The C. elegans p53 Family Gene cep-1 and The Nondisjunction Gene him-5 Are Required for Meiotic Recombination

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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Abstract

p53 promotes maintenance of genetic information either by causing apoptosis of damaged cells, or by altering the cell cycle and repair pathways such that damage can be accurately repaired. The nematode *Caenorhabditis elegans* possesses only one p53 family member, CEP-1, that controls apoptosis and the cell cycle in response to genotoxic stress.

Mutation in the meiotic gene *him-5* increases nondisjunction of the X chromosome, resulting in increased frequencies of XO male and XXX Dpy progeny, and it affects the frequency of meiotic recombination on X. *him-5* is allelic to the ORF *D1086.4*, which encodes a putative basic protein with no clear homologues or domain structure. The modest embryonic lethality (Emb) of *him-5* mutants is dramatically increased by mutation of *cep-1* but no change is seen in the proportion of XO male or XXX Dpy progeny. The synergistic effects of *cep-1* and *him-5* mutation are independent of CEP-1's DNA damage regulators and other meiotic mutants, and they do not involve deregulated apoptosis.
cep-1; him-5 double mutants have abnormal chromatin morphology in diakinesis-arrested oocytes reminiscent of that seen in double strand break (DSB) repair mutants. This phenotype depends on the presence of SPO-11-induced meiotic DSBs, suggesting CEP-1 and HIM-5 function together to promote accurate recombination during meiosis. In support of this hypothesis, cep-1; him-5 show a significant reduction in crossover frequency between autosomal markers compared to wild-type or either single mutant alone, suggesting they function together to promote meiotic crossing over.

The X chromosome nondisjunction in both him-5 and cep-1; him-5 is a result of failure of DSB formation and subsequent chiasma formation on the X. However, the embryonic lethality phenotype of him-5 and cep-1; him-5 is caused by a defect either downstream or in parallel to meiotic DSB formation. The diakinesis chromatin phenotype of cep-1; him-5 suggests this defect may be in meiotic DSB repair. This is confirmed by the fact that cep-1; him-5 animals show more persistent meiotic DSB-associated RAD-51 foci staining compared to wild-type, suggesting CEP-1 and HIM-5 may function in efficient resolution of SPO-11-induced DSBs during meiosis.

A role for CEP-1 in promoting accurate repair of DSBs during meiosis may be related to p53's function in promoting faithful meiotic recombination in mammalian cells. HIM-5's role in DSB formation and repair suggests another mechanistic link between these recombination steps. Meiotic recombination is vital for genome stability, and characterization of the role of CEP-1 and HIM-5 will increase our understanding of the p53 family and genetic redundancy at multiple steps in this process.
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<tbody>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CO</td>
<td>crossover</td>
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<tr>
<td>C-terminus, C-terminal</td>
<td>carboxy-terminus, carboxy-terminal</td>
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<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
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<td>Dpy</td>
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<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>Emb</td>
<td>embryonic lethal</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>Him</td>
<td>high incidence of males</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
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<td>HRR</td>
<td>homologue recognition region</td>
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<tr>
<td>IR</td>
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<tr>
<td>Lon</td>
<td>long</td>
</tr>
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<td>MMR</td>
<td>mismatch repair</td>
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<td>NCO</td>
<td>noncrossover</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
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<td>NHEJ</td>
<td>non-homologous end joining</td>
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<td>N-terminus, N-terminal</td>
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</tr>
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<td>OD</td>
<td>oligomerization domain</td>
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<td>programmed cell death</td>
</tr>
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<td>pI</td>
<td>isoelectric point</td>
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</tr>
<tr>
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<td>SDSA</td>
<td>synthesis-dependent strand annealing</td>
</tr>
<tr>
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<tr>
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1 Introduction

1.1 p53 and Genomic Stability

p53 was originally discovered as a cellular protein bound to the SV40 T antigen in SV40-transformed cell lines (Lane & Crawford 1979; Linzer & Levine 1979). Although p53 was originally thought to be an oncoprotein because it was present in large quantities in transformed cells and could transform normal cells, it was later found that these initial experiments were performed with a dominant-negative mutant version of the protein (Harris 1996). Since then, the p53 protein has been shown to function as a tumour suppressor of great medical importance, since mutations in the p53 gene are found in approximately 50% of all human cancers and most or all of the remaining tumours show abrogation of p53 pathway function (Brown et al. 2009).

In terms of domain structure, the p53 protein consists of an N-terminal transactivation (TA) domain followed by a proline-rich domain and a DNA binding domain. The carboxy-terminus (C-terminus) contains an oligomerization domain (OD) that facilitates tetramerization of the protein and a C-terminal regulatory region required for controlling stability and DNA binding (Belyi et al. 2010). p53's mammalian paralogues p63 and p73 are similar in structure but have an extended C-terminus containing a sterile-alpha motif (SAM) domain following the OD. The transcripts encoding p53, and especially p63 and p73, are extensively alternatively spliced (Belyi et al. 2010). Different amino-terminal (N-terminal) splicing of these transcripts gives rise to protein isoforms that are capable of transactivation (TA isoforms) and those that inhibit transactivation by acting in a dominant negative manner (ΔN isoforms), while alternative splicing affecting the C-terminus regulates DNA binding and stability.

One of the main roles of p53 is to maintain the integrity of the genome. The p53 literature is vast and heterogeneous, with tissue specificity, the presence of partial redundancy between p53, p63 and p73, and the medical interest in studying various pathological conditions all adding complexity to our understanding of the role of the p53 family. Here, I will briefly outline current
understanding of the role of these proteins in genomic stability. Some of p53's important functions in maintaining genomic stability are outlined in Figure 1-1.

1.1.1 Abrogation of p53 Correlates with Increased Genomic Instability

1.1.1.1 Evidence from Li-Fraumeni Syndrome

One of the first suggestions that p53 was required to maintain genome stability was a report that showed fibroblasts from patients with Li-Fraumeni syndrome, an inherited cancer-prone condition stemming from heterozygous mutation of the p53 gene, had increased aneuploidy compared with normal cells (Bischoff et al. 1990). Later it was found that this may not solely be a result of haploinsufficiency but rather heterozygous mutant p53 from some Li-Fraumeni patients had acquired gain-of-function or dominant negative properties that promoted an increased frequency of mutation at other sites (Liu et al. 1996). In addition, loss of the remaining wild-type p53 allele in cells derived from Li-Fraumeni patients resulted in even greater levels of genomic instability (Burt et al. 2000).

1.1.1.2 Evidence from Mouse Models

To test the role of p53 as a tumour suppressor in vivo mouse knockout models that lacked wild-type p53 function were created. Although p53−/− mice develop normally, they acquire spontaneous tumours early in life that exhibit high rates of aneuploidy and mutation (Donehower et al. 1992). Bone marrow cells isolated from p53−/− and p53+/− mice had dramatically increased frequencies of chromosomal aberrations compared with wild-type controls, and p53−/− mice have large, polyploid multinucleated cells in their prostates (Bouffler et al. 1995; Colombel et al. 1995). Even young p53−/− mice exhibit increased aneuploidy and gene amplification in many tissues, with cells that have multiple centrosomes and abnormal mitotic spindles (Fukasawa et al. 1996). p53−/− embryos also show enhanced telomere attrition compared to littermates that contain one or two copies of wild-type p53 (Bekaert et al. 2005). As these mice age, they also accumulate mutations faster than their wild-type and heterozygous littermates, a process that can be exacerbated by treatment with the mutagen N-ethyl-N-nitrosourea (ENU) (Giese et al. 2002).
Figure 1-1: p53 functions to maintain genomic stability by promoting apoptosis, cell cycle arrest and DNA repair after genotoxic insult.

After DNA damage or in genetic backgrounds to lead to genomic instability, p53 is activated, primarily by a diverse set of kinases, and then functions to promote apoptosis, cell cycle arrest and/or various DNA repair pathways. For some of these responses, p53 directs transcription of target genes, such as the proapoptotic BH3-only gene PUMA or the CKI cell cycle regulator p21. Other functions do not require p53's transcriptional activity, such as promoting apoptosis at the mitochondria by interacting with Bcl-2 and Bcl-XL and ensuring faithful homologous recombination by limiting the function of Rad51 and its cofactor Brca2.
After exposure to ionizing radiation (IR), p53\(^{-/-}\) mice show increased aneuploidy and polyploidy that result from aberrant mitosis, and these abnormalities persist far longer than those in p53\(^{+/+}\) or p53\(^{+/-}\) animals (Chang et al. 2000). Irradiated p53\(^{-/-}\) mice also showed higher rates of loss of heterozygosity due to interstitial deletions than mice that retained at least one wild-type copy of p53 (Liang et al. 2002).

1.1.1.3 Evidence from Cell Lines

Cell lines have also been used to explore the relationship between p53 and genomic stability. A pair of seminal early studies showed that mutation of p53 created conditions permissive for gene amplification that were suppressed by expressing wild-type p53 in cells from Li-Fraumeni patients (Livingstone et al. 1992; Yin et al. 1992). p53 inactivation in human cells causes spontaneous mutations and in a repeated sequence stability assay, cells with mutant p53 had a much higher deletion rate than those with only wild-type p53 (Havre et al. 1995; Gebow et al. 2000). Both p53 deficient fibroblasts and those transfected with mutant p53 have high degrees of genomic instability, including aneuploidy and dicentric chromosomes (Harvey, Sands et al. 1993; Tainsky et al. 1995). Similar to what was observed in mice, IR significantly exacerbates the karyotype defects of cells with mutant p53 compared with isogenic p53\(^{+/+}\) controls (Moore et al. 2005). In human osteosarcoma cells, p53 even played a protective role in maintaining the integrity of the mitochondrial DNA (Zuffa et al. 2008).

1.1.1.4 Evidence from Human Cancers

The study of human cancers has also provided evidence that p53 is required to maintain genome stability. Surveys of various cancers, including those of the gastric system, esophagus, ovaries, colorectum, pharynx, breast, lung, bladder, head and neck, prostate and liver, as well as osteosarcoma and leukemia, have shown that cases with mutant or dysregulated p53 were much more likely to display aneuploidy and chromosome instability (Tamura et al. 1991; Ramel et al. 1992; Kihana et al. 1992; Remvikos et al. 1992; Bourhis et al. 1994; Eyfjörd et al. 1995; Sauter et al. 1995; Dalquen et al. 1997; Tang et al. 1998; Shin et al. 2001; Al-Maghrabi et al. 2001;
Overholtzer et al. 2003; Guido et al. 2008). In some human neoplasms, including Barrett's esophagus, colorectal carcinoma and skin cancer, p53 mutation or dysregulation has been shown to precede and predict progression from diploidy to aneuploidy, often through an intermediate tetraploid stage (Carder et al. 1993; Blount et al. 1994; Burnworth et al. 2007; Galipeau et al. 1996). p53 abrogation or impaired p53 regulation is also correlated with other types of genomic instability, including loss-of-heterozygosity of specific loci in breast cancer, proto-oncogene amplification in urothelial cancer and chromosome fragility in head and neck cancer (Deng et al. 1994; Habuchi et al. 1994; Gallo et al. 1995). In hepatocellular carcinoma, abnormal increases in centromere number, which can lead to polyploidy or aneuploidy, are associated with p53 mutation (Nakajima et al. 2004).

1.1.1.5 Sensitivity to DNA damage

It has also been shown that p53 deficient mice or cell lines are less efficient at recovering from genotoxic insults. For instance, p53 mutant or deficient mice have a greater likelihood of developing cancer and reduced latency of tumour formation after exposure to mutagens, such as the liver carcinogen dimethylnitrosamine, γ-IR, and asbestos (Harvey, McArthur et al. 1993; Lee et al. 1994; Vaslet et al. 2002). p53 also contributes to DNA damage protection early in life, since p53 deficient mice exposed to mutagens before or during pregnancy show higher rates of death or morphological defects among their offspring than wild-type controls (Nicol et al. 1995; Armstrong et al. 1995; Baatout et al. 2002). Induction of p53 in mouse embryonic fibroblasts protects against ultraviolet (UV)-induced mutagenesis, whereas ENU treatment induced a higher frequency of mutations in the liver of $p53^{+/}$ mice compared with $p53^{+/-}$ animals (Yuan et al. 1995; Giese et al. 2002). Cells with reduced p53 function are particularly susceptible to genotoxins that impair mitosis and give rise to polyploidy and centrosome amplification, defects which often lead to aneuploidy in future cell generations (Wang et al. 1998; Tsuiki et al. 2001; Shinmura et al. 2008).

