, in the hermaphrodite worms it is possible to notice that always the same 131 cells, out of the 1090 generated, are doomed to die by apoptosis. This process has been deeply characterized in *C. elegans*, leading to the identification of the main genes which drive apoptosis pathway: *ced-3 (C. elegans abnormal death -3)*, *ced-4 (C. elegans abnormal death -4)*, *ced-9 (C.elegans abnormal death -9)*, *egl-1 (egg laying defective -1)*. The *ced-3*, *ced-4* and *egl-1* genes are pro-apoptotic factors, and their knock-out leads to the survival of the 131 somatic cells that normally die (Ellis and Horvitz, 1986). The *ced-9* gene has instead an anti-apoptotic function, so a gain of function mutation causes a block of apoptosis, while a loss of function mutation determines a precocious death during early development, due to the the miseregulation of the apoptotic pathway (Hengartner et al., 1992). In order to elucidate the interaction existing among the "death-genes", epistasis studies have been performed, highlithing the mechanism at the base of the apoptotic mechanism execution. When death signals trigger the activatation of the apoptotic program, the caspases belonging protein CED-3 is switched from an inactive state (pro-caspase) to an active state (caspase) by the CED-4 protein, a homologous to mammalian Apaf-1, with whom CED-3 forms a tetramer. CED-4 functions as a positive regulator of CED-3 and they together form the worm version of apoptosome, which in mammalians is composed of three proteins: caspase 9, Apaf-1 and cytochrome-c (discovered years later) (Li et al., 1997). When instead survival signals are present, CED-4 is kept in a blocked status by its interaction with CED-9, homologous to mammalian 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 review, Lettre and Hengartner, 2006). Under normal conditions, when no death signals are present, CED-4 can not activate CED-3, since it is associated in an inactive state with CED-9, but when apoptosis is

triggered, another factor plays its role, EGL-1 (that also belongs to Blc-2 family, but, differently from CED-9, which has an anti-apoptotic function, it has a pro-apoptotic action): EGL-1 binds CED-9, that this way is unable to block CED-4, and therefore CED-4 can associate and activate CED-3, achieving the apoptotic program. The identification of these key genes regulating cell death program in *C. elegans* that are conserved in humans has represented a significant advance in the 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 of the Nobel prize in Physiology or Medicine in 2002.

1.5.7. Germline apoptosis and DNA damage induced apoptosis

The developmental apoptosis, which is achieved in the somatic tissues, is performed into two waves during worms' life: in the embryonic tissues and in the L2 larval stage. Adult worms do not show apoptosis in the somatic cells, as the only compartment in which apoptosis works in the adult life is represented by the germline (Gumienny et al., 1999), in which half of the cells produced during meiosis die by apoptosis. The criteria for selection of germ cells that are designated to be eliminated, however, remain still unclear. As a result of this phenomenon, it is possible to see that also under physiological conditions, a basal level of apoptosis is always present in the germ line and occur at the late pachytene stage, This physiological apoptosis level can be perturbed by deleterious stimuli onto the DNA such as after genotoxic stresses (ICL exposure, ionizing radiations, unrepaired DSBs), that activate the damage checkpoint and consequently lead to an increase in apoptosis again restricted at late pachytene stage. As in all organisms in which apoptosis is present, the nematode cells have systems in charge to sense, monitor and transmit the signals caused by DNA damages. *hus-1*, *mrt-2* and

rad-5 are “rad” mutants, defective for the radiation-induced apoptosis (Ahmed et al., 2001; Hartman and Herman, 1982; Hodgkin et al., 1979). All three mutations abrogate the cell cycle arrest (that occurs in the mitotic compartment) and pachytene apoptosis induced by DNA damages. *hus-1* and *mrt-2* encodes proteins homologous to *S. pombe* Hus1 and Rad1 checkpoint proteins (Ahmed and Hodgkin, 2000). Hofmann’s work has shown how HUS1::GFP associates with chromatin under normal condition, while, after induction of DNA damages, HUS-1 relocates in particular sites, signalling probably un-repaired damages. For a correct localization on nucleus, HUS-1 has to interact with MRT-2 and the Rad9 homolog HPR-9, forming a complex, marker of DNA damage (Hofmann et al., 2002). Differently from *mrt-2*, *rad-5* is dispensable for the localization of *hus-1*. Moreover, *rad-5/clk-2* mutant shows a more serious phenotype compared to *mrt-2* and *hus-1* mutants: the complete elimination of *rad-5* gene function leads to developmental arrest and embryonic lethality (Ahmed et al., 2001). However, all these proteins, when knocked off, are unable to induce the cell cycle arrest and the damage-dependent apoptosis, indicating their roles as damage signalling. Although the precise molecular mechanism of action of these proteins remains still unclear, these three *C. elegans* checkpoint proteins activate a cascade signal that converges onto the activation of CEP-1 protein, which triggers the apoptotic machinery. CEP-1 is the only one member of p53 family codified in the *C. elegans* genome. The depletion of CEP-1 (by RNAi or gene deletion) does not affect the physiological apoptosis during the worm development and in germ cell line. By contrast, as p53, CEP-1 is a regulatory factor for responding to genotoxic stresses on DNA in germline. It is required for activation of DNA damage apoptosis, but not for the cell cycle arrest (Derry et al., 2001; Schumacher et al., 2001). Works indicate how the DNA damage induced apoptosis in germ cell line is transcriptionally regulated. The proposed model sees the CEP-1 protein as transactivator between the damage sensor proteins and their substrates.

1.6. Fanconi Anaemia/BRCA Pathway

Fanconi Anaemia (FA-OMIM#227646) is a rare genetic disorder, characterized by genomic instability, developmental defects and cancer predisposition. Thirteen genes have been isolated so far, as causative of this syndrome: FANC-A, -B, -C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J, -L, -M and –N. Among the several phenotypes associated with this disease, one of the characteristic feature common to all the mutations in different complementation groups is represented by the high sensitivity of the cells deriving from FA patients to DNA-crosslinking drugs (Inter-strand cross linking agents, ICL), including mitomycin C (MMC) and cis-platin (CDDP). The FA cells show also an abnormal progression of the cell cycle: in fact after ICL treatment, they show a prolonged S phase, since the ICL drugs basically inhibit DNA replication and transcription, so in order to allow the cells to survive, this kind of insult must be resolved or bypassed. In mammals, ICL repair has not been clearly understood, but the specific hyper-sensitivity of FA cells to this drugs, implies a defect that is specific for ICLs. Early studies about FA, highlighted a situation in which the FA proteins worked together the BRCA proteins (BRCA-1 and BRCA-2), that are responsible of non sporadic breast/ovarian cancer susceptibility syndrome, since some of the FA proteins interact with both BRCA-1 and BRCA-2. The BRCA2 gene indeed is isoallelic with FANCD1. The FANCD2 protein, as for several of the other proteins of this pathway, does not show any functional conserved domain involved in DNA repair, furthermore, it is not present among bacteria or yeast. FANC-D2 is in the middle of the big pathway in which FA proteins work: in fact, when cells are treated with genotoxic substances a multicomplex (FA core complex), formed by several FA proteins (FANC-A, -B, -C, -E, -F, -G, -L and –M) is in charge to promote the monoubiquitylation of FANCD2 protein, that is believed to be switched in its active form and than loaded onto chromatin foci, in which it co-localizes with other proteins

involved in homologous DNA repair such as RAD51 and where, somehow, promotes the DNA repair. Recently another FA protein has been indentified, which works with FA-D2 in a heterodimer, and that is called FANC-I. These two proteins are interdependent for monoubiquitylation, since the cells lacking FANC-I miss also the ubiquitilated pool of FANCD2 and *vice-versa*. These two proteins are visible as chromatin foci, assumed to be the repair sites, and the formation of these foci seems to be dependent on the FA core complex, ATR and BRCA1 (Garcia-Higuera, I. *et al.*,2001). In mammals, the loss of FANCD2 causes defects in the resolution of DSBs, a large spectrum of solid cancers, developmental defects, sterility and at sub-cellular level, a strong sensitivity to inter-strand cross linking agents and chromosomal abnormalities. All these phenotypes highlight a role for FAND-D2 in DNA repair and genomic stability but however, the exact molecular function and the role of this gene in the DNA repair process still remains elusive. It has been pointed out that the FA proteins work in a large pathway in which also the BRCA proteins (BRCA-1 and BRCA-2/FANCD1) exert their function in the DNA repair, composing a finely regulated network aiming to the resolution of damages.

1.7. The Fanconi/BRCA pathway in C. elegans and aim of the thesis

Some of the FA pathway genes are conserved in Metazoan and plants (FANCD2, FANCI, FANCD1, FANCJ, and FANCM) while no orthologues have been found in yeast. In *C. elegans* four orthologues of the FA proteins have been identified so far: *fcd-2* (FANCD2), *fci-1* (FANCI), *dog-1* (FANCJ), *fncm-1* (FANCM), and both BRCA proteins *brc-1* (BRCA-1) and *brc-2* (BRCA-2/FANCD1). Two mutants of *C. elegans* FANCD2 orthologue *fcd-2* have been generated (Collis *et al.*; 2006), *tm1298* and *ok1145*, both displaying the same phenotypes. The *fcd-2* gene is dispensable for meiotic double strand breaks

generation and loading of synaptonemal complex, and in the nuclei arrested in diakinema stage, six DAPI-stained bodies are visible, indicating that FCD-2 protein is dispensable for cross-over generation. However, as in human cells, the *fcd-2* mutants show an exquisitely sensitivity to ICLs (Inter-strand Cross Linking agents), but not to IR (Ionizing Radiation), furthermore the S-phase checkpoint the *fcd-2* mutant appears to be activated after ICL treatment, suggesting that the hypersensitivity characteristic of this mutant is due to the inability to repair damages rather than sensing or transmitting the signal. Also in C. elegans as in mammals the FCD-2 protein is monoubiquitylated in response to ICL damages.

It has been shown that the *dog-1* mutant (orthologue of mammals FANCJ) is a putative helicase involved in the maintenance of genomic stability as in its absence, extensive deletions of G/C DNA sequences occur; moreover this mutant is characterized by a mutator phenotype and hypersensitivity to ICL agents but not to X-ray or UVC-irradiation (Youds et al.; 2006, 2008). It has been shown that this gene is involved in the unwinding of G-quadruplex DNA structures that form at hot spots of recombination such as telomeres or during DNA replication. In *C. elegans* also the orthologues of mammalian BRCA-1 and BRCA-2 are conserved (*brc-1* and *brc-2* respectively). The *brc-1* mutant shows a normal meiotic progression, although the quantity of RAD-51 foci and the germline apoptosis level both increase in this mutant (Adamo et al.; 2008 see publication attached). Both phenotypes are *spo-11* dependent, suggesting an exquisitely meiotic defect in DSBs repair, moreover the apoptosis level is rescued to wild type levels in the double mutant *brc-1;cep-1/p53*, indicating that the apoptosis observed is induced by DNA damage (Adamo et al.; 2008). Furthermore it has been shown that *brc-1* is required for an efficient DSBs repair *via* inter-sister, since its absence in genetic backgrounds in which crossovers are depleted (*brc-1;him-14/MSH-4* and *brc-1;syp-2* double mutants) a strong chromosomal fragmentation occurs (Adamo et al.; 2008). The *C. elegans brc-2* mutant has been generated in 2005

(Martin et al.; 2005) and it has been shown that the *brc-2* mutation leads to a maternal lethal phenotype. This protein is necessary for RAD-51 loading, that starts to be recruited at the DSBs at the end of transition zone and disappears by the end of middle pachytene stage, in order to allow the homologous DNA repair: in the *brc-2* mutant the RAD-51 foci are dramatically reduced, also after X-rays irradiation, suggesting that BRC-2 protein is required for the RAD-51 localization, while its localization is independent by RAD-51 presence, since BRC-2 is loaded onto DNA also in its absence (Martin et al.; 2005). After have been discovered in *C. elegans* the above mentioned function of these proteins have been confirmed in mammalian cells.As it has been highlighted before, *C. elegans* offers several advantages for the DNA repair study, since all its features make of this animal model a particularly suited system for the study of DSBs metabolism in both mitosis and meiosis. Furthermore it is possible to study the effects of mutations in the context of a whole organism and also, making possible to overcome the limitations of unicellular systems in which some of these pathways are not conserved (as in yeast) or avoiding the potential artefactual response coming out from the study in *ex vivo* systems such as cell cultures. So the aim of this thesis is to identify the function of the the *C. elegans* gene *fcd-2* during meiosis and mitosis, clarifying its role in DSB repair and in the maintenance of genomic stability.