Many anticancer therapies function by damaging the DNA of quickly growing cancer cells in hopes of halting or reducing tumour growth. Often the efficient function of genotoxic anticancer
therapies depends on an intact p53 pathway, as has been reported for radiotherapy against lung
and prostate cancer as well as doxorubicin treatment of breast cancer patients (Langendijk et al.
1995; Prendergast et al. 1996; Aas et al. 1996). Similar conclusions were reached in a study
comparing the sensitivity of a panel of cell lines to a set of frequently used anticancer drugs:
those with p53 mutation showed less growth inhibition than those with wild-type p53 (O'Connor
et al. 1997). It has also been shown that cancers that were initially sensitive to an anticancer
drug can acquire p53 mutation and become resistant to that drug. This is especially true of
ovarian cancers and osteosarcomas treated with the alkylating agent cisplatin (Petty et al. 1998;
Asada et al. 1999). Importantly, sensitivity to cisplatin in resistant osteosarcoma and ovarian cell
lines can be restored by re-introduction of wild-type p53 (Okaichi et al. 1998; Branch et al.
2000).

1.1.1.6 Genetic Interactions with Other Genomic Instability Mutants

Ablation of p53 compounds the genomic instability in mice with mutations in DNA repair genes.
For instance, mice mutant for the DNA mismatch repair (MMR) gene Msh2, like those mutant
for p53, are viable but cancer prone, but p53<sup>−/−</sup> Msh2<sup>−/−</sup> show complete inviability of female
offspring and reduced tumour latency in males (Cranston et al. 1997). p53 deficiency accelerates
the development of genomic instability and induces cancer in mice with mutations in the DSB
repair protein BRCA1, the telomere maintenance enzyme telomerase, crosslink repair protein
Fancc, DNA damage-dependent nuclease Mre11, the non-homologous end joining (NHEJ)
protein XRCC4, the MMR protein Msh6 and the DNA repair helicase Blm (Xu et al. 1999;
Artandi et al. 2000; Freie et al. 2003; Theunissen et al. 2003; Yan et al. 2006; Young et al. 2007;
Babbe et al. 2009). The combination of mutation in p53 and genes required for MMR causes
microsatellite instability, a phenotype not seen in cells with either mutation alone (Lin et al.
2000).

Conversely, p53 mutation can rescue the viability of some DNA repair mutants, though it also
exacerbates their susceptibility to tumours. Since p53's role is to halt cell growth in the presence
of significant DNA damage (see below), some DNA repair mutants show developmental blocks
that can be rescued by mutation of the p53 gene. Mice with mutations in the Rad51 recombinase, the BRCA1 and BRCA2 DSB repair genes, the XRCC4 NHEJ component, and the Lig4 DNA ligase all show increased survival when p53 is also mutated (Lim & Hasty 1996; Ludwig et al. 1997; Gao et al. 2000; Frank et al. 2000). Despite the increased survival of these mice, their cells have increased levels of genomic instability. Similar results have been found for cell lines: fibroblasts from Werner's Syndrome patients, characterized by premature aging due to telomere attrition, senesce prematurely in the background of wild-type p53, but continue cycling when p53 is mutated (Davis et al. 2003).

This evidence showing that lack of p53 engenders greater genomic instability in mouse models, cell lines and in humans strongly suggests that p53 plays a major role in maintaining the integrity of the genetic material. What are the molecular functions of p53 and how does it respond to genotoxic insult?

1.1.2 p53 Protein Expression and Function are Induced by DNA Damage

The first indication that p53 was a DNA damage response gene came when Maltzman and Czyzyk (1984) showed that cellular levels of p53 increase after UV irradiation. This increase in p53 levels is due almost entirely to an increase in the protein's half-life, but some groups have also found an increase in p53 mRNA production (Liu et al. 1994; Sun et al. 1995). The induction of p53 after UV does not occur when cells express a p53 mutant with dominant negative function (Zhan et al. 1993). p53 is primarily known to function as a transcription factor, and its elevated protein levels after UV are associated with increased transcriptional activity (Lu & Lane 1993).

In addition to UV, DSBs caused by IR, bleomycin or restriction enzyme activity also increase p53 protein levels, protein half-life and transactivation activity (Lu & Lane 1993; Price & Calderwood 1993; Nelson & Kastan 1994). Most point mutations that inactivate p53 also prevent its degradation, so cells that have mutant p53 generally have higher levels of p53 protein that are not further increased upon DNA damage (Unger et al. 1994). Some evidence has also been found for increases in p53 mRNA levels after DSB damage (Sakhi et al. 1994).
Other types of DNA damage also induce p53. In ovarian cancers, p53 accumulation was found only in those cell lines that show growth inhibition in the presence of cisplatin (Perego et al. 1996). Tetraploid cells, which have been shown to be at risk for developing aneuploidy, also have an enhanced baseline of p53 activation (Castedo et al. 2006).

### 1.1.2.1 Regulation by Post-Translational Modification

Various post-translational modifications are required to stabilize p53 and stimulate its transcriptional activity in response to DNA damage. p53 accumulation and function is modulated by the actions of several protein kinases. For instance, cells lacking the ATM kinase, associated with the disorder ataxia-telangiectasia, are sensitive to IR damage and defective in stabilizing p53 (Xu & Baltimore 1996). ATM phosphorylates p53 at serine 15 (Ser15) in response to IR, and this modification is well correlated with p53 accumulation (Nakagawa et al. 1999). After IR, ATM is also required for the dephosphorylation of p53 at Ser376, though the phosphatase directly responsible for this modification is not known. Dephosphorylation at this site reveals a consensus binding site for 14-3-3 proteins, whose binding to p53 increases its affinity for its consensus binding sites in target genes (Waterman et al. 1998). In UV irradiated cells, the ATM-related ATR replaces ATM as the kinase responsible for Ser15 phosphorylation (Mohanty et al. 2008).

After DNA damage caused by IR, UV or genotoxic drugs such as genestein and etoposide, phosphorylation of Ser15 promotes p53-dependent transactivation of target genes and p53 accumulation by decreasing its interaction with the major p53 negative regulator Mdm2 (Siliciano et al. 1997; Shieh et al. 1997; Ye et al. 2001). Mdm2 is an E3 ubiquitin ligase whose main function is to down-regulate p53 by sequestration and ubiquitination which leads to p53 degradation or reduced function (Coutts et al. 2009). After DNA damage, interruption of the interaction between Mdm2 and p53 by various protein modifications reduces p53 ubiquitination, allowing it to accumulate and activate transcription (Maki & Howley 1997). DNA damage also induces phosphorylation of p53 at Ser20, which also inhibits the p53-Mdm2 interaction. ATM/ATR do not phosphorylate this site directly, but are required for Chk2 phosphorylation of...
this site after genotoxic stress (Chehab et al. 1999; Chehab et al. 2000; Hirao et al. 2000). p53 is also phosphorylated at its C-terminus. In particular, Ser392 is phosphorylated in response to UV but not IR and may play a role in inducing a UV-specific transcriptional program (Kapoor & Lozano 1998). Both p38 and CK2 kinases are implicated in phosphorylating p53 at this site (Huang et al. 1999; Keller et al. 2001). Other kinases implicated in modulating p53 activity in response to DNA damage are SMG-1 and DNA-PK, and cells with deficiencies in these kinases show a reduced p53 response after genotoxic stress (Brumbaugh et al. 2004; Boehme et al. 2008).

p53 is also acetylated at several lysine residues in its C terminus in response to UV or IR damage, increasing its sequence-specific DNA binding (Sakaguchi et al. 1998). Mutation of these lysines leads to decreased transactivation of p53 target genes after DNA damage (Feng et al. 2005).

It is clear that p53 is activated and stabilized by genotoxic stress, and that this is accomplished by many different mechanisms, most of which are post-translational. Collectively, these modifications enable p53 for its central role as guardian of the genome. Activated p53 promotes genome stability after DNA damage in three major ways: by halting the cell cycle to allow time for repair, by inducing apoptosis of damaged cells, and by directly promoting DNA repair.

1.1.3 p53 Induces Cell Cycle Arrest After DNA Damage

Early work on p53 showed that cells damaged by IR undergo cell cycle arrest and inhibit DNA synthesis, and that this process was dependent on the presence of functional p53 protein (Kastan et al. 1991; Kuerbitz et al. 1992). In human tumour cell lines the ability for IR to induce cell cycle arrest was directly correlated with radiosensitivity of the cell line, and knockdown of p53 or expression of a dominant negative p53 mutant after IR prevented cell cycle arrest, and increased cellular survival and the incidence of reciprocal translocations (O'Connor et al. 1993; McIlwrath et al. 1994; Gupta et al. 1996). In some cell types, including human fibroblasts, IR-
induced, p53-dependent cell cycle arrest appears to be irreversible, whereby cells undergo senescence and show long-term up-regulation of p53 (Di Leonardo et al. 1994).

Other types of genotoxic stress can also induce cell cycle arrest, including UV, cisplatin, etoposide and mitomycin C (Tsuji & Ogawa 1994; Eliopoulos et al. 1995; Dudley et al. 2008). Even introduction of a linear plasmid, circular gapped plasmid or ssDNA creates a DNA damage signal that can lead to cell cycle arrest (Almasan et al. 1995; Huang et al. 1996). Severe telomere shortening or deprotection and aberrant mitosis caused by spindle inhibiting drugs or mutations in spindle checkpoint genes also lead to p53-dependent cell cycle arrest (Chin et al. 1999; Casenghi et al. 1999; Smogorzewska & de Lange 2002; Jung et al. 2005). Cells which become tetraploid or acquire supernumerary centromeres arrest in a p53-dependent manner (Borel et al. 2002; Pieroni et al. 2008).

The most important downstream regulator for p53-dependent cell cycle arrest is the cyclin dependent kinase inhibitor (CKI) p21/Cip1/Waf1. After DNA damage, wild-type p53 transcriptionally induces p21, increasing the cellular levels of p21 transcript and protein (Dulić et al. 1994; el-Deiry et al. 1994; Slebos et al. 1994). Highlighting the importance of this cell cycle regulator, the degree of cell cycle arrest in a cell line is highly correlated to the degree of p21 accumulation (Fan et al. 1994). p21<sup>-/-</sup> mice show no defects in p53-dependent apoptosis after DNA damage, but fibroblasts from these mice are defective for cell cycle arrest after DNA damage (Brugarolas et al. 1995). Like p53, mutation of p21 extends the survival of mice deficient for Brca1, suggesting that p53- and p21-dependent arrest of the cell cycle plays a role in the early death of these embryos (Hakem et al. 1997). After treatment with spindle inhibiting drugs, p21 acts downstream of the spindle checkpoint and prevents reentry into the cell cycle after failure of mitosis, causing the cells to senesce with a 4N content (Khan & Wahl 1998; Fang et al. 2006). p21 also plays a major role in the senescence response to telomere attrition (Saretzki et al. 1999).

One of the main targets of the p21 CKI is the cyclin-dependent kinase Cdc2 (Salaun et al. 2008). In response to IR, p53 and p21 are required for down-regulation of Cdc2 mRNA and protein,
halting the cell cycle (Azzam et al. 1997). p53 can also inhibit Cdc2 function by sequestering it to the nucleus to inhibit cell cycle progression (Winters et al. 1998).

In addition to p21, p53 induces transcription of several other targets that can halt the cell cycle, including Gadd45, 14-3-3σ and the miR-34 family of miRNAs (Kastan et al. 1992; Hermeking et al. 1997; He et al. 2007).

The fact that p53 halts the cell cycle after DNA damage prompted Lane to hypothesize that p53 reduces genomic instability by allowing extra time for DNA to be repaired before cell division, dubbing it 'the guardian of the genome' (Lane 1992). There is evidence to support his hypothesis: when mice experience genotoxic stress as a result of a mutation in a gene required for NHEJ or because of shortened telomeres, p53-mediated cell cycle arrest in the absence of apoptosis is sufficient to protect them against tumorigenesis (Feldser & Greider 2007; Van Nguyen et al. 2007).

p53-induced cell cycle arrest is a major method used by the cell to maintain genomic stability after genotoxic insult. However, in certain cell types, or when damage becomes too severe, the p53 response is not arrest but activation of apoptosis, eliminating the damaged cell and preserving genome integrity.

1.1.4 p53 Induces Apoptosis in DNA-damaged Cells

It soon became clear that cell cycle arrest is not the only p53 response to DNA damage. In certain cell types, including skin cells, hematopoietic cells, intestinal cells and thymocytes, wild-type p53, but not mutant p53, leads to increased apoptosis after genotoxic stresses such as IR, UV, 5-fluorouracil, etoposide, and adriamycin (Lowe, Schmitt et al. 1993; Lee & Bernstein 1993; Clarke et al. 1993; Lowe, Ruley et al. 1993; Lotem & Sachs 1993; Ziegler et al. 1994; Merritt et al. 1994; Clarke et al. 1994). Reintroduction of p53 into p53 mutant hematopoietic cells also induces apoptosis after IR damage (Guillouf et al. 1995). Tumours with normal p53 activity contain a high proportion of apoptotic cells and respond better to chemotherapy or radiation treatment than those without p53 (Lowe et al. 1994). In a human lymphoid cell line,
p53-induced apoptosis protects cell populations from genomic instability in the form of dicentric and acentric chromosome fragments (Schwartz & Jordan 1997). Severe telomere shortening can also lead to p53-dependent apoptosis and this apoptosis, not cell cycle arrest, protects against intestinal failure in telomerase-deficient mice (Chin et al. 1999; Begus-Nahrmann et al. 2009).