2. EXPERIMENTAL PROCEDURES

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)II/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)III*;
- RB873, *lig-4(ok716)III;*
- RB1128*, fcd-2(tm1298)IV* ;
- VC13 *dog-1* (gk10) I

Three of these strains (AV106, AV308 and 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 sequences and PCR protocol

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

fcd-2 (tm1298)

5'- TCGCTCCGCCCTCTTTTCTA -3' and 5'-CGACGAGCAGCTAACAACATTGG -3'

cep-1 (gk138)

5'-TAAAATGGGATGTCTAGTGC-3' or 5'-TAAAATGGGATGTCTAGTGC-3' and 5'-GAATGTCTTGGGAATTAGAG-3'

lig-4 (ok76)

5' – AAAAAAAGTCGGCTCAAAAT- 3' and 5' – ACACCACTAACACAGACCAG

dog-1(VC13)

5'-GGA CTA TAG AAC GTG TTT CG-*3'*

5'-GCT CTT CTT TCA ATG TGA CGG-*3'* or *5'*-CGT CCA CAT CAA CAG AAC C-*3'*

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 (20U 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 *fcd-2* mutation with a PCR mix with 0.5 µl DNA, 1 µl of *fcd-2* primers [10µM], 2 µl dNTP [2.5mM], 2.5 µl 10x buffer containing 17.5mM $MgCl_2$, 0.4 µl AmpliTaq Gold [Roche], 17.6 µl H_2Odd. The reactions were heated at 94°C for 8min and cycled 35 times: 30 sec at 94°C, 1 min at 59°C and 1 min at 72°C, followed by 10 min 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 *fcd-2* mutation was used for *cep-1*, but with an annealing temperature of 60°C and also for lig-4 deletion, with an annealing temperature of 55°C. The PCR products with the internal and external primers gave the wild type pattern in an electrophoretic run on 1% agarose gel.

2.2.2. Genetic crosses

To generate *fcd-2;spo-11, the spo-11(ok79)*IV homozygous hermaphrodite worms, were crossed with *fcd-2 (tm1298)IV homozygous* males. *spo-11(ok79)*IV hermaphrodites were crossed with 3-4 *fcd-2* 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 *fcd-2* deletion and the other was immuno-stained with α-RAD-51.

To generate *fcd-2;him-14/MSH-4* worms, *him-14 (it21)/mnC1* hermaphrodites were crossed with *fcd-2 (tm1298)IV* homozygous 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/MSH-4* embryonic lethal phenotype. Adult hermaphrodites that laid a majority of unviable eggs were analyzed by PCR for the *fcd-2* deletion and the double mutant progeny were DAPI stained and their diakinesis nuclei were analyzed. Similar strategies were used to generate *fcd-2;syp-2*. Double mutant progenies were DAPI stained and their diakinesis nuclei were analyzed.

To generate *fcd-2;cep-1* worms, *fcd-2 (tm1298)IV* homozygous hermaphrodites were crossed with *cep-1* males and the worms carrying the two deletions in the F2 progeny, were isolated by PCR. The same genetic strategy was used in order to generate the double mutant *fcd-2;lig-4*.

To study recombination frequency in *fcd-2* mutant, *fcd-2* males 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 *fcd-2* deletion. The same method was performed for analysis of recombination frequency on the I chromosome with the genetic cross between *fcd-2* and *dpy-5(e61) unc-29(e403)I*.

2.2.3. 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 autosomal chromosomes can lead to aneuploidies, causing embryonic lethality in the next generation. If a defect of segregation affects the sexual chromosomes, the effect will be visible by the appearance of male individuals (Him phenotype) 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.

2.3. Quantitative analysis of DAPI-staining bodies in diakinesis nuclei

At the end of the deposition window (about 72 hours since eggs lying started), adult hermaphrodites were picked out for quantitative analysis of DAPI-staining bodies in diakinesis nuclei. The worms were transferred and suspended in 15 µl of M9 solution (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml of 1mM $MgSO_4$ in 1 liter) on glass slides. The samples were permeabilized and fixed through 15 µl of absolute ethanol. Once the samples had been air dried, ethanol was added again. To visualize the DNA in the fixed animals, 15 µl of the 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI) (2ng/µl) diluted in M9 were added. The observed nuclei were collected from both arms of each gonad, and about 20 worms in different genetic backgrounds (wt, *fcd-2*, *him-14/MSH-4*, *syp-2, fcd-2;him-14/MSH-4*, *fcd-2;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.

2.4. Immunostaining of meiotic nuclei

Gravid hermaphrodites were dissected in 15 μl M9 solution (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml of 1 mM $MgSO_4$ in 1 liter) on poly-lisine coated slides. Coverslips, treated previously with sigmacote (a special silicone solution), were added and freeze cracked on dry ice. The samples were permeabilized and fixed through three steps at -20°C in methanol, methanol/acetone (1:1), and acetone for 5 minutes 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). The anti-RAD-51 antibody was used at 1:200 dilution. 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 texas-red conjugated anti-rabbit secondary antibody was used at 1:200 dilution (Molecular Probes) in Ab buffer and carried out for 55 min at room temperature in the dark. Finally, the slides were washed and mounted in anti-fading medium (5 mg phenylenediamine, 500 μl PBS, 4.5 ml glycerol, 20 μl NaOH for a final pH 6-9) containing DAPI (1 ng/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 six zones(tip, mitotic zone,

transition zone, early pachynema, middle pachynema, late pachynema), according to their cytological features.

2.5. Fluorescence In Situ Hybridization (FISH)

2.5.1. Probes Preparation

The DNA chosen as probe for the chromosome III was the cosmid T17A3. The stab clone was streaked onto a 25 µg/ml kanamicin plate and a medium size colony was picked and grown in 15 ml of LB containing 25 µg/ml kanamicin vigorously shacking for 24 hours at 37°C. The cosmidic DNA was extracted using the Quiaprep Kit (Quiagen) and eluted in 50 µl of bi-distilled water. The DNA used as probe for the chromosome V was amplified by PCR amplifying the 1 Kb locus of 5s rDNA using the forward primer 5'-TACTTGGATCGGAGACGGCC –3' and the reverse primer 5'-CTAACTGGACTCAACGTTGC –3' using the TaqGold (Applied Biosystem) with the following protocol:

-1,0µl genomic DNA (100 ng)

-2,5µl 10x Buffer containing 15 mM MgCl2

-2,0µl dNTP 2,5 mM

-1,0µl forward oligo

-1,0µl reverse oligo

-0,4µl TaqGold (5U/µl)

-bi-distilled water up to 25µl.

The amplification program was:

94°C for 8' 1 cycle

94°C for 45"
56°C for 1' } 35 cycles

72°C for 1’
72°C for 10’ 1 cycle

2.5.2. Probes labelling

The DNA from the cosmidic clone T17A3, used as probe for the chromosome III, and the PCR amplified DNA for the 5s rDNA, used as probe for the chromosome V, were labelled using the *Digoxigenin-Nick Translation Mix* (for the T17A3 cosmid) and *Biotin-Nick Translation Mix* (for the 5s rDNA) both purchased by Roche. The reaction was set as follow:

-1µg of cosmidic DNA or PCR product
-4µl of Digoxigenin- or Biotin-Nick Translation Mix
-15µl of double distilled water

The reaction was incubated for 90 minutes at 15°C and then stopped by adding 1µl of 0,5 M EDTA, and heating to 65°C for 10 minutes: 2,5 µl of the probes so produced, were used to hybridize each slide.

2.5.3. Hybridization Procedure

L4 worms for all analyzed genotypes were picked out and transferred onto fresh plates. After 24 hours, worms were picked up and gonads were dissected in 15 µl of EGG 1x buffer (10x: 118 mM NaCl, 48 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM Hepes, pH 7.3) with tween 0,1%. Once all gonads were out of worms bodies, the fixation step was carried out by adding 15 µl of 7,4% parafomaldehyde to the 15 µl of EGG+tween in which worms were cut, so that the final concentration of fixative was 3,7%. The fixation time was of 2

minutes. A cover slip was applied and the slides were immersed in liquid nitrogen for 30 seconds. Cover slips were freeze cracked and samples were put for 5 minutes in –20°C methanol. After the methanol step, slides were placed 1 minute in 50% methanol+1x SSCT (SSC with 0,1% tween) and then transferred in 2x SSCT for 5 minutes. Slides were washed three times in 2x SSCT for 5 minutes each one and then dehydrated in a gradient of 70, 90, 100% ethanol, 3 minutes in each one. After 3 minutes in 100% ethanol, the slides were let air-dried and then the probe was added. Each probe, 2,5 µl of each one, was added to 12,5 µl of hybridization solution (2x SSCT, 50% formamide, 10% w/v dextran sulfate) in order to have 15 µl of total volume to apply for each slide. After the probe was added, a cover slip was applied and the slides were subjected to a denaturation step at 93°C for 3 minutes onto a heated block surrounded by wet towels and closed on the high side, so to create a humid chamber. After the denaturation step, the slides were removed and placed in Petri dishes at 37°C over night. Two post-hybridization washes were carried out in 2x SSCT+ 50% formamide at 37°C each one for 20 minutes. After the second wash, three washes of 5 minutes each one at room temperature were done in 2x SSCT. Secondary antibodies directed against digoxigenin (anti DIG-rhodamine conjugated, ROCHE) and biotin (anti biotin AlexaFluor-488 conjugated, Molecular Probes) were used for the detection of the probes signals, diluted 1:100 in 1% BSA+2x SSCT, and 50 µl of antibodies solution was added to each slide. The incubation of the secondary antibodies was conducted for 1 hour at room temperature in the dark. The slides were then washed three times 10 minutes each one in 2x SSCT in the dark at room temperature, and 40 µl of 2 µg/ml of DAPI was added. After 10 minutes, the DAPI solution was removed and the slides rinsed in 2x SSCT and then mounted with 15 µl of vectashield (SIGMA) and observed.

Images were acquired as stacks of optical sections at 0.3 µm intervals using a DeltaVision deconvolution microscopy system.

2.6. 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 phenotypic markers 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 *fcd-2 (tm1298)IV* were crossed with *fcd-2(tm1298)IV; 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). *fcd-2(tm1298)IV; dpy-5(e61)unc-29(e403)I* were crossed with *fcd-2(tm1298)IV* 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.

2.7. SYTO12 staining for 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, *fcd-2*, *cep-1*, *fcd-2;cep-1*, *spo-11*, *fcd-2;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 (an average of 70 gonad arms have been used for the apoptotic corps quantification).