Robust p53 apoptotic response after DNA damage requires BLM and WRN, two RecQ helicases. Mutations in these helicases are associated with human syndromes characterized by increased genomic instability (Spillare et al. 1999; Blander et al. 2000; Wang et al. 2001).

Downstream of p53, much of the apoptotic response to genotoxic stress is mediated by p53-dependent transcription. In response to DNA damage, p53 transcriptionally upregulates the cell death receptors KILLER/DR5 and Fas/Apo1 (Wu et al. 1997; Bouvard et al. 2000). p53 also upregulates the mRNA encoding the proapoptotic BH3-only proteins Puma, Noxa, Bax and Bid in response to IR (Bouvard et al. 2000; Fei et al. 2002; Sax et al. 2002). In some cell types, p53-dependent induction of Puma is required for DNA damage-induced apoptosis (Wang et al. 2007). The mitochondrially-located p53-regulated apoptosis-inducing protein 1 (p53AIP1) is also transcriptionally activated by p53 after DNA damage. Phosphorylation of p53 on Ser46 is required for p53AIP1 induction and the resultant apoptotic cell death (Oda et al. 2000; Okamura et al. 2001). The DNA damage-induced apoptotic program is also mediated by p53-dependent transcription of the microRNA miR-34, and abrogation of this small RNA strongly attenuates apoptosis after IR (Chang et al. 2007; Raver-Shapira et al. 2007).

The proapoptotic activity of p53 does not depend entirely on transactivation. Caelles et al. (1994) showed that p53 could promote apoptosis in the absence of new RNA or protein synthesis. p53 appears to function directly at the mitochondria, forming complexes with the protective proteins Bcl2 and BclX_L triggering outer mitochondrial membrane permeabilization, caspase activation and apoptosis in response to DNA damage (Ding et al. 1998; Erster et al. 2004; Mihara et al. 2003).
Given that p53 has the capacity to promote either cell cycle arrest or apoptosis to protect against genomic instability, how is the choice between these very different outcomes made? Cell type appears to play a key role, with some tissues tending toward one outcome or the other (Song & Lambert 1999). However, growth conditions can also play a role. Cells with favourable growth factor conditions have been found to undergo cell cycle arrest when challenged with low levels of IR, while increasing the degree of damage or decreasing the permissiveness of the growth medium tends to cause apoptosis (Gottlieb et al. 1996). Prolonged induction of p53 tends to activate apoptosis rather than induce cell cycle arrest (Andera & Wasylyk 1997). The apoptosis vs arrest choice may simply be due to absolute levels of p53 or influenced by the presence of various cell-specific p53 cofactors. For example, the presence of Brca1 pushes p53 responses toward growth arrest rather than apoptosis (MacLachlan et al. 2002).

It is clear that many of the 'guardian' activities of p53 are mediated by cell cycle arrest and apoptosis. However, p53 also functions to promote various DNA repair pathways after DNA damage, but how p53 engages in DNA repair is not as well understood.

1.1.5 p53 Plays a Direct Role in Promoting DNA Repair

Early work in the p53 field found that, in addition to its sequence-specific DNA binding and transactivation capability, p53 had a non-sequence-specific propensity for binding abnormal and damaged DNA, including DNA regions that are single-stranded (ssDNA), have insertion/deletion mismatches, DSBs, or heteroduplex joints such as Holliday junctions (Steinmeyer & Deppert 1988; Lee et al. 1995; Carbone et al. 2002; Janz et al. 2002; Subramanian & Griffith 2005). In fact, the affinity of p53 for DNA mismatches is as high as that of the MMR complex MSH2-MSH6 (Degtyareva et al. 2001). In addition, p53 was found to have some intrinsic DNA modification capabilities. For instance, p53 has a strong DNA and RNA annealing activity and catalyzes strand transfer, both of which are specific to particular p53 isoforms and require its C-terminus (Oberosler et al. 1993; Bakalkin et al. 1994; Brain & Jenkins 1994; Reed et al. 1995; Mummenbrauer et al. 1996; Huang 1998).
This exonuclease activity maps to the DNA binding domain of p53 and is especially active when the 3’ end contains a base mismatch or is single-stranded, further supporting its ability as a proofreader (Mummenbrauer et al. 1996; Skalski et al. 2000; Bakhanashvili 2001).

p53 plays a key role in regulating nucleotide excision repair (NER). Cells mutant for p53 have reduced NER removal of cyclobutane pyrimidine dimers and various DNA adducts from genomic DNA, and an increased rate of mutation after damage (Smith et al. 1995; Ford & Hanawalt 1995; Yagi et al. 1998; Lloyd & Hanawalt 2000; Torino et al. 2001). NER can be divided into two partially independent subpathways, global genomic repair (GGR) and transcription-coupled repair (TCR) (Fousteri & Mullenders 2008; Shuck et al. 2008). There is evidence that wild-type p53 is required for both to operate efficiently, though other studies report only a defect in GGR and not TCR (Mirzayans et al. 1996; Wani et al. 1999). Some mutant p53 proteins have dominant negative activity in NER (Zhu et al. 2000). p53 binds several components of the NER pathway, including XPB and XPD, recruiting them to the DNA and inhibiting their helicase activities (Wang et al. 2003). CSB, required only for the TCR subpathway of NER, also binds p53 (Wang et al. 1995). Human p53 is required for a DNA damage-dependent transactivation of the XPE damage recognition protein, which is required to target components of the GGR machinery to the site of damage (Hwang et al. 1999; Tan & Chu 2002; Wang et al. 2004). Another damage recognition protein, XPC, is also induced in a p53-dependent manner after UV (Amundson et al. 2002). These roles for p53 in promoting NER lead to the counter-intuitive finding in some cell systems that p53 mutation can increase apoptosis after UV damage due to defective DNA repair (Chouinard et al. 2002).

p53 also augments base excision repair (BER), independent of its transactivation activity (Offer et al. 2001). Two BER enzymes, 8-oxoguanine glycosylase and Ref-1, the abasic (A/P) endonuclease bind with p53 (Achanta & Huang 2004). After DNA damage, p53 enhances the activity of 3-MeAde DNA glycosylase, one of the enzymes that initiates BER (Zurer et al. 2004). p53 also augments BER repair of mitochondrial DNA (de Souza-Pinto et al. 2004). The DNA repair pathway MMR is also enhanced by p53. This function requires p53’s transcriptional
activity, upregulating MMR proteins MSH2, MLH1 and PMS2 after UV (Scherer et al. 2000; Warnick et al. 2001; Chen & Sadowski 2005).

p53 participates in cell controls against polyploidy and the aneuploidy that often results from division of polyploid cells. Although much of this protection stems from its ability to induce p21 and cell cycle arrest after cells become tetraploid, there is also some evidence that p53 may play a more direct role (Lanni & Jacks 1998). Some of these roles required transactivation by p53. For instance, the spindle checkpoint protein BubR1 plays a crucial role in suppression of centrosome hyperamplification and is under p53 transcriptional control (Oikawa et al. 2005). Unlike wild-type p53, expression of some p53 mutants results in polyploidy after treatment with spindle inhibitors, independent of any transactivation activity (Gualberto et al. 1998). p53 associates with centrosomes, dependent on functional ATM, suggesting it may have a direct role in regulating centrosome duplication (Brown et al. 1994; Tritarelli et al. 2004).

There is some evidence that p53 functions to maintain the fidelity of homologous recombination (HR) repair in mammalian cells. I will summarize these data in Chapter 3, where I will also present my data suggesting a role for the \textit{C. elegans} p53 family member \textit{cep-1} in meiotic recombination by HR that involves cooperation with the \textit{him-5} gene.

As I have summarized here, mammalian p53 plays an important role in protecting the integrity of the genome by halting the cell cycle, promoting apoptosis and directly regulating repair mechanisms in the wake of DNA damage. The vast majority of work in this field has focused on mammalian p53, due to its important role in human cancers. But emerging studies indicate that the p53 paralogues p63 and p73 also play important roles in maintaining genomic stability. Analysis of p53 family genes in non-mammalian animals has also increased our understanding of the various roles this protein family plays in the maintenance of genome integrity.

\textbf{1.1.6 p53 Family Members Play a Role in Genomic Stability}

Cells from mice lacking TAp73 isoforms show enhanced aneuploidy, suggesting that p73 plays a role in genomic stability (Tomasini et al. 2008). Similarly, overexpression of $\Delta$Np73 isoforms
leads to tetraploidy due to evasion of the mitotic spindle checkpoint. In the absence of p53, p73 plays a compensatory role in protecting against polyploidy and aneuploidy (Talos et al. 2007). p73 functions downstream of the spindle checkpoint to initiate caspase-independent cell death during mitosis after treatment with spindle inhibiting drugs (Niikura et al. 2007). p73 can also associate with spindle checkpoint proteins, including Bub1, Bub3, and BubR1, and can influence their sub-cellular localization (Tomasini et al. 2009).

Like its parologue p53, p73 can induce apoptosis after DNA damage, including cisplatin and IR (Gong et al. 1999; Yuan et al. 1999). p73 is transcriptionally upregulated in a Chk1- and Chk2-dependent manner in response to DNA damage. p73 can induce both the proapoptotic p53AIP1 and the cell cycle arrest CKI p21 (Costanzo et al. 2002). Like p53, p73 is induced by chemotherapeutic agents, and p53 and p73 can also work synergistically, since dominant negative forms of p53 can abrogate p73 cytotoxicity in response to chemotherapeutic drugs (Bergamaschi, Gasco et al. 2003; Irwin et al. 2003). One isoform of p73, p73α is phosphorylated by Chk1 in response to DNA damage and subsequently activates a proapoptotic transcriptional program (Gonzalez et al. 2003). p73α also upregulates genes involved in NER and MMR (Vikhanskaya et al. 2001). The MMR protein PMS2 binds p73 and promotes p73's proapoptotic function after treatment with cisplatin (Shimodaira et al. 2003). p73 is downregulated by ubiquitination by the E3 ligase Itch, and p73 accumulation requires Yap1, which binds to and stabilizes p73 by inhibiting its interaction with Itch (Levy et al. 2007).

p63 also plays a role in genome stabilization. One isoform, TAp63γ, accumulates after DNA damage by UV doxorubicin, etoposide or actinomycin D and it trans-activates p21, PIG3, 14-3-3σ and GADD45, all known p53 targets (Katoh et al. 2000; Petitjean et al. 2005). Conversely, the dominant negative ΔNp63α isoform decreases after genotoxic stress due to proteasomal degradation, which is predicted to increase transcriptional activity of p53 family isoforms that retain the TA domain (Fomenkov et al. 2004). In the absence of p53, p63 is required for rapid GGR of thymine dimers after UV irradiation, and when p63 is absent, expression of both XPC and XPE is reduced (Ferguson-Yates et al. 2008).
Understanding of the role of the p53 gene family in maintaining genome stability has also been informed by investigating the functions of p53 family members in non-mammalian model organisms. *Xenopus laevis* p53 is induced by DNA damage and can induce cell cycle arrest and apoptosis (Cox et al. 1994; Bensaad et al. 2001). Inhibition of *Drosophila melanogaster* p53 (Dmp53) renders cells resistant to DNA damage-induced apoptosis (Brodsky et al. 2000; Ollmann et al. 2000). After IR, Dmp53 transcriptionally activates the proapoptotic genes *hid, reaper* and *sickle*, and irradiated Dmp53 mutants show increased genomic instability (Brodsky et al. 2000; Sogame et al. 2003; Brodsky et al. 2004). On the other hand, Dmp53 protects cells from UV-induced apoptosis, likely by playing a role in DNA repair (Jassim et al. 2003). Dmp53-dependent pro-apoptotic transactivation requires DNA-dependent phosphorylation by the *D. melanogaster* Chk2 homologue MNK (Brodsky et al. 2004). Cells with broken chromosomes are eliminated by Dmp53-dependent apoptosis, controlled by the homologues of Chk1 and Chk2 (Titen & Golic 2008). Interestingly, Dmp53 is also induced by the programmed DSBs created during meiosis, suggesting Dmp53 plays a role during meiotic recombination (Lu et al. 2010).

Our understanding of the p53 family has also been significantly aided by work on the sole p53 family member in *C. elegans*, cep-1. This thesis will describe data suggesting a role for *cep-1* in maintenance of genomic stability.

### 1.2 CEP-1, the Sole *C. elegans* p53 family member

It was originally thought that the p53 family was confined to vertebrate lineages, but subsequently p53 genes have been found in invertebrates as primitive as sea anemones (Belyi et al. 2010). When CEP-1, the *C. elegans* p53 family protein was first described in 2001 it created much excitement, since the presence of a single p53-like gene in a genetically tractable organism had the potential to significantly enhance our understanding of this important gene family (Frantz 2001; Derry et al. 2001; Schumacher et al. 2001).
Derry et al (2001) reported that *C. elegans* contains a p53 homologue that is required for DNA damage-induced apoptosis in the germline, but not for physiological germline apoptosis nor developmental apoptosis in the soma. CEP-1 was found to be ubiquitously expressed in embryos, but when overexpressed from a heterologous promoter it could cause widespread cell death. Worms mutant for *cep-1* are also more sensitive to hypoxia and have reduced lifespan upon starvation-induced diapause than wild-type. Schumacher et al. (2001) confirmed that CEP-1 is required for DNA damage-induced apoptosis and showed that p53 was a transcription factor able to bind DNA at consensus sites for mammalian p53.

CEP-1 is a 644aa protein with sequence similarity to other p53 family members mainly confined to the DNA binding region, located around 223-418aa (Huyen et al. 2004). The *cep-1* locus also produces a more truncated RNA that encodes CEP-1B, a putative 197aa protein of unknown function that lacks the DNA binding domain of full-length CEP-1 ([www.wormbase.org](http://www.wormbase.org)). Although conservation between CEP-1 and mammalian p53 is only 15% in the DNA binding region, conserved residues include those that are most frequently mutated in human cancers, including four out of five arginine residues in the DNA binding domain, while the fifth is substituted in a conservative manner (Derry et al. 2001; Schumacher et al. 2001). The structure of the DNA binding domain of CEP-1 has been solved and is very similar to mammalian p53 in overall shape despite its low sequence homology, though there are differences in which specific loops are predicted to contact DNA (Huyen et al. 2004). Given these differences it is somewhat surprising that CEP-1 and p53 have almost identical preferred binding sites (Huyen et al. 2004). Unlike p53, however, CEP-1 contains a cryptic C-terminal SAM domain that was not recognized until the protein structure was solved and appears to form dimers instead of tetramers (Ou et al. 2007). Mammalian p63 and p73 also contain a SAM domain, reinforcing the idea that CEP-1 may resemble an ancestral p53 family gene with composite structural features and functions that later became separated upon gene duplication leading to creation of the three paralogues found in mammals.
The main way in which CEP-1 promotes DNA damage-induced apoptosis is by trans-activating the BH3-only homologues EGL-1, and, to a lesser extent, CED-13 (Hofmann et al. 2002; Schumacher et al. 2005). I will outline the apoptosis pathway downstream of EGL-1 in Section 2.2.2. Briefly, upregulated EGL-1 displaces the Apaf-1 homologue CED-4 from its inhibitor, the Bcl-2 homologue CED-9 (Spector et al. 1997; Wu, Wallen & Nuñez 1997; Conradt & Horvitz 1998; del Peso et al. 1998). CED-4 then goes on to promote auto-activation of the CED-3 protease which initiates apoptosis (Qi et al. 2010; Xue et al. 1996). There is also some indication that nuclear pore restructuring may be important for CEP-1-dependent (and not CEP-1-independent) cell death since inhibiting various nuclear pore genes abrogates CEP-1-induced apoptosis but not CEP-1-independent physiological cell death (Pinkston-Gosse & Kenyon 2007).

Upstream of CEP-1, DNA damage-induced apoptosis is regulated both positively and negatively by various factors. Among these is the Inhibitor of Apoptosis Stimulating Protein for p53 (iASPP) homologue APE-1, which inhibits apoptosis in a CEP-1-dependent manner (Bergamaschi, Samuels et al. 2003). This inhibition is probably direct, similar to the interaction between mammalian p53 and iASPP, since APE-1 and CEP-1 interact. CEP-1's ability to promote apoptosis is also inhibited by ABL-1, homologue of the mammalian kinase c-Abl (Deng et al. 2004). This inhibition is specific to IR-induced apoptosis and does not affect the number of apoptotic events after treatment with DNA alkylating agents. Another inhibitor of CEP-1 is GLD-1, the translational repressor (Schumacher et al. 2005). Mutation of gld-1 increases CEP-1 dependent apoptosis, and GLD-1 functions by binding the 3'UTR of the cep-1 mRNA and repressing its translation. The neddylation pathway and the Skp1/cullin/F-box E3 ubiquitin ligase components skr-1, cul-1, rbx-1, rpm-1 and fsn-1 function together as negative regulators of CEP-1 after damage with ENU, which was recently shown to be a conserved mechanism by which p73 is regulated in human cells (Gao et al. 2008; Peschiaroli et al. 2009). The transcription factor HIF-1 also modulates the germline's response to DNA damage by upregulating the tyrosinase family member TYR-2, a secreted factor which antagonizes CEP-1-dependent apoptosis (Sendoel et al. 2010).
The homologues of the 9-1-1 DNA damage checkpoint complex, MRT-2 and HUS-1, and the Tel2 homologue RAD-5/CLK-2 are required upstream of CEP-1 for DNA damage-induced apoptosis (Harris et al. 2006). Unlike mutations in these proteins, loss of CEP-1 does not cause a mutator phenotype, suggesting this DNA damage checkpoint activates DNA repair pathways independently of CEP-1. Other conserved positive regulators of CEP-1 include the ATM homologue ATM-1, the ATR homologue ATL-1 and the Akt homologue AKT-1 (Garcia-Muse & Boulton 2005; Quevedo et al. 2007). Different sorts of DNA damage also activate CEP-1 via different pathways. For instance, UV-induced apoptosis is completely dependent on CHK-2, CLK-2 and ATL-1, while ATM-1 is only required at low UV doses (Stergiou et al. 2007). The genes of the NER pathway also function upstream of CEP-1-induced apoptosis after UV damage (Stergiou et al. 2007). The C. elegans MAML homologue LAG-3 and the ING3 homologue ING-3 promote CEP-1-induced apoptosis, perhaps by acting as CEP-1 coactivators, as their homologues do for p53 in mammalian cells (Zhao et al. 2007; Luo et al. 2009).

In addition to damage induced by exogenous agents, CEP-1 can also increase germline apoptosis in worms with mutations that lead to increased endogenous DNA damage. This has been reported for him-6 and uri-1 mutants (Kim et al. 2005; Parusel et al. 2006). Lifespan-extending mutations suppress germline tumours by inducing CEP-1 dependent apoptosis, perhaps in an analogous fashion to wild-type p53’s protection against mammalian tumour growth (Pinkston et al. 2006).

CEP-1 can also induce germ cell cycle arrest after UV damage, though not after IR (Stergiou et al. 2007). Like UV-induced apoptosis, this involves the upstream activators, HUS-1, CLK-2, CHK-2 and ATL-1, as well as components of the NER pathway (Stergiou et al. 2007). CEP-1 also plays a role in somatic cell cycle control. clk-2 mutants show decreased rates of development during larval stages, and this decrease is mediated by CEP-1 and its downstream target PHG-1, the C. elegans Gas1 homologue (Derry et al. 2007). Generally, CEP-1 integrates several signalling pathways to regulate life-or-death decisions.
Two studies have used microarrays to study CEP-1-dependent gene expression with somewhat disparate results. Greiss et al. (2008) reported that CEP-1 did not significantly affect gene expression in the absence of DNA damage and only induced the expression of three genes in the presence of damage, the proapoptotic BH3-only homologues egl-1 and ced-13 and one uncharacterized gene, leading the authors to hypothesize that the main ancestral function of p53 genes is to induce apoptosis after genotoxic stress. On the other hand, (Derry et al. 2007). found that CEP-1 both activates and represses many genes both normally and after DNA damage, and that many of these CEP-1 regulated genes have homologues that are regulated by mammalian p53 or p63. Some of these discrepancies may be explained by the use of different genotoxic damage (IR vs. UV) and different cep-1 alleles, as well as differences in experimental procedure. Still, these differences highlight how much we still have to learn about the function of CEP-1.

Since CEP-1 has conserved functions in promoting apoptosis and cell cycle arrest after genotoxic stress, it is possible that it also has conserved roles in promoting DNA repair directly to maintain genomic stability. Evidence for a direct roles in genomic stability have not been reported as yet, except for a low but significant increase in X chromosome nondisjunction during meiosis, resulting in increased male offspring after cep-1 RNAi (Derry et al. 2001). In this thesis, I characterize a novel role for CEP-1 in genome stability in conjunction with the meiotic protein HIM-5, and suggest that CEP-1 may play a conserved role in promoting accurate recombinational repair during meiosis in C. elegans.

1.3 The Meiotic Gene him-5

The free living soil nematode C. elegans occurs in two sexes in wild-type populations: self-fertile hermaphrodites and males. Wild-type hermaphrodites give rise almost exclusively to hermaphrodite progeny, with males occurring rarely at a frequency of approximately 1 in 500 worms (Brenner 1974; Hodgkin et al. 1979). Wild type hermaphrodites have two X chromosomes while males have only one X chromosome, in addition to the five pairs of autosomes seen in both sexes (Nigon 1949). Sex is determined by karyotype, with the ratio of sex chromosomes to sets of autosomes being the primary signal (Madl & Herman 1979). Early
on in the genetic analysis of *C. elegans*, various mutant strains were isolated that produced much higher frequencies of male progeny than did wild-type animals (Hodgkin et al. 1979). This was called the 'Him' phenotype, an acronym for 'high incidence of males', and it is caused by an increased frequency of X chromosome nondisjunction during meiosis. These *him* mutants generate increased numbers of progeny with a single X chromosome and also segregate a small number of progeny containing three X chromosomes, which develop into hermaphrodites that exhibit a Dumpy (Dpy) phenotype (Hodgkin et al. 1979). The XXX Dpy hermaphrodites are much rarer among progeny of *him* mutants than are XO males despite the fact their viability is not greatly reduced compared to XX hermaphrodites. This suggests that X chromosomes are lost more often than they are segregated unequally (Hodgkin et al. 1979). Hodgkin (1983) later showed that XXX hermaphrodites have a Dumpy phenotype due to the inability of the dosage compensation machinery to compensate for the presence of three X chromosomes rather than two. In addition to XO males and XXX Dpy males, most *him* mutants produce a significant number of dead embryos. This has been suggested to be due to increased nondisjunction of autosomes, resulting in increased embryonic lethality (Hodgkin 2005).

Hodgkin et al. (1979) published an analysis of the first batch of meiotic mutants, *him-1* through *him-9* as well as *unc-86*. Mutations in each of these genes resulted in a high frequency of males and XXX Dpy hermaphrodites. With the exception of *him-8*, all of the other Him mutants produce an increased frequency of inviable embryos relative to wild-type, which was suggested to be due to autosomal nondisjunction. All of the *him* mutant male and hermaphrodite progeny described were superficially wild-type in appearance and behaviour, except *him-4* and *unc-86*, which also have morphological and behavioural defects.

*him-5* is one of the original meiotic mutants characterized by Hodgkin et al (1979). Mutations in it, along with those in *him-1* and *him-8*, were thought to induce meiotic nondisjunction of the X chromosome more readily than the autosomes, since all three generate a higher frequency of males among their progeny than they do inviable embryos. *him-5*, unlike *him-8*, was found to segregate a significant number of dead eggs, but much fewer than would be expected if
autosomes were disjoining at a frequency similar to the X chromosome. The same paper reported that him-5 significantly reduced recombination on the X chromosome while leaving autosomal recombination frequencies largely unaffected. This was the first indication that him-5 played a role in meiotic recombination.

The three putative X-specific him genes, him-1, him-5 and him-8, were subsequently characterized further (Broverman & Meneely 1994). These authors found that him-1; him-5 showed an additive Him phenotype while him-8; him-5 had a Him phenotype not significantly different from him-8 alone. It was later shown that only weak alleles of him-1 were X chromosome specific, with null alleles showing complete sterility (A. Chan, D. Pasqualone, B. Meyer, personal communication). him-8, on the other hand, is indeed X chromosome specific, being required specifically for the X chromosome homologue pairing that precedes crossing over (Phillips et al. 2005). him-8 mutants therefore produce oocytes with normal recombination between autosomal homologues but achiasmate X chromosomes. The lack of additive effects on the Him phenotype of mutations in him-8 and him-5 suggest these genes are involved in the same process, such as recombination on the X chromosome (Broverman & Meneely 1994). In support of this hypothesis, Broverman & Meneely (1994) also found that him-5 and him-8 mutants all show reduced recombination on the X chromosome. Later still, the same group showed that the X chromosomes in him-5 meiotic nuclei are achiasmate despite the fact that early homologue pairing events occur normally (P.Meneely, J. Havassy, K. Crozier, personal communication).