2.8. ICL Sensitivity Assay and Developmental Defects Quantification

After 24 hours from L4 stage, young adults worms were picked out and cloned onto single fresh plates containing 180 µM *cis*-diamminedichloroplatinum(II) (CDDP, Sigma) and they were let to lay eggs. Each worm has been transferred onto a fresh plates every 24 hours and the eggs laid after 48 and 72 hours were counted. The ICL sensitivity was calculated as the number of hatched eggs versus dead eggs in the *wt, fcd-2, dog-1, dog-1;lig-4, fcd-2;lig-4* and *lig-4* mutants. The same strains were used to quantify the developmental defects, in both untreated and treated conditions. In treated conditions, single worms were picked out and cloned onto single plates with 180 µM *cis*-diamminedichloroplatinum(II). Every 24 hours the worms were transferred onto fresh plates and the whole progeny was scored for mutants with developmental defects. The same procedure was performed for the untreated worms, on plates without CDDP.

2.9. Statistical tools

Statistical analyses of DAPI stained bodies in diakinesis 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.

3. RESULTS

3.1. The fcd-2 mutant is viable, fertile and competent for crossing-over formation

We screened several individuals from a population of *fcd-2* mutants, and the analysis of fertility and viability of the progeny did not show any significant difference compared to the wild type (Table 3.1).

	N2	*fcd-2*
worms screened	*15*	*14*
Laid eggs	*4597*	*3649*
Dead embryos	*10*	*19*
Hatched eggs	*4587*	*3630*
Males	*3*	*2*
% of dead embryos	*0.22*	*0.52*
% of males	*0.07*	*0.06*

Table 3.1. Screening of N2 and *fcd-2* mutant

We also performed a DAPI-staining of the *fcd-2* mutants' gonads in order to check the presence of alterations in chromosome structure: we analyzed a large number of diakinesis nuclei from *fcd-2* mutants (N2 *wt*= 107, *fcd-2*= 94, Table 3.2)

	<7	7--11	12	>12	Nuclei observed
wt	106	1	0	0	107
fcd-2	90	4	0	0	94

wt=fc d-2	P value = 0.52

Table 3.2. Quantification of DAPI-stained bodies in *wt* and *fcd-2* mutant (upper) and χ^2 square value (down)

and most of them show six DAPI-stained bodies as in wt (figure 3.1), implying that FCD-2 protein is not required for chiasma-formation.

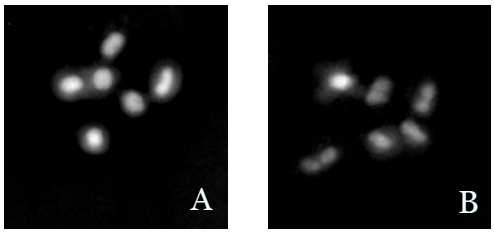


Figure 3.1- Diakinesis nuclei from *wt* (A) and *fcd-2* (B) worms, showing six DAPI-stained bodies

By FISH analysis we also checked the pairing/synapsis level of *fcd-2* mutant (Figure 3.2): 290 pachytene nuclei for the *wt* and 313 for *fcd-2* have been quantified, finding that the percentage of unsynapsed chromosomes is 1,03% (3/290) and 0,96% (3/313) respectively, indicating that pairing and synapsis are normal in the *fcd-2* mutant, therefore the FCD-2 protein is not required for these two processes.

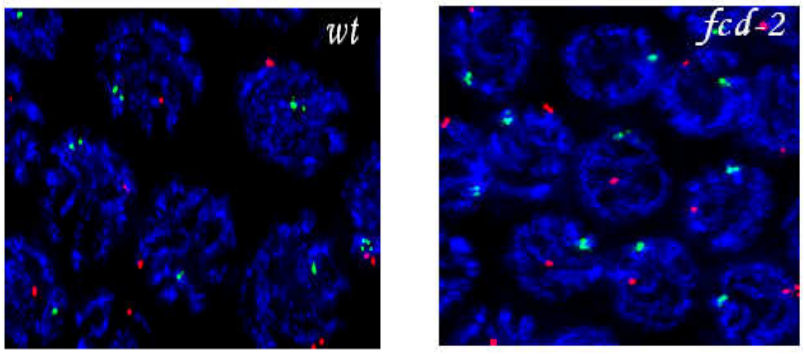
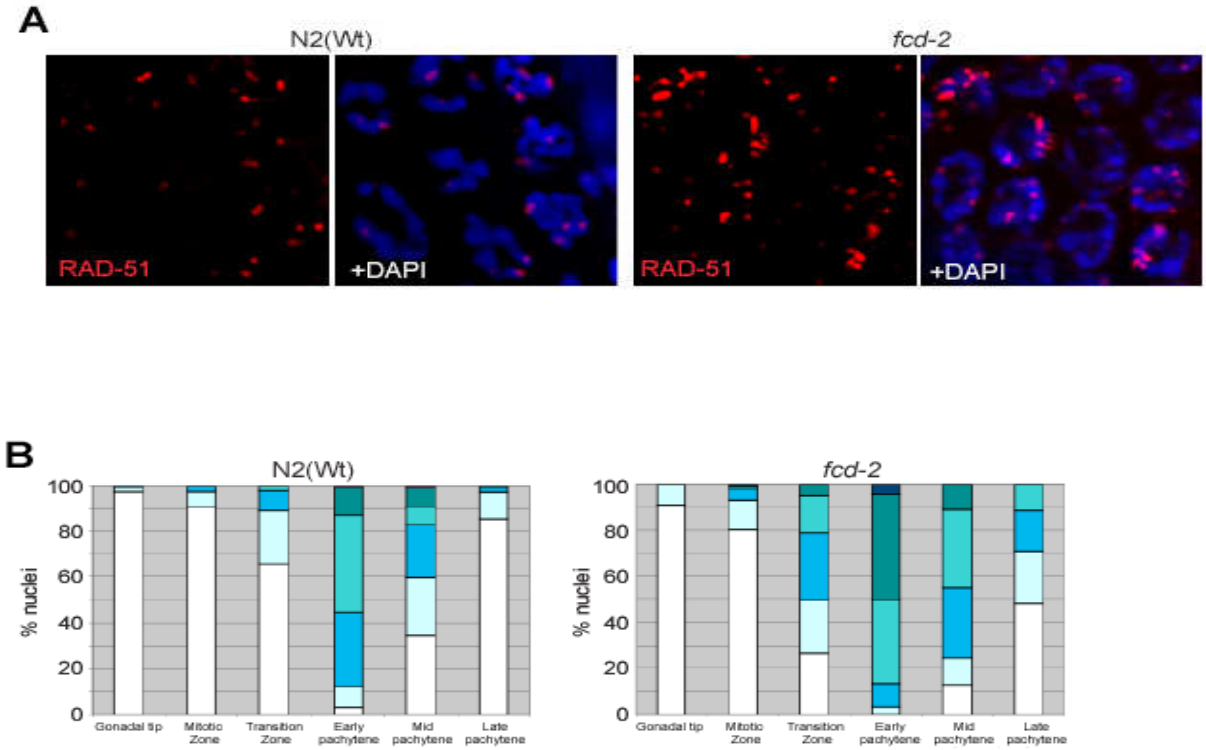


Figure 3.2 – FISH hybridization of pachytene nuclei from *wt* and *fcd-2* mutants. The probes used were cosmidic clone T17A3 for chromosome III (in red) and 5s rDNA locus for chromosome V (in green).

3.2. The fcd-2 mutant shows a spo-11 dependent increase in RAD-51 foci.

We performed an immunostaining analysis on whole mounted gonads of *fcd-2* mutant, using antibodies directed against the RAD-51 protein, in order to monitor the DSBs repair progression: in the *fcd-2* mutant, the loading and disappearing of RAD-51 foci resembles the wildtype profile, however, the number of these foci is increased in the *fcd-2* mutant compared to the wildtype levels (Figure 3.3 A and B). We wanted also to check if this increase was due to genuine meiotic defects or to un-repaired double strand breaks inherited from mitotic divisions, so to address to this question, we generated the double mutant *fcd-2;spo-11* and detection of RAD-51 foci was again conductedon the double mutant gonad: in the figure 3.3 C, it is possible to see that the RAD-51 expression in the *fcd-2;spo-11* double mutant's gonads traces the same profile observed in the *spo-11* single mutant, indicating that, in the *fcd-2* mutant, the DSBs involved in the repair foci were of meiotic origin.



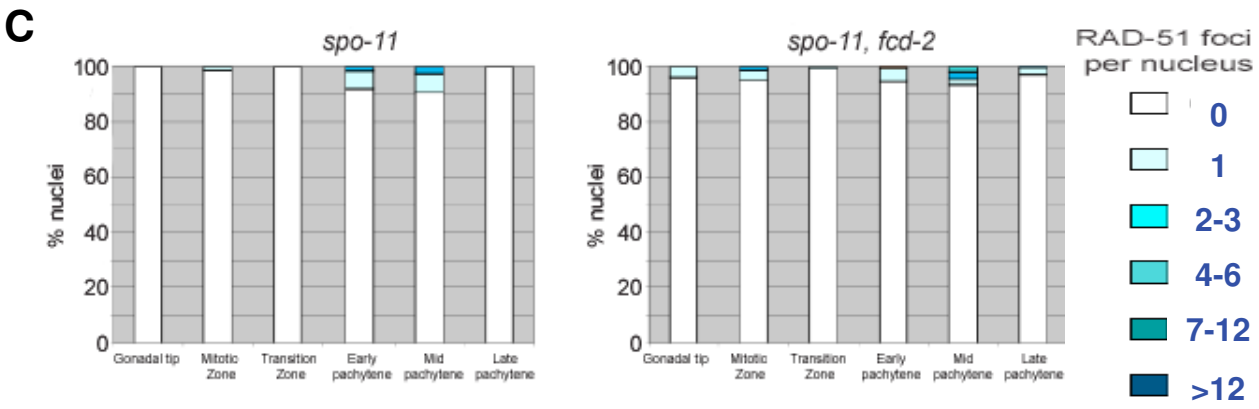


Figure 3. 3 – (A) RAD-51 immunostaining in *wt* and *fcd-2* gonads. In (B) and (C) is shown the quantification of RAD-51 foci during prophase I stages in *wt*, *fcd-2(tm1298)*, *spo-11(ok79)* and double mutant *fcd-2;spo-11*. On the X axis the stages of prophase I are indicated and on the Y axis the percentage of nuclei with a given number of RAD-51 foci.

3.3. fcd-2 mutation does not affect crossover frequency or distribution

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 *fcd-2* germ line does not lead to an abnormal level of embryonic lethality. Given that *fcd-2* is dispensable for the crossover formation, because at diakinesis six normal bivalents occur, the next question was whether or not the RAD-51 pattern in the *fcd-2* mutant may reflect an up-regulation of the crossover pathway, increasing the frequency of crossover events or their distribution**.**

We answered this question testing the frequency of recombination in the *fcd-2* genetic background. In *C. elegans*, crossovers preferentially tend to accumulate into 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 center 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). We used these two different intervals to check whether frequency and distribution of crossovers throughout the length of chromosomes were altered. *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.

In both intervals, the observed frequencies of recombination, 3.17% and 20.10% respectively (Fig. 3.4), were not significantly different compared to the frequencies observed in wild-type controls and to the genetic map units reported previously in literature (Edgley & Riddle, 1993).

Therefore, the *fcd-2* mutation does not influence either the frequency of recombination or the distribution of crossovers along the chromosomes.