In 2005 the Meneely lab reported at the International Worm Meeting that they had cloned him-5 and it corresponded to the ORF D1086.4 (P. Meneely, N. Favini, K. Crozier, J.-J.Wang, J. Havassy, personal communication). This ORF is predicted to encode a protein of 252aa that contains no obvious homology to any known proteins and no recognizable domain structure. It does have a high predicted isoelectric point (pI) of 10.8, perhaps suggesting a role in contacting DNA (www.wormbase.org).
Meiotic recombination in eukaryotes is initiated by DSBs catalyzed by a member of the Spo11 topoisomerase-like nuclease family (Keeney 2001). Spo11 mutants of various species show no meiotic recombination and severe meiotic defects and/or catastrophic homologue nondisjunction results. In yeast, nematodes and other organisms, Spo11 mutants can be rescued with a moderate dose of IR administered during early meiosis (Celerin et al. 2000; Dernburg et al. 1998; Bowring et al. 2006; Thorne & Byers 1993). In the absence of Spo11-catalyzed DSBs, IR can provide a source of DSBs to be used as substrate by the meiotic recombination repair machinery, rescuing crossing over and offspring viability. As I will outline in Chapter 3, Spo11 does not work alone. In *Saccharomyces cerevisiae*, a number of other genes are required along with Spo11 for programmed meiotic DNA breaks to occur efficiently (Malone et al. 1991). The phenotypic consequences of mutation of these genes can also be rescued by moderate doses of IR (Liu et al. 2002). Recently, the Yanowitz and Meneely labs reported at a conference that they could rescue the Him and Emb defects of *him-5* with a moderate IR dose (J. Yanowitz, F. Heinis, F. Favani, S. DiRienzi, C. Wagner and P. Meneely, personal communication). This suggests that both the Him and Emb phenotypes of *him-5* mutants are due to a defect in initiation of Spo11-induced DSBs. Failure of recombination initiation on the X chromosome would explain the Him phenotype and the presence of achiasmate X chromosomes in *him-5* mutants.

*him-5*, along with *him-8*, has been used extensively as a laboratory tool to generate strains with a high frequency of male progeny to allow the study of male behaviour and development (Burdine et al. 1998; Srinivasan et al. 2008; Liu et al. 2007; Gower et al. 2005). Despite its extensive use, *him-5*'s molecular role was completely unknown until recently. The work of the Yanowitz and Meneely labs, as well as the work I present here, begins the process of characterizing *him-5*'s roles in recombination initiation and repair.

### 1.4 Meiotic Recombination in *C. elegans*

Sexually reproducing organisms produce haploid gametes by a specialized cell division pathway called meiosis (Marcon & Moens 2005). Meiosis consists of replication followed by two rounds of cell division, resulting in four haploid cells. In *C. elegans*, the distal end of the hermaphrodite
gonad arm contains mitotically replicating syncitial germ cell nuclei, which act as a stem cell population that both self-renews and also provides germ cells to mature into oocytes (Kimble & Crittenden 2005). Figure 1-2 shows a schematic of chromosome behaviour during the stages of meiotic prophase.

Germ cells destined to become oocytes leave the mitotic region and enter the transition zone, where entry into meiosis is initiated. As oocytes move along the gonad, they progress through meiosis, providing a convenient experimental system where germ cell position in the gonad correlates well with meiotic stage (Greenstein 2005). Downstream of the transition zone, chromosomes pair with their homologues and in the pachytene region crossing-over occurs. Also during pachytene a significant number of germ cells are culled by 'physiological' apoptosis (Gumienny et al. 1999). Under conditions of DNA damage, faulty homologue pairing or defective meiotic recombination, the pachytene checkpoint is activated, and the number of cells undergoing apoptosis increases (Gartner et al. 2000). Those cells that are not culled continue to progress through meiosis I until they reach diakinesis where they arrest with maximally condensed bivalents connected by chiasmata. Chromosome condensation is coupled with increasing cellularization of the oocytes until the nuclei are fully enclosed (Greenstein 2005). Oocytes are signalled to restart meiosis upon fertilization (McCarter et al. 1999). Bivalents separate in meiosis I (the reductional division) and sister chromatids separate in meiosis II (the equational division).

Accurate segregation of homologous chromosomes during meiosis I requires that they become paired then linked by crossover recombination. Meiotic recombination occurs in several steps (Székvölgyi & Nicolas 2010). First, programmed DSBs are created, then processed and resected to form 3’ ssDNA ends. One of these ends then invades the homologous DNA sequence on the paired homologue, forming a heteroduplex structure which is finally resolved as a crossover, physically linking the chromosomes. This physical linkage, or chiasma keeps the homologues together so they can be accurately distributed during anaphase I. Meiotic recombination is an evolutionary innovation common to most sexually reproducing organisms, though the details of
Figure 1-2: During meiotic prophase I, homologous chromosomes pair, synapse and become linked by crossing over.

A schematic of the behaviour of one pair of homologues (one shown in red and the other in blue) as they progress through meiosis I. During the leptotene stage, chromosomes begin to condense in preparation for pairing to begin in zygotene. During pachytene, chromosomes synapse and are held in close proximity by the proteinacious synaptonemal complex (SC), allowing crossing over to occur. The SC dissociates during diplotene, and homologue pairs are held together by the chiasma, the physical linkage that results from crossing over. *C. elegans* oocytes arrest in diakinesis with the linked homologues maximally condensed, and only reenter meiosis I after fertilization (Greenstein, 2005). A more in-depth schematic outlining the process of crossing over is shown in Figure 1-3.
how it is accomplished frequently differ between species. Here, I will outline the current understanding of meiotic recombination in *C. elegans*. A schematic of some of the key proteins and processes discussed in this section is shown in Figure 1-3.

1.4.1 General Features of Meiotic Recombination in *C. elegans*

Unlike in *Drosophila*, meiotic recombination occurs in the germline of males, as well as in both sperm and eggs of hermaphrodites, though spermatocytes in both males and hermaphrodites have lower meiotic recombination frequencies than oocytes (Zetka & Rose 1990; Brenner 1974). Rates of meiotic recombination change with temperature and parental age (Rose & Baillie 1979; Zetka & Rose 1990). Recombination frequencies are highest on chromosome arms rather than toward the middle of the chromosome, resulting in apparent clustering of genes in the centre of each chromosome where recombination frequencies are lowest (Barnes et al. 1995).

Early in prophase I, homologous chromosomes are paired, beginning at specialized homologous recognition regions (HRRs) on one end of each chromosome (McKim et al. 1993). In the absence of these pairing regions, homologues remain unpaired and unable to form chiasmata (Herman & Kari 1989). For this reason, free duplications that lack an HRR show significantly less recombination with homologous regions on intact chromosomes (Herman et al. 1976). Due to difficulties in homologous pairing, chromosome translocations also suppress crossing over (Herman 1978).

Meiotic crossovers are tightly controlled in *C. elegans*, with each chromosome receiving one crossover, irrespective of gaps in homology (Hammarlund et al. 2005). For example, inverted regions show little crossing over due to difficulties in homologous pairing, but the remainder of the chromosome containing the inversion has higher rates of recombination to compensate (Zetka & Rose 1992). With similar logic, whole-chromosome fusions only show one meiotic crossover, even though both component chromosomes would normally cross over once each in the absence of fusion (Hillers & Villeneuve 2003). This work suggests that a continuous
Figure 1-3: Meiotic Recombination in *C. elegans*.

A schematic of some of the major events in meiotic recombination in *C. elegans*. Homologous chromosomes are shown as paired red and blue lines and the major steps in crossing-over are labelled along the right. Recombination proteins are shown as coloured shapes with their names listed on the right of the figure. Specific functions for each protein are outlined in the text. For simplicity, only a single chromatid is shown for each homologue. Adapted from Garcia-Muse and Boulton (2007).
chromosome axis, and not chromosome size or sequence, is important for regulating numbers of crossovers.

Some genes globally affect recombination frequencies or patterns, but it is still unknown at which point in recombination they function. One of these is rec-1, an uncloned gene that gene alters the pattern of recombination by making crossovers as frequent in the middle of the chromosome as on the chromosome arms (Zetka & Rose 1995; Rose & Baillie 1979). In rec-1 mutants, each homologue pair still undergoes one crossover event, but the pattern is randomized such that the genetic map in rec-1 mutants resembles the physical map (Zetka & Rose 1995).

1.4.2 Formation of Meiotic DSBs

A cross-species overview of meiotic recombination initiation will be given in Chapter 3. In all eukaryotes characterized so far, meiotic recombination is normally initiated by programmed DSBs catalyzed by a member of the Spo11 type topoisomerase-like family (Keeney 2001). In C. elegans, meiotic DSBs are catalyzed by SPO-11 (Dernburg et al. 1998). Meiotic recombination is absent in spo-11 mutants, though it can be rescued by IR-induced DSBs.

SPO-11, however, does not work alone. Animals with mutations in the mre-11 nuclease are also defective for programmed meiotic DSB formation as well as for their subsequent repair (Chin & Villeneuve 2001). This is similar to the situation in budding yeast, where Mre11 is required for meiotic DSBs, while in other organisms Mre11 homologues are dispensable for this process (Borde 2007). C. elegans MRE-11 binds to the HORMA domain-containing protein HTP-1, which is also required for DSB formation (Goodyer et al. 2008). HTP-1 links DSB induction and SC formation, since it is required to direct the SC assembly by directing the structural protein HIM-3 to meiotic chromosome axes. The nematode-specific protein HIM-19 may act in a similar process to HTP-1, since it is also required for DSB and SC formation, especially as animals age (Tang et al. 2010). The putative chromatin-modifying protein HIM-17 is also required for DSB formation, and chiasmata are rescued by IR in him-17 mutants (Reddy & Villeneuve 2004). HIM-17 is required for the meiosis-specific accumulation of histone H3.
methylation at lysine 9 during prophase I, suggesting that its function is required to prepare chromatin for DSB formation.

The number and position of programmed DSBs is a significant source of crossover regulation in *C. elegans*, since increasing the number of DSBs by IR increases the frequency of crossover formation (Kim & Rose 1987). Despite the fact that the number of DSBs induced by SPO-11 is limited, pachytene nuclei can tolerate and accurately repair many more DSBs than are normally created (Takanami et al. 2003). One of the proteins required to limit the number of DSBs that are formed is DPY-28, a condensin-like subunit best known for its role in X chromosome dosage compensation (Tsai et al. 2008). In its meiotic role, DPY-28 acts in a novel complex that contains subunits from both the dosage compensation and condensin complexes required for mitotic chromosome compaction (Mets & Meyer 2009). Mutations in *dpy-28* increase crossover frequency and abolish crossover interference, the control that allows only one crossover per homologue pair, and they partially rescue the DSB formation defects of HIM-17 (Tsai et al. 2008). This DPY-28 complex and HIM-17 appear to direct chromosome organization during early meiosis and oppositely affect the ability of the chromatin to accept SPO-11-induced DSBs.

Similar to what is seen in *Drosophila*, meiotic DSBs are not required for SC formation in *C. elegans* as they are in most organisms (Dernburg et al. 1998). DSB formation is dependent on homologue pairing, and mutants defective in genes required for this process, such as the Chk2 kinase homologue *chk-2* show no meiotic DSBs (Higashitani et al. 2000). A second, less characterized Chk2-like gene, T08D2.7, may contribute to this phenotype (Oishi et al. 2001). DSBs are also formed on the single unpaired X of male or *fem-3* XO transformed germ cells, and some SC-associated components are loaded, but synapsis is incomplete and DSBs are repaired accurately in its absence, though with delayed kinetics compared to that seen with the paired X chromosomes of hermaphrodites (Jaramillo-Lambert & Engebrecht 2010).
1.4.3 Meiotic DSB Processing

In addition to its role in DSB formation, MRE-11 also likely plays a role in DSB resection as it does in other organisms (Chin & Villeneuve 2001). Certainly, it is required for meiotic recombination at a step after DSBs are created, and homology would suggest this is the resection step (Borde 2007). Also likely required for DSB resection is the Com1/Sae2/CtIP nuclease homologue COM-1 (Penkner et al. 2007). These proteins are required for meiotic DSB resection in various organisms (Takeda et al. 2007). Loading of the RAD-51 recombinase (see next section) onto meiotic DSBs is impaired in com-1 mutants, suggesting COM-1 is required to create the ssDNA at SPO-11-induced breaks that are the substrate for the HR machinery (Penkner et al. 2007).

1.4.4 Meiotic DSB Repair

The central enzyme required for HR in C. elegans is the Rad51 recombinase homologue RAD-51 (Rinaldo et al. 1998; Takanami et al. 1998). Abrogation of RAD-51 causes total embryonic lethality of progeny and aggregated, malformed chromatin at diakinesis of prophase I (Takanami et al. 1998). These rad-51 defects are dependent on the presence of SPO-11-induced DSBs, suggesting RAD-51 functions in repair of these breaks (Rinaldo et al. 2002). RAD-51 is highly expressed during meiosis and is required for germ cell resistance to exogenous IR as well as for repair of endogenous meiotic DSBs (Takanami et al. 1998; Takanami et al. 2000). During zygotene and early pachytene, RAD-51 localizes to chromatin-associated foci in a SPO-11, CHK-2 and MRE-11-dependent manner and these foci disappear by the end of pachytene, when DSB repair is expected to be completed (Alpi et al. 2003).

In addition to the RAD-51 recombinase, the functions of several other proteins are required for meiotic DSB repair. Functional Rad50 homologue RAD-50 is required for RAD-51 loading onto meiotic DSBs (Hayashi et al. 2007). The BrcA2 homologue BRC-2 interacts directly with RAD-51 and is required to direct the recombinase to the nucleus and then to the ssDNA surrounding resected DSBs, where it stimulates recombinase activity (Martin et al. 2005; Petalcorin et al. 2006). BRC-2 also contributes to the aggregation phenotype seen in rad-51 and com-1 mutants,
since animals mutant for brc-2 and one of the components of the NHEJ pathway show chromosome fragmentation rather than aggregation. The aggregation phenotype therefore is a result of defective repair using back-up repair pathways, one of which is NHEJ and another of which requires BRC-2 (Martin et al. 2005). The MutS family protein MSH-5 also shows a similar phenotypic interaction. When MSH-5 and RAD-51 are both depleted, diakinesis chromosomes show fragmentation rather than the aggregation phenotype seen in rad-51 alone (Rinaldo et al. 2002). msh-5 mutants show persistent RAD-51 foci and MSH-5, along with another MutS family member HIM-14, has been implicated for a role in converting resected DSBs into stable post-strand exchange intermediates (Colaiácovo et al. 2003; Carlton et al. 2006).

While DSB formation does not rely on a fully formed SC in C. elegans, completion of meiotic recombination does. Thus, animals with mutations in the SC components SYP-1 or SYP-4 have severe reductions in crossing over and accumulate recombination intermediates, as indicated by persistent RAD-51 foci (MacQueen et al. 2002; Alpi et al. 2003; Smolikov et al. 2009). While a functional SC is required for recombination, the reverse is not true: rad-51 mutants form a normal SC (Alpi et al. 2003). However, completion of recombination does signal the dismantling of the SC. The SC regulatory proteins HTP-1 and HTP-2 are removed in a crossover dependent manner from the vicinity of the chiasmata while ZHP-3 localizes to chiasmatic regions (Martinez-Perez et al. 2008; Bhalla et al. 2008). This asymmetric dismantling of the SC and localization of ZHP-3 are required for efficient cohesion of both the homologous chromosomes and sister chromatids.

Once RAD-51 has performed its recombinase function it then must be removed from the DNA for crossovers to be resolved. The Rad51 parologue RFS-1 and the HELQ helicase homologue HELQ-1 act in a redundant manner to remove RAD-51 from chromatin (Ward et al. 2010). In their absence, RAD-51 persists on chromatin and crossovers fail to be resolved. The MUS312/Slx4 orthologue HIM-18 is also required for a late step in resolving HR intermediates into crossovers (Saito et al. 2009). Its specific role is unknown but is likely related to its
interaction with the SLX-1 and XPF-1 nucleases, which may be required to create the ssDNA nicks required to resolve heteroduplex DNA at Holliday junctions.

When animals bear mutations that inhibit completion of meiotic DSB repair, recombination intermediates accumulate, triggering the pachytene checkpoint and apoptosis (Gartner et al. 2000). While the particular recombination intermediate required to trigger the checkpoint response is unknown, it may include regions of ssDNA, since com-1 mutants, which contain unrepairred DSBs that remain unreseected, do not have increased germline apoptosis, while those with mutations that affect later recombination steps do (Gartner et al. 2000; Penkner et al. 2007).

1.4.5 The Crossover/Noncrossover (CO/NCO) Decision

Despite the fact some amount of crossover control occurs at the level of meiotic DSBs in *C. elegans*, not all SPO-11-generated breaks are converted into crossovers by the recombination machinery. Work in other organisms has shown that meiotic DSBs can be repaired without crossing over in two ways: by resolution of the heteroduplex DNA formed with the homologous chromosome in such a way as to create a non-crossover event, or by HR repair using the sister chromatid (Baudat & de Massy 2007). The CO/NCO decision in *C. elegans* involves a delicate balance of regulation that ultimately results in each bivalent pair receiving exactly one crossover event (Hammarlund et al. 2005). The meiotic crossover process is highly controlled developmentally, since germline nuclei have the competence to convert a DSB into a crossover only during early meiotic prophase I and lose that competence by late pachytene (Hayashi et al. 2007).

COs and NCOs in *C. elegans* occur by overlapping but different pathways, as evidenced by genetic mutations that separate the two pathways. Recent work has shown that the DEAD superfamily helicase RTEL-1 is required to prevent excess COs by promoting DSB repair by synthesis-dependent strand annealing (SDSA), an HR subpathway that is not normally associated with crossing-over (Youds et al. 2010). The Brca1 homologue BRC-1 is probably required for SDSA since it is dispensable for creation of crossovers, but in backgrounds where DSB repair is
functional but chiasmata are lacking, \textit{brc-1} mutation causes chromosome fragmentation (Adamo et al. 2008). The SMC superfamily proteins SMC-4 and SMC-5 may also be required for SDSA, since mutations in these genes do not affect crossing over but are defective for noncrossover DSB repair (Bickel et al. 2010). Other DSB repair pathways must be kept in check, rather than promoted, to allow accurate repair and sufficient numbers of crossovers. For instance, the FANCD2 homologue FCD-2 is dispensable for normal recombination but is required in crossover defective mutants to prevent repair of DSBs by the error-prone NHEJ pathway (Adamo et al. 2010).

Both the MutS family proteins HIM-14 and MSH-5 and the Blm helicase homologue HIM-6 have also been implicated in the CO/NCO decision (Kelly et al. 2000). Mutation in any of these genes reduces the frequency of crossing over, with the result some bivalents fail to form a chiasma, leading to increased nondisjunction. HIM-14 and MSH-5 may function to inhibit branch migration of Holliday junctions, promoting COs over NCOs (Zalevsky et al. 1999). IFE-1, an mRNA cap-binding protein homologue of IF4E is required to increase translation of \textit{him-14} and \textit{msh-5} mRNAs in response to increased temperatures, a situation when recombination intermediates require extra stabilization to be effectively converted into crossovers (Song et al. 2010).

There is also evidence that some aspect of synapsis is required for the CO/NCO decision. Non-null mutations affecting SC-associated Hop1 homologue HIM-3 appear to synapse normally but show either severe reductions or increases in frequency of crossing-over, suggesting HIM-3 contributes to control of the number of crossovers per homologue pair (Zetka et al. 1999; Nabeshima et al. 2004). Despite defects in homologue pairing and SC assembly, \textit{him-3} null mutants repair meiotic DSBs with wild-type kinetics, presumably using the sister chromatid as template (Couteau et al. 2004). Similar phenotypes have been noted for mutants defective in the SC-associated RING finger protein ZHP-3, the SC component SYP-3 and the HIM-3 paralogue HTP-1, where meiotic recombination is initiated normally and repair occurs, but without chiasmata formation (Jantsch et al. 2004; Martinez-Perez & Villeneuve 2005; Smolikov et al. 2010).
HTP-1 appears to be required to prevent the repair of meiotic DSBs using sister chromatids, thereby encouraging chiasmata formation (Martinez-Perez & Villeneuve 2005). Meiotic recombination is an active area of research in *C. elegans*, and while work in this organism lags significantly behind that in *S. cerevisiae*, its convenient germline organization, transparency, extensive genetic tools and large numbers of progeny per self-fertile hermaphrodite recommend it for examination of this vital meiotic process.

The work presented in this thesis outlines a role for the meiotic gene HIM-5 and the p53 family member CEP-1 in accurate meiotic recombination in *C. elegans*. Mutation in *him-5* had previously been shown to affect meiotic recombination frequency but CEP-1 had no previous known role in recombination during meiosis (Broverman & Meneely 1994; Hodgkin et al. 1979). This work opens new lines of enquiry into the mechanism by which CEP-1 and HIM-5 affect meiotic recombination and genomic stability.
2 cep-1 and him-5 Are Required in Parallel in the Germline to Promote Embryonic Viability

2.1 Abstract

him-5 is a \textit{C. elegans} nondisjunction gene required for proper segregation of the X chromosome during meiosis. Recent work from the Meneely lab and confirmed here shows that \textit{him-5} is allelic to the ORF \textit{D1086.4} and encodes a basic protein with no clear homologies or domain structure. CEP-1 is the sole \textit{C. elegans} member of the p53 family of transcription factors. It is well known for its role in promoting apoptosis in the germline in response to DNA damage. A low level of X chromosome nondisjunction and embryonic death in the offspring of these worms suggests that CEP-1 may also play a more direct role in genomic stability.

I found that, in contrast to \textit{cep-1} and \textit{him-5} single mutants, which show low and moderate rates of embryonic lethality respectively, \textit{cep-1; him-5} double mutants give rise to a significantly increased frequency of unhatched eggs. Despite the increased frequency of embryonic lethality, the rates of male and XXX Dpy offspring remain unchanged compared to \textit{him-5} alone. The defect leading to decreased survival appears to occur specifically in the germline of \textit{cep-1; him-5} mothers, since the \textit{cep-1; him-5} offspring of \textit{cep-1/+; him-5/+} heterozygous mothers and the offspring of \textit{cep-1; him-5} fathers do not show increased lethality compared to wild-type. These results demonstrate that \textit{cep-1} and \textit{him-5} have overlapping function in embryonic survival.

Unlike \textit{cep-1} and \textit{him-5} double and single mutants, \textit{cep-1; him-6} double mutants do not show increased lethality compared to \textit{him-6} alone, suggesting that \textit{cep-1}'s role in promoting survival in \textit{him-5} mutants is not general to all meiotic mutants but is at least somewhat specific to \textit{him-5}. The embryonic lethality of \textit{him-5} is also not enhanced by \textit{hus-1}, a known upstream regulator of \textit{cep-1} in the DNA damage response pathway or by \textit{ced-3} or \textit{ced-4}, components of the core apoptotic machinery required for germline apoptosis induced by \textit{cep-1}. These results suggest that CEP-1 does not function within its known genetic pathway to promote survival in \textit{him-5} mutants and that this represents a new cellular function for CEP-1. In support of this hypothesis,
him-5 mutants do not show increased germline apoptosis compared to wild-type and cep-1; him-5 mutants, suggesting CEP-1 does not promote survival in him-5 mutants by promoting apoptosis, but by a novel mechanism. The enhanced lethality seen in double mutants between cep-1 and the meiotic mutant him-5 may reveal a new role for CEP-1 in promoting genomic stability in C. elegans.

2.2 Introduction

2.2.1 Meiotic Nondisjunction Mutants of C. elegans

The work outlined in this thesis focuses on an interaction I uncovered between the C. elegans p53 family member cep-1 and the meiotic mutant him-5. The previous work done on him-5 that informed this work was outlined in Chapter 1. Briefly, him-5 is one of the original meiotic nondisjunction genes initially characterized by Hodgkin et al. (1979). Unlike most other him genes, it affects nondisjunction of the X chromosome much more strongly than the autosomes. But, unlike him-8, the other gene known for its X chromosome-specific effect, him-5 mutations cause significant embryonic lethality that may be due to autosomal nondisjunction or to another defect. Early evidence suggested that him-5 may play a role in meiotic recombination on the X chromosome, and the X chromosome was found to be achiasmate in him-5 mutants (Hodgkin et al. 1979; Broverman & Meneely 1994).

Besides him-5, two other nondisjunction mutants are used in this work. Like him-5, both him-2 and him-6 are among the original him mutants described by Hodgkin et al. (1979). him-2 is a weak Him mutant, generating only 2% male progeny and mapped to a region on chromosome I that includes cep-1 (www.wormbase.org). It is represented by only one allele (e1065) and remains uncloned (Hodgkin et al. 1979; www.wormbase.org). Since cep-1(lg12501) and cep-1(RNAi) have a modest Him phenotype similar to him-2, it was possible that cep-1 and him-2 were allelic (Derry et al. 2001).

him-6 encodes a RecQ helicase and has been fairly well characterized (Wicky et al. 2004). The strong him-6 allele e1423 causes the highest frequency of embryonic lethality reported in
Hodgkin et al. (1979). Unlike him-5, him-6 mutation caused similar nondisjunction frequencies for all chromosomes, accounting for their severe embryonic lethality and mild Him phenotypes. Because of the high rate of nondisjunction in both the sperm and oocytes of him-6 mutants, diploid him-6 progeny can be recovered that inherited both copies of any particular chromosome from either the sperm or the egg. For this reason it has been used to test for genomic imprinting in C. elegans (Haack & Hodgkin 1991). No evidence for major chromosomal imprinting was found in this analysis. Mutation of him-6 leads to a significant reduction in the amount of crossing over, which could account for its high rates of meiotic nondisjunction and embryonic lethality (Zetka & Rose 1995). The him-6 gene encodes a RecQ helicase most closely related to Bloom helicase (BLM) in humans (Wicky et al. 2004). Mutations affecting BLM are associated with a syndrome characterized by elevated risk of cancer, immunodeficiency and low fertility (German 1993). Mammalian BLM appears to play a role in suppressing hyperrecombination and sister chromatid exchanges in somatic cells (Chu & Hickson 2009). him-6 worms are also sensitive to ionizing radiation and have a defective DNA damage-induced checkpoint in germline mitotic cells (Wicky et al. 2004). Mutants for him-6 also appear to show increased likelihood of spontaneous mutation and a mortal germline at 25°C (Grabowski et al. 2005). him-6's role in germline apoptosis is unclear, since one group found that him-6 mutants have normal germline apoptosis in the absence of damage and reduced germline apoptosis induction after damage, and another found that him-6 mutants show a cep-1-dependent increase in germline apoptosis in the absence of damage (Wicky et al. 2004; Kim et al. 2005). Notwithstanding this disagreement it appears that HIM-6 plays a vital role in genomic stability in the germline.