	Parental chromosomes					**Recombination frequency**	**(cM)**
	dpy+ unc+	dpy unc	dpy unc+	dpy+ unc	tot	Observed	Expected
fcd-2(tm1298); unc-60(m35)dpy-11(e224)V	355	396	84	105	940	20.1%	18.79
unc-60(m35)dpy-11(e224)V	404	369	97	90	960	19.5%	18.79
fcd-2(tm1298); dpy-5(e61)unc-29(e403)I	1027	1047	31	37	2142	3.17%	3.32
dpy-5(e61)unc-29(e403)I	629	652	25	24	1330	3.68%	3.32

Figure 3.4 - Table with the number of observed worms for each genotype and recombination frequency expected and observed.

3.4. Germline apoptosis is increased in the fcd-2 mutant, as result of the DNA damage checkpoint activation, and it is dependent on spo-11 activity

In most meiotic mutants, such as *syp-2* (Colaiacovo et al.; 2003), *brc-1* (Adamo et al.; 2008, Boulton et al.; 2004) and others, perturbations in RAD-51 expression are often coupled with a variation in the physiological levels of apoptosis in the germline, so we wanted to check the average of the apoptotic corps in the *fcd-2* gonads: as it is shown in the figure 3.5, the quantification of the apoptosis in the *fcd-2* mutant revealed an increase in the number of cells dying by apoptosis compared to the *wt*. In order to understand whether the triggering of the apoptotic program was caused by the DNA damage checkpoint, we generated the double mutant *fcd-2; cep-1/p53* and the apoptosis was scored: in the double mutant the apoptosis level was restored to the *cep-1* one (Fig. 3.5), indicating that in the *fcd-2* mutant, the apoptosis is driven by DNA damages. Moreover, these damages are most likely generated during meiotic prophase, as the *spo-11* depletion, in a *fcd-2* background, leads to the rescue of wild-type levels of apoptosis (Fig. 3.5).

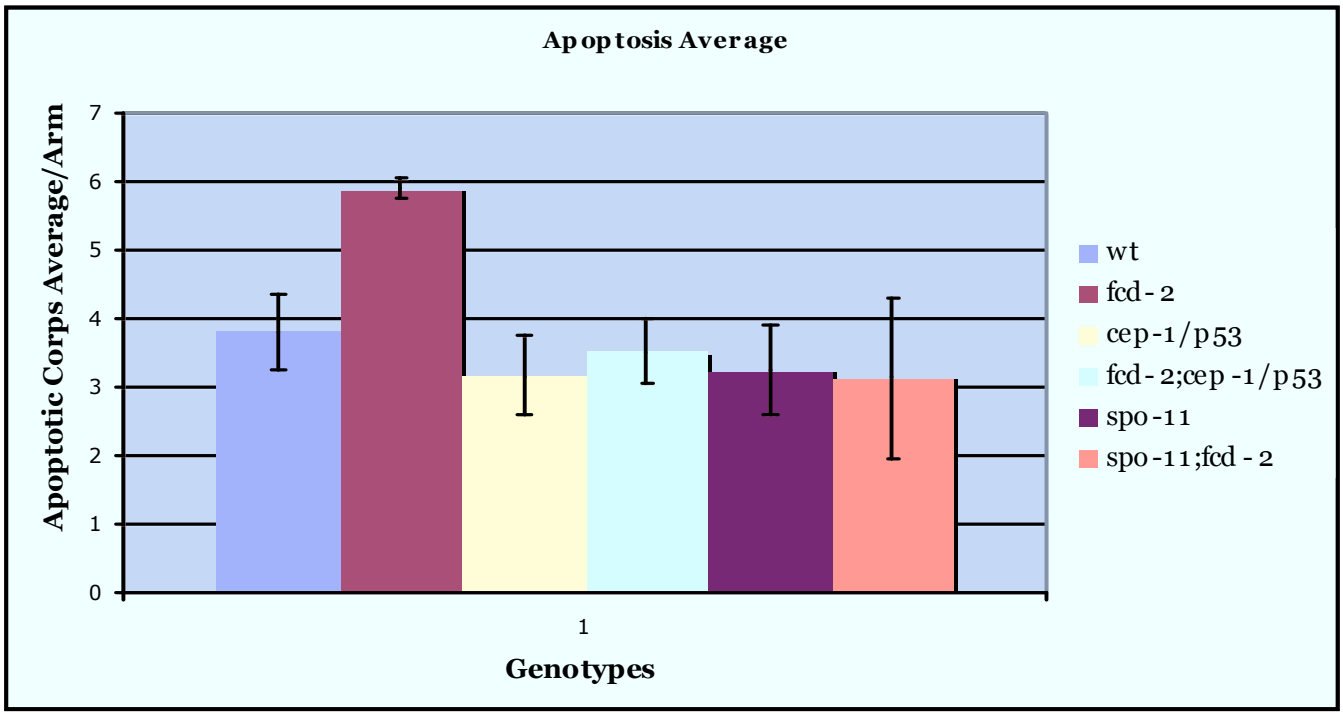


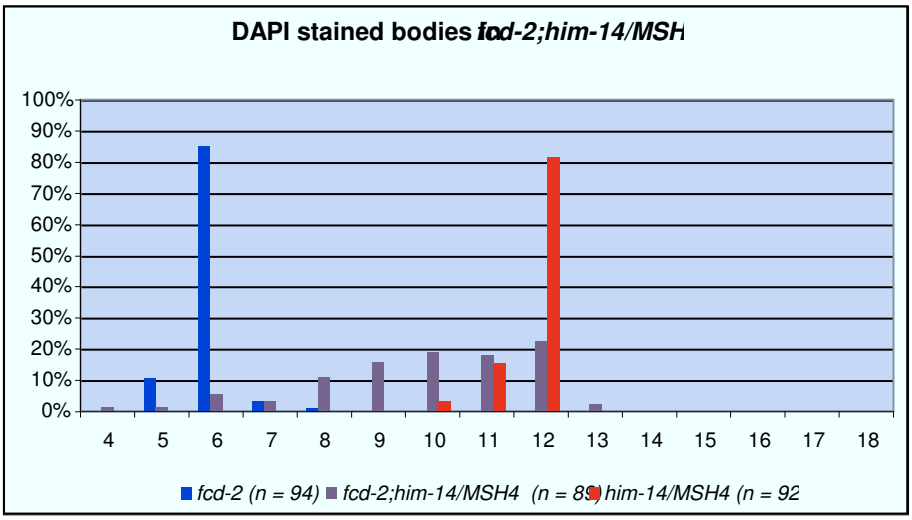
Figure 3.5 - Average of apoptotic corps per arm calculated in worms growing under physiological conditions.

3.5. Simultaneous depletion of FCD-2 and COs-promoting genes him-14/MSH-4 or syp-2 activate lead to chromosome association at diakinesis

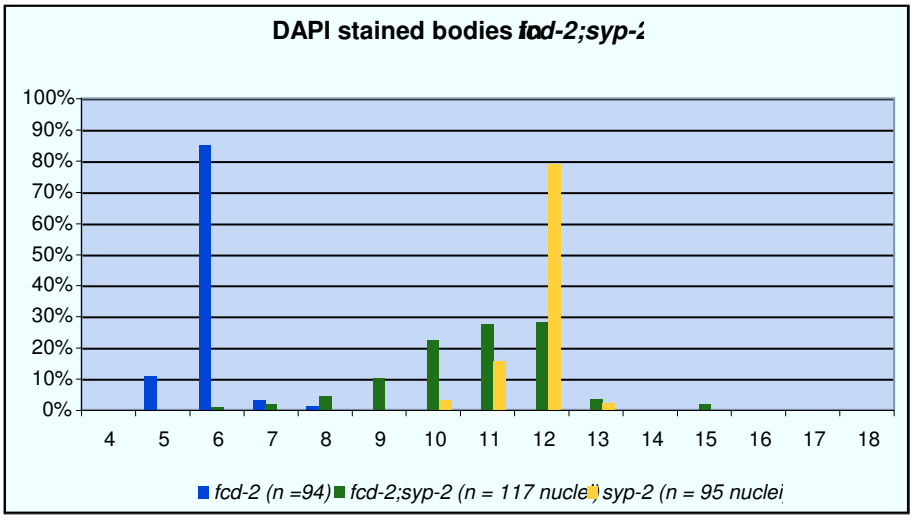
Since the *fcd-2* mutant was competent in crossovers formation, we generated double mutants with genes involved in crossing-over formation, in particular with *syp-2,* lacking the synaptonemal complex, and *him-14/MSH-4,* in which the pro-crossovers complex is impaired. Since both of these mutants are unable to generate crossovers, but competent to repair *spo-11* induced DSBs by non crossover HR, they show twelve DAPI-stained bodies at diakinesis (figure 3.6 A and B). If FCD-2 were required for the DSBs repair in absence of crossovers, in the diakinesis nuclei, chromosomes fragmentation would have

been observable, while if *syp-2* and/or *him-14/MSH-4* were epistatic on *fcd-2*, twelve DAPI-stained bodies would have been found. As shown in figure 3.6 (A and B), we surprisingly found that either in *fcd-2*; *syp-2* or in *fcd-2*; *him-14/MSH-4* double mutants more than the half of the scored diakinesis nuclei showed less than twelve DAPI-stained bodies (Number of diakinesis observed: *him-14/MSH-4*= 92, *syp-2*=95, *fcd-2;him-14/MSH-4*=89, *fcd-2;syp-2*=117).

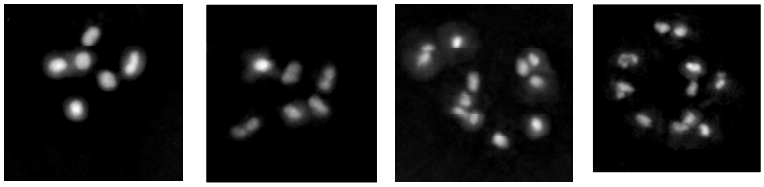
(A)



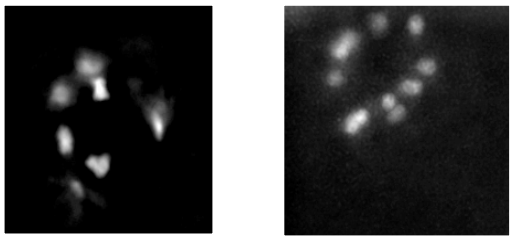
(B)



(C)



wt ***fcd-2*** ***him-14/MSH-4*** ***syp-2***



syp-2;fcd-2 ***fcd-2;him-14/MSH-4***

(D)	*him-14/MSH-4 = fcd-2; him-14/MSH-4*	P < 0.0001
	syp-2 = fcd-2; syp-2	P < 0.0001

Figure 3.6 - (A) Quantification of DAPI-stained bodies in *fcd-2*, *him-14/MSH-4*. The Y axis represents the percentage of nuclei in each class and the X axis indicates the number of DAPI-stained bodies. (B) Quantification of DAPI-stained bodies in *fcd-2;syp-2*. (C) Representative images of DAPI-stained oocytes nuclei at diakinesis of the indicated genotypes are shown. (D) Statistical analysis of DAPI stained bodies in diakinesis nuclei obtained by T-student test for independent samples. The difference in DAPI-stained bodies between *fcd-2;him-14/MSH4* and *fcd-2*, and *syp-2;fcd-2* and *fcd-2* are statistically significant.