C. elegans him-6 exhibits several interesting synthetic genetic interactions. HIM-6 protein interacts with TOP-3, and top-3; him-6 double mutants exhibit germline mitotic failure due to extensive chromosome fragmentation, a phenotype that is suppressed by mutation of the rad-51 recombinase or its paralogue rfs-1, suggesting the mitotic catastrophe is a result of indiscriminate recombination (Wicky et al. 2004; Kim et al. 2002; Ward et al. 2007). There are also synergistic interactions between him-6 and dog-1, the C. elegans FANC-J orthologue required for maintenance of poly-G tracts in DNA during replication. dog-1; him-6 double mutants show
increased replication stress and an increased frequency of deletions in small DNA regions containing poly-G tracts (Youds et al. 2006; Youds et al. 2008). In addition, strong genetic interactions have been observed between him-6 and the MUS312/Slx4 homologue him-18, where him-18; him-6 double mutants exhibit enhanced lethality (Saito et al. 2009). When I began to characterize the enhanced lethality between him-5 and the p53 homologue cep-1, I wanted to know if this phenotype was due to a general effect of cep-1 mutation on genomic stability or if this phenotype was specific to him-5. The interactions between him-6 and other genes that regulate genomic stability and germline maintenance prompted me to test whether the him-6 embryonic lethality phenotype was also modified by cep-1.

The sex determination system of C. elegans, based on X chromosome dosage, and the large number of progeny generated from a single hermaphrodite animal make this organism ideal for performing genetic screens to identify genes that regulate chromosome segregation during meiosis (Hodgkin et al. 1979). Various him genes are involved in genomic stability during meiosis and mitosis, and characterization of these genes has deepened our understanding of how these conserved processes function in vertebrates (Zetka et al. 1999; Wicky et al. 2004; Howe et al. 2001). The genetic interaction I characterize here between C. elegans p53 family member cep-1 and the meiotic mutant him-5 may lend similar insights into eukaryotic meiosis.

2.2.2 The CEP-1 DNA Damage-Induced Apoptosis Pathway

Although p53 was originally thought to be restricted to mammals, a single family member, cep-1, was found in C. elegans (Frantz 2001; Derry et al. 2001; Schumacher et al. 2001). CEP-1 diverges substantially in sequence from other known p53 family members, so it was somewhat surprising that it shows considerable conservation of function. In particular, it induces apoptosis in C. elegans germ cells that have undergone DNA damage by various mutagens (Derry et al. 2001; Schumacher et al. 2001). Of interest, the pathway by which it promotes apoptosis in response to damage is well conserved, both up- and downstream of cep-1 (Hofmann et al. 2002; Gartner et al. 2000).
The somatic cell lineage of *C. elegans* is essentially invariant, with mitosis proceeding according to a strict developmental program (Sulston & Horvitz 1977; Sulston et al. 1983). The germline, however, consists of a population of mitotic cells that serve as self-renewing stem cells and also give rise to cells which enter meiosis and become gametes (Kimble & Crittenden 2005). Unlike somatic cells, the mitotic and meiotically active germ cells are subject to various kinds of checkpoint control in response to DNA damaging agents (Gartner et al. 2000).

Gartner et al. (2000) found that late larval stage worms subjected to γ-IR showed increased levels of germ cell apoptosis in the pachytene region, providing evidence for a pachytene checkpoint that removes damaged germ cells before they become mature oocytes. The same apoptotic response was also seen for the DNA modifying drug ENU and for genotoxic damage caused by UV light (Gartner et al. 2000). Since processes required for apoptotic clearance of damaged cells are vital to maintaining healthy mitotic populations and defects in these processes are related to human disease states, interest in understanding DNA damage checkpoints in the simple organism *C. elegans* increased (Halazonetis et al. 2008; Lovell & Markesbery 2006; Mercer et al. 2007).

In mammalian cells, p53 plays a key role in protecting genome integrity by inducing apoptosis in cells with DNA damage (Levine 1997). *C. elegans* p53 family protein CEP-1 was found to play similar roles in the germline (Derry et al. 2001; Schumacher et al. 2001). Our knowledge of cep-1’s roles in the worm was reviewed in Chapter 1. This section will focus on cep-1’s relationship to its upstream regulator *hus-1*, the core apoptotic machinery and the engulfment gene ced-1, since these play a key role in the experiments described in this chapter.

When they found that DNA damage caused apoptosis in the germline of *C. elegans*, Gartner and colleagues demonstrated that these cell deaths required the same core apoptotic machinery required for developmentally programmed cell death (PCD) of somatic cells (Gartner et al. 2000; Lettre & Hengartner 2006). The *C. elegans* core apoptotic machinery consists of four genes: egl-1, ced-9, ced-4 and ced-3 (Lettre & Hengartner 2006). To promote apoptosis after DNA damage, CEP-1 transcriptionally upregulates the BH3-only domain gene *egl-1* (Hofmann et al.
This is similar to the mammalian pathway, where p53 transcriptionally regulates the expression of BH3-only domain proteins NOXA and PUMA to induce apoptosis in response to DNA damage (Nakano & Vousden 2001; Oda et al. 2000). In unstressed conditions, the anti-apoptotic Bcl-2 homologue CED-9 binds and sequesters proapoptotic Apaf-1 homologue CED-4 to the outer surface of the mitochondrial membrane preventing it from activating the CED-3 caspase (Spector et al. 1997; Wu, Wallen & Nuñez 1997). When CEP-1 induces EGL-1 expression, the EGL-1 BH3 only domain binds CED-9, which undergoes a conformational change that interrupts the interaction between CED-9 and CED-4 (Conradt & Horvitz 1998; del Peso et al. 1998). Once released from CED-9, CED-4 translocates to the nuclear membrane and binds and activates the CED-3 caspase (Yang et al. 1998; Chen et al. 2000; del Peso et al. 1998; Seshagiri & Miller 1997). In order to activate CED-3, several pro-CED-3 molecules must associate with a CED-4 octamer, leading to proteolytic cleavage of the proCED-3 molecules to the active CED-3 enzyme (Wu, Wallen, Inohara et al. 1997; Yang et al. 1998; Seshagiri & Miller 1997; Qi et al. 2010). The CED-3 caspase is the master regulator of apoptosis, presumably causing cell death by a proteolytic cleavage cascade (Xue et al. 1996).

Once killed by the core apoptotic machinery, the germ cell assumes a distinct morphology visible by differential interference contrast optics (Gumienny et al. 1999). The cell 'corpse' must then be engulfed by surrounding cells and its cell components recycled. In the germline, this is done by the gonadal sheath cells of the somatic gonad (Gumienny et al. 1999). Cell corpse engulfment is governed by a set of genes distinct from the core apoptotic machinery, and engulfment mutants can be recognized by the persistence of cell corpses long past the time when they would be phagocytosed and broken down in wild-type worms (Reddien & Horvitz 2004). One of these genes required for engulfment is ced-1, which encodes a large transmembrane protein required in the engulfing cell (Zhou et al. 2001). It is thought that ced-1 acts as a receptor that helps recognition of the dying cell, though the specific molecular identity of its ligand is unknown (Zhou et al. 2001). One idea is that it recognizes phosphatidylserine, a component of cell membranes that normally only occurs on the interior of cell membranes.
During apoptosis, the normal asymmetry of phosphatidylserine is interrupted, and it appears on the outside of the cell. The appearance on phosphatidylserine on the exterior of the cell membrane is a well-known 'eat me' signal that induces engulfment, and it has been hypothesized that CED-1 recognizes this signal as well (Zhou et al. 2001; Chaurio et al. 2009). Whatever its specific molecular signal, it is clear that CED-1 recognizes and binds to apoptotic cell corpses, initiating their engulfment (Zhou et al. 2001). A CED-1::GFP transgene shows a bright fluorescent ring around apoptotic cell corpses and has become an important tool for quantifying the number of cell corpses, particularly in the germline (Zhou et al. 2001; Gartner et al. 2008).

The activation of apoptosis in response to DNA damage downstream of CEP-1 by the core apoptosis machinery and engulfment proteins is reasonably well understood. The factors that function to transduce the DNA signal and activate CEP-1 are less well understood, although several of the genes involved are known and are conserved (Ahmed & Hodgkin 2000; Ahmed et al. 2001; Hofmann et al. 2002; Boulton et al. 2002; Stergiou & Hengartner 2004). One of these DNA damage checkpoint genes that acts upstream of cep-1 is hus-1, which, like cep-1, is required for all DNA damage-induced apoptosis in the germline (Hofmann et al. 2002). HUS-1 physically interacts with MRT-2, and MRT-2 and HPR-9 are required for HUS-1 localization to the nucleus (Hofmann et al. 2002). Together, HUS-1, MRT-2 and HPR-9 make up the 9-1-1 complex, an evolutionarily conserved part of the DNA damage checkpoint (Parrilla-Castellar et al. 2004; Ahmed & Hodgkin 2000; Hofmann et al. 2002; Boulton et al. 2002). Under normal conditions, HUS-1 is associated with chromatin, but after DNA damage HUS-1 staining is concentrated in nuclear foci that may coincide with sites of DNA damage (Hofmann et al. 2002). The C. elegans 9-1-1 complex mutants also have defects in telomere maintenance and a spontaneous mutator phenotype, neither of which have been observed in cep-1 mutants (Ahmed & Hodgkin 2000; Hofmann et al. 2002; Harris et al. 2006). The molecular mechanism by which HUS-1 as part of the 9-1-1 complex signals to CEP-1 is not known, but in the absence of HUS-1, CEP-1 fails to upregulate egl-1 to promote apoptosis, suggesting that HUS-1 signals to CEP-1, either directly or indirectly, to promote PCD after DNA damage (Hofmann et al. 2002).
2.2.3 Summary

In this chapter, I discuss an enhanced embryonic lethality interaction between cep-1 and him-5 that I show to be a result of a defect in the germlines of cep-1; him-5 mothers. Moreover, I show that cep-1's role in promoting embryonic survival in him-5 mutants is independent of its ability to promote apoptosis in the germline, and independent of its upstream regulator hus-1. cep-1's role is also specific to him-5 mutant backgrounds, since cep-1 mutation does not modify the embryonic lethality phenotype of the meiotic mutant him-6. This suggests a novel role for CEP-1 outside of its proapoptotic function, and may provide a model system that will allow further understanding of the meiotic mutant him-5.

C. elegans has been a powerful model system for understanding many conserved biological processes common to multicellular organisms. Important examples include the understanding that work in C. elegans has brought to the fields of PCD, DNA damage response and evolution of the p53 family (Kinchen & Hengartner 2005; Stergiou & Hengartner 2004; Lu & Abrams 2006). The work presented here showing the C. elegans sole p53 family member CEP-1 playing a critical role for survival in the background of the HIM-5 meiotic mutant may provide greater understanding of how these genes function in basic meiotic processes.

2.3 Materials and Methods

2.3.1 Nematode Strains and Culture

All strains used were derivatives of the N2 wild-type strain and were cultured on NGM plates at 20°C as described (Brenner 1974). Listed below are the mutant alleles used. Sources for and information on all mutations listed can be found at Wormbase (www.wormbase.org). A schematic of the molecular nature of the cep-1 and him-5 alleles used is shown in Figure 2-1.

LG I: cep-1(gk138), cep-1(lg12501), him-2(e1065), hus-1(op244)

LG III: ced-4(n1162), sma-3(e491)
Figure 2-1: The molecular identity of the *cep-1* and *him-5* alleles used.