3.6. Appearance of illegitimate chromosome fusion/translocation in absence of crossovers and FCD-2 protein

The chromosome bodies observed at diakinesis in *fcd-2*; *syp-2* and in *fcd-2*; *him-14/MSH-4* double mutants could either derive from a rescue of crossing over or from illeggitimate DNA repair between non-contigous chromosomal fragments. To clarify the nature of these DNA structures, we performed *FISH* (***F****luorescence* ***I****n* ***S****itu* ***H****ybridization*) analysis on diakinesis nuclei of the above mentioned double mutants. The figure 3.7, shows that in all the diakinesis nuclei observed in the N2 (wild-type =45/45) probes label two separate bivalents, and the same happens for *fcd-2* single mutant (*fcd-2*= 75/75). In the *syp-2* and *him-14/MSH-4* mutants, both lacking crossovers, in the diakinesis nuclei the two probes identify a total of four different univalents (*syp-2*= 40/40, *him-14/MSH-4*= 45/45). Both in *fcd-2;syp-2* and *fcd-2;him-14/MSH-4* double mutants instead, some chromosomal aggregates were composed of non-homologous chromosomal fragments (*fcd-2;syp-2*= 6/63, *fcd-2;him-14/MSH-4*= 1/56), indicating that in the contemporary absence of FCD-2 and crossovers, chromosomes undergo illegittimate DNA repair phenomena between non homologous chromosomes.

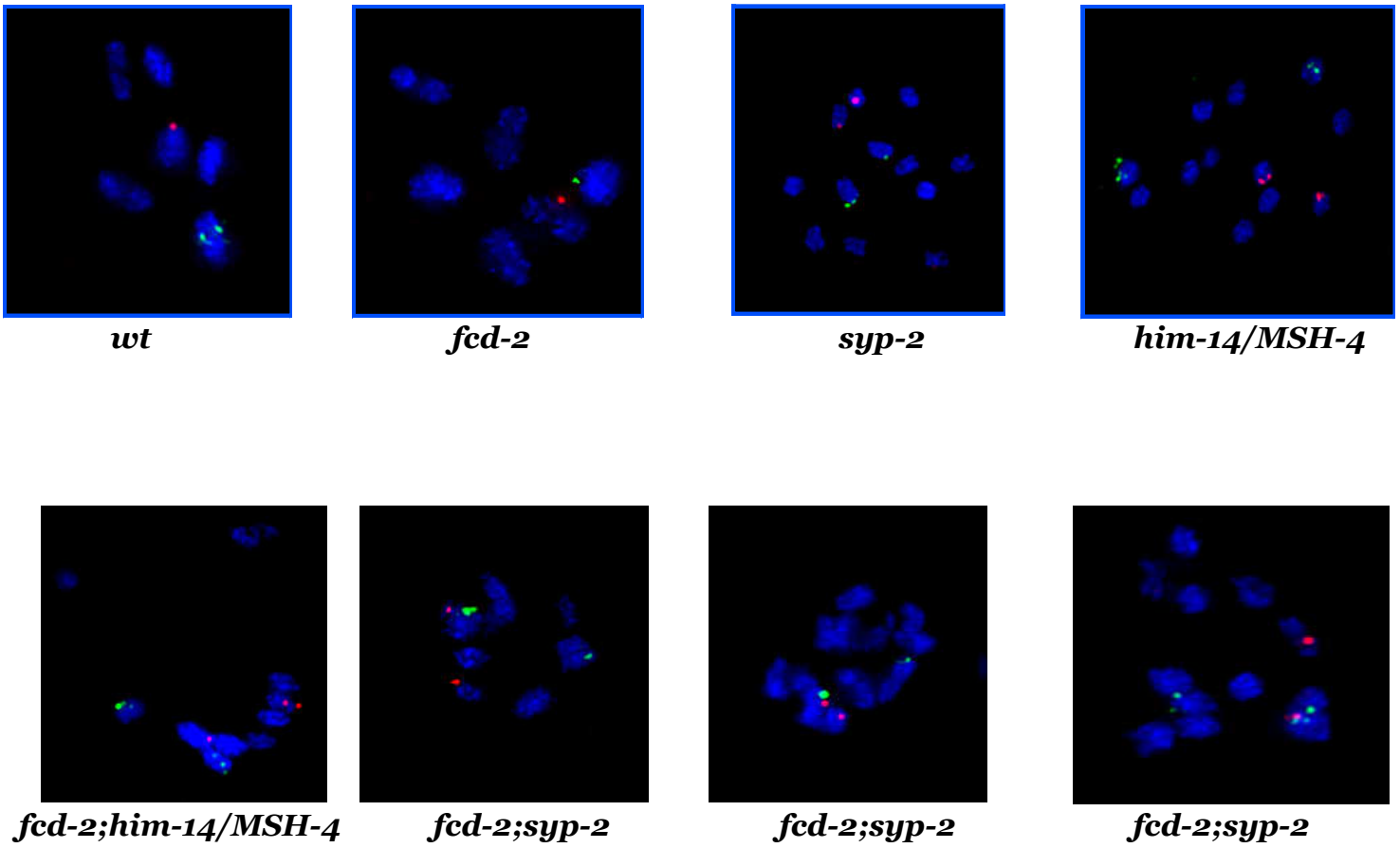
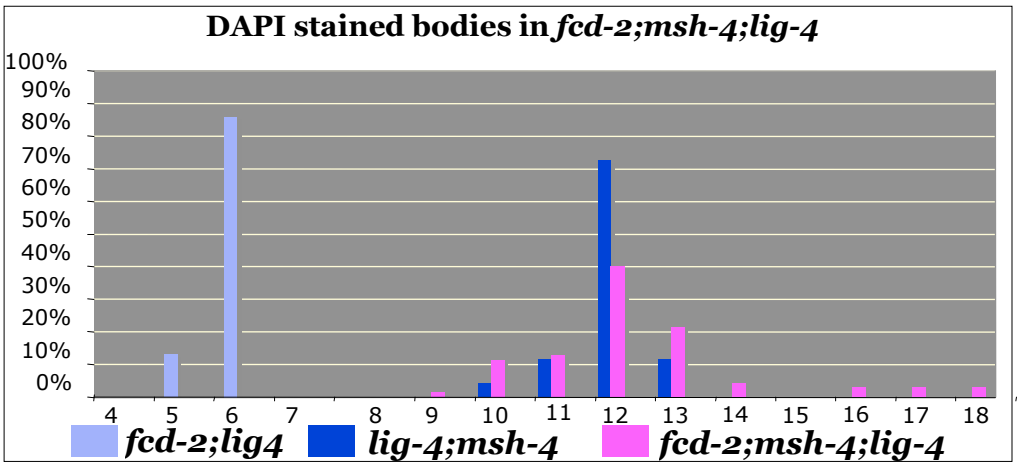


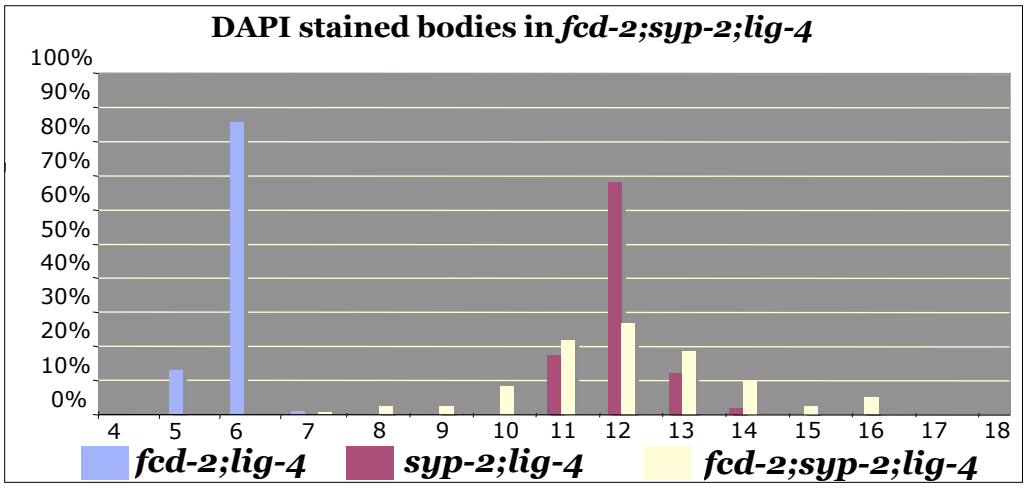
Figure 3.7 - Representative images of FISH hybridizations on diakinesis nuclei of the indicated genotypes. The red probe (cosmid T17A3) recognizes chromosome III and the green one (5s rDNA) the chromosome V.

3.7. In absence of crossovers and FCD-2 protein NHEJ is up-regulated

In order to understand whether the NHEJ pathway were involved in this process, we generated the triple mutants *fcd-2;syp-2;lig-4* and *fcd-2;him-14/MSH-4;lig-4*, depleted of the ligase protein responsible during NHEJ for the re-ligation of the broken ends flanking the double strand break.

As we show in the Figure 3.8, in the triple mutants a high number of diachinesis nuclei with chromosomes fragmentation is present. So effectively, the Non-Homologous End Joining is activated in genetic backgrounds contemporary depleted for the *fcd-2* gene and either *syp-2* or *him-14/MSH-4* genes.





	χ2 test P value
fcd-2 = wt	P = 0.52
fcd-2 = *fcd-2; syp-2*	P< 0.0001
syp-2 = *fcd-2; syp-2*	P< 0.0001
fcd-2 = *lig-4*	P = 0.47
fcd-2; syp-2= fcd-2; syp-2;lig-4	P< 0.0001
syp-2;lig-4 = fcd-2; syp-2;lig-4	P< 0.0001
fcd-2;lig-4 = fcd-2; syp-2;lig-4	P< 0.0001
fcd-2;him-14= fcd-2;lig-4;him-14	P< 0.0001
lig-4;him-14 = fcd-2;lig-4;him-14	P= 0.0003
fcd-2;lig-4 = fcd-2;lig-4;him-14	P< 0.0001

Figure 3.8- Quantification of DAPI-stained bodies in the triple mutants *fcd-2;him-14/MSH-4;lig-4* and *fcd-2;syp-2;lig-4*: a high level of chromosomal fragmentation is present when NHEJ is depleted together with CO and fcd-2, indicating that this pathway is hyper-activated when fcd-2 activity is impaired.

3.8. The fcd-2 mutation confers NHEJ dependent ICL sensitivity

ICL sensitivity, is a characteristic feature displayed by *Fanconi Anaemia* patients'cells and it is also one of the tools applied for diagnosis. The *fcd-2* mutation causes hypersensitivity to ICL agents also in worms, so we wanted to check if the NHEJ could have a role in the genesis of this phenomenon. In order to address this point, we treated *wt*, *fcd-2*, *lig-4* and *fcd-2;lig-4* mutants with 180 mM CDDP for 72 hours: in the figure 3.9, it is possible to notice that the *fcd-2* worms, show a strong reduction in the embryos viability percentage compared to the *wt*, and more surprisingly, this phenotype is dramatically reduced in the *fcd-2;lig-4* double mutant, indicating that after ICL insult, in absence of FCD-2 protein, the NHEJ pathway is hyper-activated, leading to an abnormal DNA repair that causes embryonic lethality.

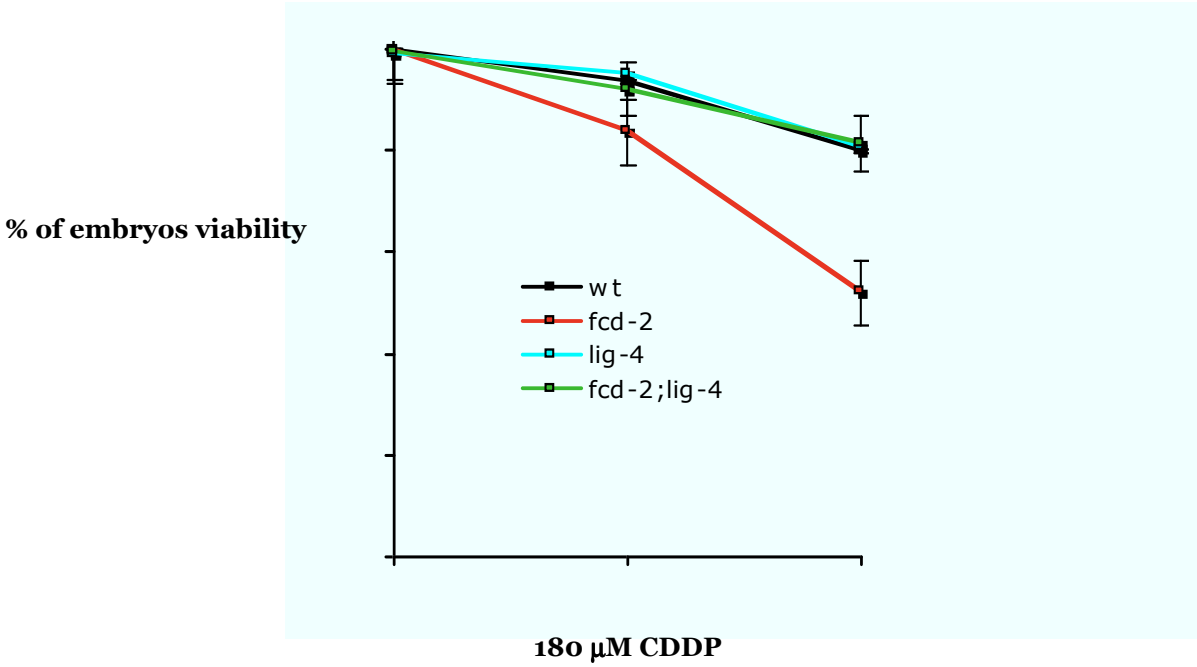


Figure 3.9 – Profile of embryos viability after CDDP treatment

3.9. The fcd-2 mutation confers NHEJ dependent developmental defects

We found out that NHEJ was also responsible of another phenotype observed in *fcd-2* deleted worms. In physiological growing conditions, we noticed that occasionally, developmental defective mutants arose in the population. We quantified this phenomen in untreated wildtype, *fcd-2*, *lig-4* and *fcd-2;lig-4* mutant populations. In physiological growth condition, the percentage of developmental defects was statistically different in the *fcd-2* worms (25/3630) compared to the *wt* (0/4587), *lig-4* (0/3513) and *fcd-;lig-4* (7/4133) worms (*fcd-2 = fcd-2; lig-4* P=0,0007 (Figure 3.10).
In order to understand if the developmental defect derive from illegittimate repair of stalled replication forks we exposed worms to CDDP for 72 hours and scored again for developmental defects and, as shown in the figure 3.10, the difference between *fcd-2* worms (31/236=13.1%) and the other genotypes (*wt* 9/470=1.9% and *lig-4* 12/575=2.09%) becomes dramatically appreciable and it is suppressed to wild type or *lig-4* levels in the *fcd-2;lig-4* double mutant (6/354=1.7%).

	untreated				180µM CDDP			
	wt	*fcd-2*	*lig-4*	*fcd-2;lig-4*	*wt*	*fcd-2*	*lig-4*	*fcd-2;lig-4*
Survival progeny	4587	3630	3513	4133	470	236	575	354
Developmental defects	0	25	0	7	9	31	12	6
% dev. defects	0%	0.69%	0%	0.17%	1.91%	13.13%	2.09%	1.69%

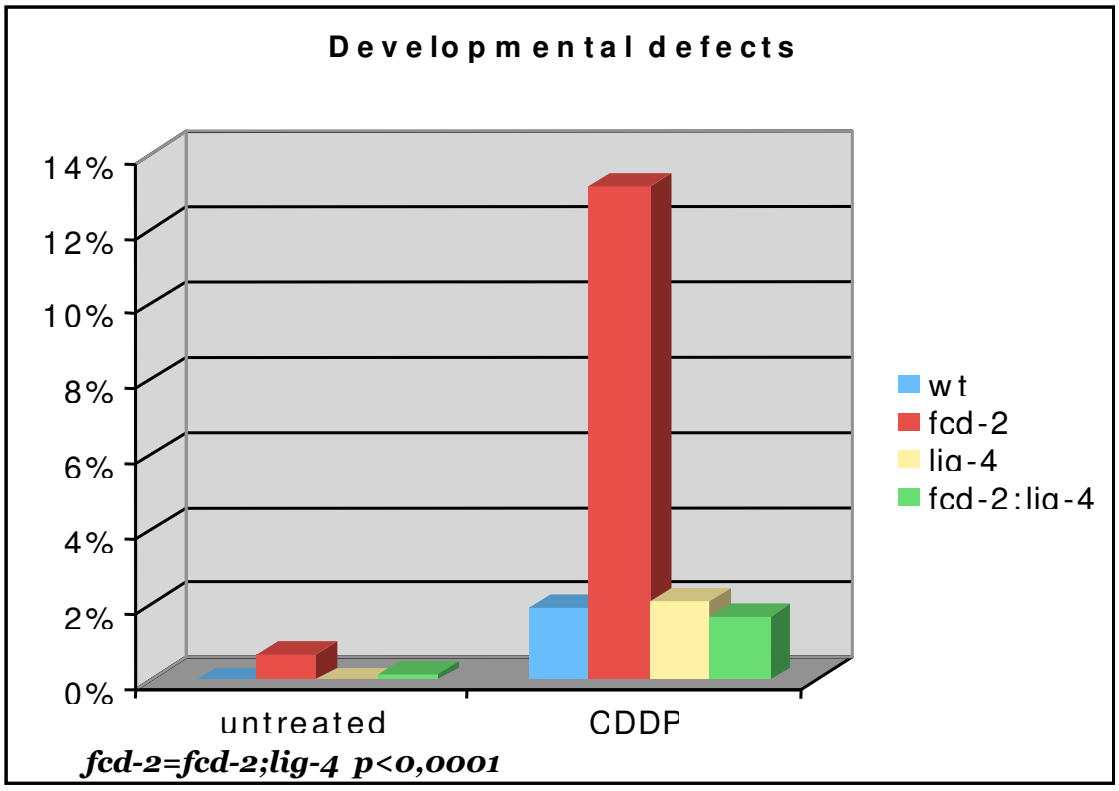


Figure 3.10 – Percentage of developmental defects found in untreated worms and after CDDP administration. All the aberrant phenotypes were collected as developmental defects.

3.10. NHEJ is not involved in the ICL sensitivity generated by dog-1 (FANCJ) mutation

We treated with CDDP also *dog-1*, the orthologue of the mammals FANCJ gene, which is required for the correct DNA repair *via* the homologous recombination downstream FCD-2 protein, and is known to be hypersensitive to ICL agents. In order to understand whether at the root of this phenomenon there was NHEJ activation, together with the *dog-1* single mutant, we also treated the *dog-1; lig-4* double mutant with CDDP: as shown in Fig. 3.11, the *dog-1* worms show hypersensitivity to CDDP that, in the double mutant *dog-1; lig-4*, is neither rescued nor increased. Therefore ICL sensitivity of *dog-1* mutants is due to inability of repairing via homologous recombination once this pathway has been determined upstream by the suppression of NHEJ operated by FCD-2 protein.

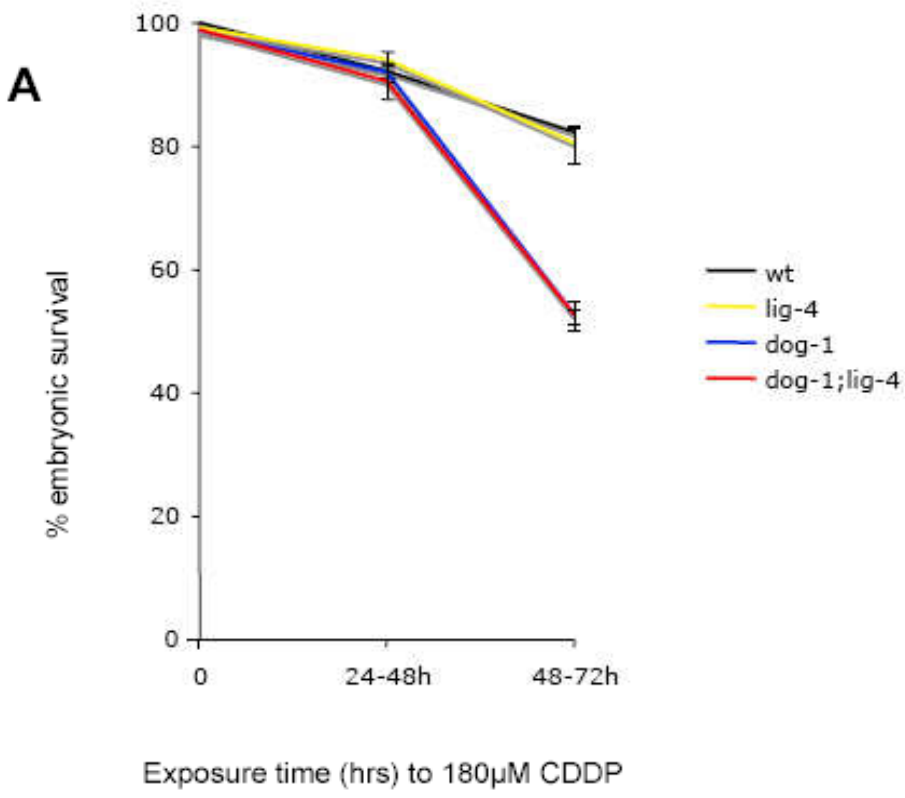


Figure 3.11 – Profile of embryos viability after CDDP treatment

4. DISCUSSION

The *C. elegans* gonad can be used as a tool-kit to study molecular function and interactions between genes involved in DSBs repair and in the safeguard of genomic stability. It has been pointed out recently that DSBs repair proceeds through activation of specific pathways that operate in specific moments during meiosis, depending on the spatial/temporal position of the meiocytes along the germline in *C. elegans* (Hayashi et al., 2007). Among the several DSBs that are generated from SPO-11, only one of them *per* homologs couple will rise a cross-over in order to generate a chiasma, required for the faithful segregation of the chromosomes, while all the other DSBs will be repaired through different mechanisms which do not involve crossovers formation. Under physiological conditions, it is assumed that the excess of DSBs may be repaired either by as inter-homologue non-crossovers in early prophase, made possible by the presence of an intact synaptonemal complex (SYP proteins) and a functional promoting complex (him-14/MSH-4-MSH-5) or later on by intersister homologous repair. In order to ensure faithful segregation of chromosomes in the next generations and the survival of the species, homologous DNA repair, the most conservative repair pathway, is the elective mechanism for DNA repair during meiosis, just because the use of homologous sequences involved as repair template, assures the fidelity of the DNA repair and therefore of the genetic information. For these reasons, DNA repair mechanisms that may threaten the conservation and fidelity of genetic information, as for example Non-Homologous End Joining (NHEJ), are strongly down-regulated or usually silenced during meiosis (Goedecke et al.; 1999). Recent evidences suggest in fact that in *C. elegans*, NHEJ plays little or no role in DNA repair in germ cells, possibly ensuring homology-based double-strand break repair and transmission of a stable genome from one generation to the next (Clejan et al.; 2006).

In this thesis, a role for FCD-2 in preserving the genomic stability through NHEJ silencing is reported. The FCD-2 protein is dispensable for crossovers

formation as suggested by normal diakinesis nuclei and recombination frequency. Nonetheless the *fcd-2* mutant shows abnormal level of germline apoptosis driven by DNA damage response, as in the *fcd-2;cep-1/p53* double mutant apoptosis is rescued to *wt* levels. This phenomenon suggests that in the *fcd-2* genetic background, the DNA repair is not totally efficient or that the *fcd-2* mutation may lead to abnormal DSBs resolution which generates aberrant gametes that are in the end eliminated by apoptosis. The aberrant level of germline apoptosis is rescued in a *fcd-2;spo-11* genetic background as well, suggesting that the same damages that activate apoptotic machinery are due to SPO-11 activity, and therefore they depend on an improper repair of meiotic DSBs. Furthermore, the relative abundance of RAD-51 foci, marking the DSBs repair intermediates, is increased in the *fcd-2* mutant, indicating a lower efficency in the meiotic DSBs repair, as this foci rise is dependent on SPO-11 activity.

As displayed by FISH analysis, when crossovers promoting factors are impaired, either at a structural level (through synaptonemal complex - depletion by SYP-2 knock-out) or by him-14/MSH-4 complex elimination, the contemporary absence of FCD-2 protein leads to the formation of abnormal chromosomal structures, identifiable in diakinesis nuclei as fusions between non-homologous chromosomes (over 70% of diakinesis nuclei observed show less than 12 univalents). This phenomenon is absent in the *fcd-2* single mutant, in which homologous recombination occurs normally, and it is also absent in both *him-14/MSH-4* and *syp-2* single mutants, indicating that FCD-2 is required for the down-regulation of DNA repair mechanisms that do not involve crossovers formation. So, the *fcd-2* mutation, in genetic backgrounds in which crossovers formation machinery is impaired, must release a block to the use of inappropriate DNA repair pathways that can eventually cause illegitimate chromosomes formation. The blocking action exerted by FDC-2 involves the silencing of Non-Homologous End Joining, that is assumed to be inhibited during meiosis in *C. elegans* (Clejan et al.; 2006). This is supported

by the fact that in the triple mutants *fcd-2;him-14/MSH-4;lig-4* and *fcd-2;syp-2;lig-4*, in which the NHEJ pathway is blocked through the knock-out of the ligase-IV gene (responsible of the re-ligation of the DNA extremities), several diakinesis nuclei show chromosomal fragmentation. As this phenotype is not present in the double mutants *fcd-2;him-14/MSH-4* and *fcd-2;syp-2*, it means that NHEJ becomes activated in these two backgrounds, leading to the illegittimate chromosomes formation displayed in the diakinesis of the double mutants.

In humans, mutations associated with whichever of the genes belonging to the Fanconi pathway, are causative of a genetic disorder called *Fanconi Anaemia (FA),* a cancer prone syndrome displaying several phenotypes such as high incidence of solid tumors, a plèthora of congenital defects, bone marrow failure and sterility. At subcellular level, FA patients' cells, display a marked hypresensitivity to Inter-trand Cross Linking agents (ICLs), a feature that is also used as a diagnostic tool for this disease. When indeed these cells are treated with ICLs such as *cis-platin* (CDDP), *hydroxyurea* (HU), *mitomicin C* (MMC) and others, they show a high level of mortality and a large spectrum of chromosomal aberrations, all characteristic of some DNA repair defects. Also in worms, mutations in the orthologs of mammalian FA genes, lead to hypersensitivity to ICLs, as in the *fcd-2* mutant (Collis et al.; 2006) and in *dog-1* mutant (Youds et al.; 2008), orthologue of mammals FANC-J. In the present thesis, for the first time has been demonstrated that the lethality observed in the *fcd-2* mutants is caused by a promiscuous use of Non-Homologous End Joining, as the lethality observed in the *fcd-2* mutants after CDDP treatment, is rescued to the wt levels in the *fcd-2; lig-4* double mutant. Worms lacking the *dog-1* gene, the *C. elegans* orthologue of the mammalians FANCJ, still show hypersensitivity to ICLs, but it is likely that loss of FANCJ, that is necessary downstream of FANCD2, will have the NHEJ option already banned, since the ICL sensitivity of *dog-1* mutants was neither rescued nor increased by the *lig-4* mutation. It is also been reported in

literature that the FA patients, can be affected by several congenital abnormalities. This phenotype is present also in the *fcd-2* mutant worms, in which developmental defects spontaneously arise in the population growing under physiological conditions. Once again the NHEJ has a role in the genesis of this phenomenon, as in the *fcd-2;lig-4* double mutant, the incidence of developmental defects are not statistically different compared to the *wt*. After CDDP treatment this phenomenon gets more evident, as in the *fcd-2* worms we found a 7-folds increase in the developmental defects frequency compared to the *wt* and *fcd-2;lig-4* double mutant.

This work for the first time proposes a role of the *fcd-2* gene in the preservation of genomic stability through the down regulation of Non-Homologous End Joining. The findings shown in this study, could have a relevant impact on the mammalian system in the understanding of the molecular interactions between *Fanconi Anaemia* proteins and their action in preserving the genomic stability, in particular explaining cancer susceptibility displayed in FA patients. The improper use of NHEJ pathway in a temporal window during which it should be switched off or its use in an uncontrolled level could be at the base of the genomic instability and the high level of tumour malignancy characterizing this syndrome, therefore NHEJ components could be used also as a new target for drugs to prevent delay cancer onset. In conclusion, this work also demonstrated 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 Fanconi anemia, to identify genetic interaction among repair genes and pathways involved in genome stability, between repair genes and checkpoint/apoptosis genes (Adamo et al., 2008; Boulton, 2006b; Collis 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.

5. ACKNOWLEDGEMENTS

I would like to thank all the people who gave me the opportunity to follow my interests and made possible to follow my scientific formation path.
In particular, I would like to thank Dr. Adriana La Volpe, who gave me the opportunity to have my PhD in her lab and Adele Adamo, who trained me in the beginning and taught me all the basics about working with *C. elegans* as model system, and with whom a personal, over than a professional interaction, has come out during these years. I want to thank Prof. John Pulitzer Finali for his trust in my job, in my skills, and for the helpful suggestions and scientific discussions, and Prof. Girolama La Mantia for her patience and availability during these years. I would like to thank also the EMBO organization that granted me a three months short term fellowship by which I could perform part of the experiments shown in this thesis and Dr. Enrique Martinez-Perez, who gave me the possibility to spend my short term EMBO fellowship in his lab at the University of Sheffield. My acknowledgements also go to Dr. Aurora Storlazzi, Dr. Silvana Gargano and Dr. Maria Ciaramella's group for the always helpful advices and scientific discussions.

6. LITERATURE CITED

Adamo A, Montemauri P, Silva N, Ward JD, Boulton SJ, La Volpe A. BRC-1 acts in the inter-sister pathway of meiotic double-strand break repair. *EMBO Rep*. 2008 Mar;9(3):287-92. Epub 2008 Jan 25.

Ahmed S, Alpi A, Hengartner MO, Gartner A. *C. elegans* RAD-5/CLK-2 defines a new DNA damage checkpoint protein. *Curr Biol*. 2001 Dec 11;11(24):1934-44.

Ahmed S, Hodgkin J. MRT-2 checkpoint protein is required for germline immortality and telomere replication in *C. elegans*. *Nature*. 2000 Jan 13;403(6766):159-64.

Ashley T, Plug AW, Xu J, Solari AJ, Reddy G, Golub EI, Ward DC. Dynamic changes in Rad51 distribution on chromatin during meiosis in male and female vertebrates. *Chromosoma*. 1995 Oct;104(1):19-28.

Barnes TM, Kohara Y, Coulson A, Hekimi S. Meiotic recombination, noncoding DNA and genomic organization in Caenorhabditis elegans. *Genetics*. 1995 Sep;141(1):159-79.

Baudat F, Manova K, Yuen JP, Jasin M, Keeney S. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. *Mol Cell*. 2000 Nov;6(5):989-98.

Bergerat A, de Massy B, Gadelle D, Varoutas PC, Nicolas A, Forterre P. An atypical topoisomerase II from Archaea with implications for meiotic recombination. *Nature*. 1997 Mar 27;386(6623):414-7.

Bishop DK, Zickler D. Early decision; meiotic crossover interference prior to stable strand exchange and synapsis. *Cell*. 2004 Apr 2;117(1):9-15.

Boulton SJ. BRCA1-mediated ubiquitylation. *Cell Cycle*. 2006 Jul;5(14):1481-6. Epub 2006 Jul 17. Review.

Brenner S. The genetics of Caenorhabditis elegans. *Genetics*. 1974 May;77(1):71-94.

Cary RB, Peterson SR, Wang J, Bear DG, Bradbury EM, Chen DJ. DNA looping by Ku and the DNA-dependent protein kinase. *Proc Natl Acad Sci U S A*.1997; 94: 4267–72.

Chin GM, Villeneuve AM. *C. elegans* mre-11 is required for meiotic recombination and DNA repair but is dispensable for the meiotic G(2) DNA damage checkpoint. *Genes Dev*. 2001 Mar 1;15(5):522-34.

Clejan I, Boerckel J, Ahmed S. Developmental modulation of nonhomologous end joining in *Caenorhabditis elegans*. *Genetics*. 2006 Jul;173(3):1301-17. Epub 2006 May 15.

Colaiácovo MP, MacQueen AJ, Martinez-Perez E, McDonald K, Adamo A, La Volpe A, Villeneuve AM. Synaptonemal complex assembly in C. elegans is dispensable for loading strand-exchange proteins but critical for proper completion of recombination. *Dev Cell*. 2003 Sep;5(3):463-74.

Calsou P, Frit P, Humbert O, Muller C, Chen DJ, Salles B. The DNA-dependent protein kinase catalytic activity regulates DNA end processing by means of Ku entry into DNA. *J Biol Chem*. 1999; 274: 7848–56.

Chan DW, Ye RQ, Veillette CJ, Lees-Miller SP. DNA-dependent protein kinase phosphorylation sites in Ku70/80 heterodimer. *Biochemistry*. 1999; 38: 1819–28.

Chan DW, Lees-Miller SP. The DNA-dependent protein kinase is inactivated by autophosphorylation of the catalytic subunit. *J Biol Chem*. 1996; 271: 8936–41.

Colaiácovo MP. The many facets of SC function during *C. elegans* meiosis. *Chromosoma*. 2006 Jun;115(3):195-211. Epub 2006 Mar 23. Review.

Collis SJ, Barber LJ, Ward JD, Martin JS, Boulton SJ.. *C. elegans* FANCD2 responds to replication stress and functions in interstrand cross-link repair. *DNA Repair (Amst)*. 2006 Nov 8;5(11):1398-406. Epub 2006 Aug 17

Couteau F, Goodyer W, Zetka M. Finding and keeping your partner during meiosis. *Cell Cycle*. 2004 Aug;3(8):1014-6. Epub 2004 Aug 7. Review.

Couteau F, Nabeshima K, Villeneuve A, Zetka M. A component of *C. elegans* meiotic chromosome axes at the interface of homolog alignment, synapsis, nuclear reorganization, and recombination. *Curr Biol*. 2004 Apr 6;14(7):585-92.

Critchlow SE, Bowater RP, Jackson SP. (1997) Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. *Curr Biol*.; 7: 588–98

Daley JM, Palmbos PL, Wu D, Wilson TE. Nonhomologous end joining in yeast.*Annu Rev Genet*. 2005;39:431-51.

Dernburg AF, McDonald K, Moulder G, Barstead R, Dresser M, Villeneuve AM. Meiotic recombination in C. elegans initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. *Cell*. 1998 Aug 7;94(3):387-98.

Derry WB, Putzke AP, Rothman JH. *Caenorhabditis elegans* p53: role in apoptosis, meiosis, and stress resistance. *Science*. 2001 Oct 19;294(5542):591-5. Epub 2001 Sep 13.

Ellis HM, Horvitz HR. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell*. 1986 Mar 28;44(6):817-29.

Feldmann E, Schmiemann V, Goedecke W, Reichenberger S, Pfeiffer P. DNA double-strand break repair in cellfree extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining. *Nucleic Acids Res*. 2000; 28: 2585–96.

Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*. 2001 Feb;7(2):249-62.

Gartner A, Milstein S, Ahmed S, Hodgkin J, Hengartner MO. A conserved checkpoint pathway mediates DNA damage--induced apoptosis and cell cycle arrest in *C. elegans*. *Mol Cell*. 2000 Mar;5(3):435-43.

Goodyer W, Kaitna S, Couteau F, Ward JD, Boulton SJ, Zetka M. HTP-3 links DSB formation with homolog pairing and crossing over during *C. elegans meiosis*. *Dev Cell*. 2008 Feb;14(2):263-74.

Grawunder U, Wilm M, Wu XT, Kulesza P, Wilson TE, Mann M, Lieber MR. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature* 1997.; 388: 492–5.

Grawunder U, Zimmer D, Fugmann S, Schwarz K, Lieber MR. DNA ligase-IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. *Mol Cell* 1998.; 2: 477–84.

Grelon M, Vezon D, Gendrot G, Pelletier G. AtSPO11-1 is necessary for efficient meiotic recombination in plants. *EMBO J*. 2001 Feb 1;20(3):589-600.

Gumienny TL, Lambie E, Hartwieg E, Horvitz HR, Hengartner MO. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. *Development*. 1999 Feb;126(5):1011-22.

Hartman PS, Herman RK. Radiation-sensitive mutants of *Caenorhabditis elegans*. *Genetics*. 1982 Oct;102(2):159-78.

Hengartner MO, Ellis RE, Horvitz HR. *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. *Nature*. 1992 Apr 9;356(6369):494-9.

Hillers KJ, Villeneuve AM. Chromosome-wide control of meiotic crossing over in C. elegans. *Curr Biol*. 2003 Sep 16;13(18):1641-7.

Hodgkin J, Horvitz HR, Brenner S. Nondisjunction Mutants of the Nematode *Caenorhabditis elegans*. *Genetics*. 1979 Jan;91(1):67-94.

Hofmann ER, Milstein S, Boulton SJ, Ye M, Hofmann JJ, Stergiou L, Gartner A, Vidal M, Hengartner MO. *Caenorhabditis elegans* HUS-1 is a DNA damage checkpoint protein required for genome stability and EGL-1-mediated apoptosis. *Curr Biol*. 2002 Nov 19;12(22):1908-18.

Holliday R. The induction of mitotic recombination by mitomycin C in Ustilago and Saccharomyces. *Genetics*. 1964 Sep;50:323-35

Hollingsworth NM, Ponte L, Halsey C. MSH5, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in *Saccharomyces cerevisiae* but not mismatch repair. *Genes Dev*. 1995 Jul 15;9(14):1728-39.

Houghtaling S, Timmers C, Noll M, Finegold MJ, Jones SN, Meyn MS, Grompe M. Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev*. 2003 Aug 15;17(16):2021-35. Epub 2003 Jul 31.

Keeney S, Giroux CN, Kleckner N. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell*. 1997 Feb 7;88(3):375-84.

Kelly KO, Dernburg AF, Stanfield GM, Villeneuve AM. *Caenorhabditis elegans* msh-5 is required for both normal and radiation-induced meiotic crossing over but not for completion of meiosis. *Genetics*. 2000 Oct;156(2):617-30.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 1997 Nov 14;91(4):479-89.

MacQueen AJ, Colaiácovo MP, McDonald K, Villeneuve AM. Synapsis-dependent and -independent mechanisms stabilize homolog pairing during meiotic prophase in *C. elegans*. *Genes Dev*. 2002 Sep 15;16(18):2428-42.

Martin JS, Winkelmann N, Petalcorin MI, McIlwraith MJ, Boulton SJ. RAD-51-dependent and -independent roles of a *Caenorhabditis elegans* BRCA2-related protein during DNA double-strand break repair. *Mol Cell Biol*. 2005 Apr;25(8):3127-39.

Martinez-Perez E, Schvarzstein M, Barroso C, Lightfoot J, Dernburg AF, Villeneuve AM. Crossovers trigger a remodeling of meiotic chromosome axis composition that is linked to two-step loss of sister chromatid cohesion. *Genes Dev*. 2008 Oct 15;22(20):2886-901.

Martinez-Perez E, Villeneuve AM. HTP-1-dependent constraints coordinate homolog pairing and synapsis and promote chiasma formation during *C. elegans* meiosis. *Genes Dev*. 2005 Nov 15;19(22):2727-43.

Moens PB, Chen DJ, Shen Z, Kolas N, Tarsounas M, Heng HH, Spyropoulos B. Rad51 immunocytology in rat and mouse spermatocytes and oocytes. *Chromosoma*. 1997 Sep;106(4):207-15.

Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, Wang ZQ, Jasin M. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc Natl Acad Sci U S A*. 2005 Jan 25;102(4):1110-5. Epub 2005 Jan 13.

Narod SA, Foulkes WD. BRCA1 and BRCA2: 1994 and beyond. *Nat Rev Cancer*. 2004 Sep;4(9):665-76.

Nick McElhinny SA, Snowden CM, McCarville J, Ramsden DA. () Ku recruits the XRCC4-ligase IV complex to DNA ends. *Mol Cell Biol*. 2000; 20: 2996–3003.

Oliver-Bonet M, Turek PJ, Sun F, Ko E, Martin RH. Temporal progression of recombination in human males. *Mol Hum Reprod*. 2005 Jul;11(7):517-22.

Pang D, Yoo S, Dynan WS, Jung M, Dritschilo A. Ku proteins join DNA fragments as shown by atomic force microscopy. *Cancer Res*. 1997; 57: 1412–5.

Papavasiliou FN, Schatz DG. Cell-cycle-regulated DNA double-stranded breaks in somatic hypermutation of immunoglobulin genes. *Nature. 2000* Nov 9;408(6809):216-21.

Pasierbek P, Jantsch M, Melcher M, Schleiffer A, Schweizer D, Loidl J. A *Caenorhabditis elegans* cohesion protein with functions in meiotic chromosome pairing and disjunction. *Genes Dev*. 2001 Jun 1;15(11):1349-60.

Resnick MA. The repair of double-strand breaks in DNA; a model involving recombination. *J Theor Biol*. 1976 Jun;59(1):97-106.

Rich T, Allen RL, Wyllie AH. Defying death after DNA damage. *Nature*. 2000 Oct 12;407(6805):777-83. Review.

Rinaldo C, Bazzicalupo P, Ederle S, Hilliard M, La Volpe A. Roles for *Caenorhabditis elegans* rad-51 in meiosis and in resistance to ionizing radiation during development. *Genetics*. 2002 Feb;160(2):471-9.

Rinaldo C, Ederle S, Rocco V, La Volpe A. The Caenorhabditis elegans RAD51 homolog is transcribed into two alternative mRNAs potentially encoding proteins of different sizes. *Mol Gen Genet*. 1998 Nov;260(2-3):289-94.

Rockmill B, Sym M, Scherthan H, Roeder GS. Roles for two RecA homologs in promoting meiotic chromosome synapsis. *Genes Dev*. 1995 Nov 1;9(21):2684-95.

Roeder GS. Meiotic chromosomes: it takes two to tango. *Genes Dev*. 1997 Oct 15;11(20):2600-21.

Ross-Macdonald P, Roeder GS. Mutation of a meiosis-specific MutS homolog decreases crossing over but not mismatch correction. *Cell*. 1994 Dec 16;79(6):1069-80.

Schumacher B, Hofmann K, Boulton S, Gartner A. The *C. elegans* homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. *Curr Biol*. 2001 Oct 30;11(21):1722-7.

Severson AF, Ling L, van Zuylen V, Meyer BJ. The axial element protein HTP-3 promotes cohesin loading and meiotic axis assembly in *C. elegans* to implement the meiotic program of chromosome segregation. *Genes Dev*.

2009 Aug 1;23(15):1763-78. Epub 2009 Jul 2.

Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. *Cell Res*. 2008 Jan;18(1):134-47.

Smolikov S, Eizinger A, Schild-Prufert K, Hurlburt A, McDonald K, Engebrecht J, Villeneuve AM, Colaiácovo MP. SYP-3 restricts synaptonemal complex assembly to bridge paired chromosome axes during meiosis in *Caenorhabditis elegans*. *Genetics*. 2007 Aug;176(4):2015-25. Epub 2007 Jun 11.

Smolikov S, Schild-Prüfert K, Colaiácovo MP. CRA-1 uncovers a double-strand break-dependent pathway promoting the assembly of central region proteins on chromosome axes during *C. elegans* meiosis. *PLoS Genet*. 2008 Jun 6;4(6):e1000088.

Storlazzi A, Tessé S, Gargano S, James F, Kleckner N, Zickler D. Meiotic double-strand breaks at the interface of chromosome movement, chromosome remodeling, and reductional division. *Genes Dev*. 2003 Nov 1;17(21):2675-87. Epub 2003 Oct 16.

Sulston JE, Schierenberg E, White JG, Thomson JN. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol*. 1983 Nov;100(1):64-119.

Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. The double-strand-break repair model for recombination. *Cell*. 1983 May;33(1):25-35. Review.

Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, Kim ST, Lane WS, Kastan MB, D'Andrea AD. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell*. 2002 May 17;109(4):459-72.

Tsuzuki T, Fujii Y, Sakumi K, Tominaga Y, Nakao K, Sekiguchi M, Matsushiro A, Yoshimura Y, MoritaT. Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. *Proc Natl Acad Sci U S A*. 1996 Jun 25;93(13):6236-40.

Usui T, Ohta T, Oshiumi H, Tomizawa J, Ogawa H, Ogawa T. Complex formation and functional versatility of Mre11 of budding yeast in recombination. *Cell*. 1998 Nov 25;95(5):705-16

van Gent DC, Hoeijmakers JH, Kanaar R. Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet. 2001*. Mar;2(3):196-206.

Weinstock DM, Richardson CA, Elliott B, Jasin M.Modeling oncogenic translocations: distinct roles for double-strand break repair pathways in translocation formation in mammalian cells. *DNA Repair (Amst)*. 2006 Sep 8;5(9-10):1065-74. Epub 2006 Jul 11.

Winand NJ, Panzer JA, Kolodner RD. Cloning and characterization of the human and *Caenorhabditis elegans* homologs of the *Saccharomyces cerevisiae* MSH5 gene. *Genomics*. 1998 Oct 1;53(1):69-80.

Yamamoto K, Ishiai M, Matsushita N, Arakawa H, Lamerdin JE, Buerstedde JM, Tanimoto M, Harada M, Thompson LH, Takata M. Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. *Mol Cell* Biol. 2003 Aug;23(15):5421-30.

Yaneva M, Kowalewski T, Lieber MR. Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies. *EMBO J*. 1997; 16: 5098–112.

Zalevsky J, MacQueen AJ, Duffy JB, Kemphues KJ, Villeneuve AM. Crossing over during *Caenorhabditis elegans* meiosis requires a conserved MutS-based pathway that is partially dispensable in budding yeast. *Genetics*. 1999 Nov;153(3):1271-83.

Zetka MC, Kawasaki I, Strome S, Müller F. Synapsis and chiasma formation in Caenorhabditis elegans require HIM-3, a meiotic chromosome core component that functions in chromosome segregation. *Genes Dev*. 1999 Sep 1;13(17):2258-70.

Zickler D, Kleckner N. Meiotic chromosomes: integrating structure and function. *Annu Rev Genet*. 1999;33:603-754. Review.