A schematic of the coding regions of *cep-1* (A) and *him-5* (B). Coloured boxes depict exons while the intervening grey line is intronic regions. Arrows show the origin and direction of transcription and grey boxes represent noncoding exon regions. For *cep-1*, coloured exon regions indicate the domain structure of the encoded protein, with the DBD shown in red (Huyen et al, 2004) and the OD and SAM domains in green and purple, respectively (Ou et al, 2007). CEP-1 is also expected to contain an N-terminal TA domain, but this remains poorly defined. The *cep-1*(gk138), *cep-1*(lg12501) and *him-5*(ok1896) alleles are deletions that remove the genomic regions shown (www.wormbase.com). If the wild-type intron/exon boundaries are used, the *gk138* mutation encodes an in-frame deletion and would encode an internally deleted CEP-1 protein lacking much of the DBD domain, and *lg12501* would create a frameshift that would render the whole C-terminal portion of CEP-1 untranslated. The *him-5*(ok196) deletion removes the predicted start codon of HIM-5 and some upstream sequences, so this mutation could only give rise to a protein product if an alternative downstream start codon were used. *him-5*(e1467) is a point mutation that changes the initiator methionine codon from ATG to ATA while *him-5*(e1490) is a mutation of the splice acceptor site at the beginning of the third coding exon of *him-5* (P. Meneely and J. Yanowitz, personal communication).
LG IV:  *ced-3*(n717), *him-6*(e1423)

LG V:  *him-5*(e1467), *him-5*(e1490), *him-5*(ok1896)

Rearrangement:  *hT2 (I;III)ream*


### 2.3.2 Genotyping by Single-Worm PCR

The two *cep-1* alleles used in this study, *cep-1*(gk138), *cep-1*(lg12501), as well as *hus-1*(op244) are all deletions of portions of the gene (www.wormbase.org). Since *cep-1* and *hus-1* worms show no gross phenotype under the dissecting microscope that would aid in identifying homozygotes during crosses (Derry et al. 2001; Schumacher et al. 2001; Hofmann et al. 2002), I used single worm PCR to genotype strains made with these alleles. Single worm PCRs were done essentially as described (Williams et al. 1992). After an initial round of PCR a second nested reaction was performed using 1µL of the primary reaction as template. The primers were:

- For *cep-1*(gk138), WBD34: 5' GGTGGACTGTTGCTTTGAAATCAAGACTGC 3' and WBD35: 5' GCTCTTGATGTTGCCAACAAGATCGGATTC 3' (primary PCR) and WBD38: 5' CAGGGTGAGTTGGCGTTAGG 3' and WBD39: 5' AATTGGTGACGCGACTTCTCTTCA 3' (secondary PCR);
- For *cep-1*(lg12501), *cep-1*(lg)OF: 5' TGCCTTCCTCTATTTCACTTG 3' and *cep-1*(lg)OR: 5' TTACTCCACTCCAACAGAAG 3' (primary PCR) and *cep-1*(lg)F: 5' ATAGGGGGTCGTGTAAGAGA 3' and *cep-1*(lg)R: 5' GCTTACGTTAGTGGGCTTC 3' (secondary PCR);
- And for *hus-1*(op244), *hus-1*(244)OF: 5' ATGGTCTTGAGGAAATAG 3' and *hus-1*(244)OR: 5' ATCCGTTAACAGTGAGATACTC 3' (primary PCR) and *hus-1*(244)IF: 5' AGGCACATACAATAATACGGTG 3' and *hus-1*(244)IR: 5' ATCACGATCATGTGAAGCCG 3' (secondary PCR). The presence of at least one copy of the deletion allele was confirmed by the presence of a band corresponding to the expected size for the deletion PCR product by gel electrophoresis. For the *cep-1* deletions, once one copy of the deletion allele was confirmed, homozygosity was assayed using another nested reaction.
Using the primary PCR reaction as template again, a reaction was performed that included one primer whose corresponding sequence is absent from the deletion allele but would be present in the wild-type allele. The primers used for this homozygosity reaction were as follows: for cep-1\((gk138)\), WBD38 (above) and AP15: 5' CGCCTGAACTGCGCCTGG 3'; and for cep-1\((lg12501)\), cep-1\((lg)F\) (above) and cep-1\(lgPOIR\): 5’ cat cca tga aga cga ctt gc 3’. Since hus-1 mutants were always crossed and maintained in the presence of the hT2 balancer, no PCR test for homozygosity was required. Representative examples of these PCR reactions are shown in Figure 2-2.

2.3.3 Brood Counts for Embryonic Lethality, Incidence of Males and XXX Dpys

For offspring counts, \(P_0\) worms were picked to individual plates as L4 larvae and allowed to lay eggs for one day. \(P_0\)s were then moved each day for all subsequent days until they failed to lay eggs in a 24h period.

For all of these plates, worms were counted in the following manner: On Day 1 (the day the \(P_0\) was removed) embryos and early larvae were counted, giving the number of Eggs Laid for the given plate. On Day 2, the number of Unhatched Eggs were scored. On Days 4 through 7, depending on the strain and the speed of development, the number of Adult Males, Adult Hermaphrodites and Adult Dpy XXX Hermaphrodites were scored.

If for an individual plate, the number of Eggs Laid was less than the sum of all other measurements \([ie.\text{ if }\text{Eggs Laid }< (\text{Unhatched Eggs }+ \text{Adult Males }+ \text{Adult Hermaphrodites }+ \text{Dpy XXX Hermaphrodites})]\) this was assumed to be due to counting error on the first day and so Eggs Laid was set to be equal to the sum of all other measurements \([ie.\text{ Eggs Laid }= (\text{Unhatched Eggs }+ \text{Adult Males }+ \text{Adult Hermaphrodites }+ \text{Dpy XXX Hermaphrodites})]\) for this plate. For each \(P_0\), the total number of Eggs Laid, Unhatched Eggs, Adult Males, Adult Hermaphrodites and Dpy XXX Hermaphrodites for the whole brood were calculated by adding the scores for each from all plates.
Figure 2-2: *gk138*, *lg12501* and *op244* were genotyped by PCR.

A. shows a schematic outlining the primers used for genotyping *cep-1(gk138)*. B. and C. show representative examples of PCR reactions used to genotype for *gk138*. Primary single-worm PCRs were done using primers WBD34 and WBD35. 1µL of this primary reaction was used as template for a secondary reaction (B.) using primers WBD38 and WBD39. In B., wild-type N2 (lane N) and *cep-1(gk138)* homozygotes (lane C) were done as positive and negative controls, respectively and show bands of differing sizes, corresponding to the wild-type and *gk138* alleles. Lanes labelled 1 and 2 are examples of worms tested for their genotype and both contain the *gk138* allele. In C., the same primary PCRs were used as template with the primers WBD38 and AP15. Wild-type worms (lane N) show a band while *gk138* homozygotes (lane C) do not, since the sequence complementary to the primer AP15 is absent. Worms 1 and 2 do not show the band, demonstrating they are homozygous for the *gk138* allele. D. shows a schematic outlining the primers used for genotyping *cep-1(lg12501)*. E. and F. show representative examples of PCR reactions used to genotype *lg12501*. Primary single-worm PCRs were done using primers *cep-1(lg)*OF and *cep-1(lg)*OR. 1µL of this primary reaction was used as template for a secondary reaction (E.) using primers *cep-1(lg)*F and *cep-1(lg)*R. In E., wild-type N2 (lane N) and *cep-1(lg12501)* homozygotes (lane C) were done as positive and negative controls, respectively and show bands of differing sizes, corresponding to the wild-type and *lg12501* alleles. Lanes labelled 1 through 5 are examples of worms tested for their genotype. Lanes 2, 3 and 5 contain the *lg12501* allele while 1 and 4 do not. Note that sometimes the wild-type band is absent, as in 1 and 4. In F., the same primary PCRs were used as template with the primers *cep-1(lg)*F and *cep-1(lg)*POIR. Wild-type worms (lane N) show a band while *lg12501* homozygotes (lane C) do not, since the sequence complementary to the primer *cep-1(lg)*POIR is absent. Worms 2, 3 and 5 were tested in this reaction to assess if they were homozygous for *lg12501*. Lanes 2 and 3 show the band, demonstrating they are heterozygous for *lg12501*. Lane 5 does not show the band, demonstrating it is a *lg12501* homozygote. G. shows a schematic outlining the primers used for genotyping *hus-1(op244)*. Primary single-worm PCRs were done using
primers hus-1(244)OF and hus-1(244)OR. 1µL of this primary reaction was used as template for a secondary reaction (H.) using primers hus-1(244)IF and hus-1(244)IR. wild-type N2 (lane N) and hus-1(op244)/hT2 heterozygotes (lane H) were done as positive and negative controls, respectively, and show bands of differing sizes, corresponding to the wild-type and op244 alleles. Lanes labelled 1 and 2 are examples of worms tested for their genotype and both contain the op244 allele. Since worms 1 and 2 were GFP-positive and were therefore hT2 heterozygotes, no homozygosity PCR test was needed.
For each P₀, the following calculations were made:

Embryonic lethality = Unhatched eggs/Eggs Laid

Larval lethality = (Eggs Laid – Unhatched Eggs – Adult Males – Adults Hermaphrodites
– Adult Dpy XXX Hermaphrodites)/Eggs Laid

Incidence of Males = Adult Males/(Adult Males + Adult Hermaphrodites + Adult Dpy
XXX Hermaphrodites)

Incidence of XXX Dpy Hermaphrodites = XXX Dpy Hermaphrodites/(Adult Males
+Adult Hermaphrodites + Adult Dpy XXX Hermaphrodites)

In all cases, averages are given for 5-9 broods for each strain. Error bars represent S.E.M. and
pairs of strains are compared using a 2-tailed Student's t Test.

2.3.4 Complementation Tests

To test for complementation between cep-1 and him-2, cep-1(lg12501); sma-3(e491)
hermaphrodites were crossed to him-2(e1065) males. The sma-3 allele was included to ensure
F1 self and cross progeny could be differentiated. F1 Non-Sma cross progeny were picked to
individual plates and their progeny were scored and compared as outlined in Section 2.3.3.

The complementation test between him-5 and D1086.4 was done in the same manner, but using
the strains sma-3(e491); him-5(e1490) and D1086.4(ok1896).

2.3.5 bcls39 Apoptosis Assay

The bcls39 transgene contains a ced-1::GFP transgene under the control of the lim-7 promoter
(Zhou et al. 2001). lim-7 drives expression in gonadal sheath cells (Voutev et al. 2009). The
bcls39 transgene marks germline cell corpses with a ring of fluorescence, allowing them to be
counted easily (Zhou et al. 2001).
I constructed cep-1 and him-5 single and double mutant strains in the bcIs39 background and synchronized worms from each strain at L4. Worms from each strain were subjected to either 0 Gray (Gy) or 60Gy of ionizing radiation 24h post-L4 using a Gammacell 1000 Elite Cesium-137 radiation source. 24h later, worms were mounted on a 2% agarose pad in 0.4% 1-phenoxy 2-propanol in M9, examined under a Leica DMR HC fluorescence microscope and the number of CED-1::GFP marked cell corpses per gonad arm were counted. At least 25 worms were scored per condition in each experiment and the results shown represent averages of 3 independent experimental trials. Error bars are S.E.M. and experimental conditions were compared pairwise using a 2-tailed Student's $t$ Test. Pictures were taken using Openlab software and images were cropped and contrast-adjusted using the program ImageJ.

2.4 Results

2.4.1 him-2 Is Not Allelic With cep-1

cep-1(lg12501) and cep-1(RNAi) both show a slight Him phenotype, suggesting cep-1 has a role in meiotic genomic stability (Derry et al. 2001). In their initial paper on nondisjunction mutants in the worm, Hodgkin et al. (1979) describe him-2, mutations in which cause a subtle Him phenotype, represented by a single allele (e1065) and mapping to a region near cep-1 (Derry et al., 2001, Schumacher et al, 2001). him-2 remains uncloned, and as I became interested in cep-1's role in meiotic chromosome stability I decided to test whether him-2 and cep-1 are allelic using a complementation test.

I crossed him-2(e1065) to cep-1(lg12501); sma-3(e491) and cloned Non-Sma F1 cross progeny at the L4 stage to individual plates and counted their offspring. I then compared the offspring of these trans-heterozygotes to those from cep-1(lg12501)/+;sma-3(e491)/+, him-2(e1065)/+;sma-3(e491)/+ as well as cep-1(lg12501) and him-2(e1065) homozygotes. As shown in Figure 2-3, cep-1(lg12501) and him-2(e1065) homozygotes produce low but significant numbers of males among their progeny compared to heterozygotes of either allele. cep-1(lg12501)/him-2(e1065); sma-3(e491)/+ generate significantly fewer males than either cep-1(lg12501) or him-2(e1065)
Figure 2-3: *him-2 and cep-1 are not allelic.*

*him-2 and cep-1* homozygotes, *him-2/+;sma-3/+* and *cep-1/+;sma-3/+* heterozygotes and *him-2/cep-1* trans-heterozygotes were cloned as L4s and the proportion of males among their offspring were calculated. *cep-1(lg12501)* and *him-2(e1065)* both have modest but significant numbers of males among their offspring (2-5%) while heterozygotes of either gene show few if any males among their offspring. Similarly, *him-2/cep-1* trans-heterozygotes produce significantly fewer male offspring than either *him-2* or *cep-1* alone. * denotes p < 0.05 by Student's t test. 5-9 whole broods were counted per condition and error bars are standard error of the mean (S.E.M.)
alone (p < 0.05 by Student's t test for both), suggesting that cep-1 and him-2 are not allelic.

2.4.2 him-5 Corresponds to the ORF D1086.4

Although him-5 mutations were originally described in the 1970s, the molecular identity of him-5 was unpublished when I became interested in it (Hodgkin et al. 1979). However, a 2005 International Worm Meeting abstract from the Meneely lab reported that him-5 corresponds to the ORF D1086.4 (P. Meneely, personal communication). Therefore I performed a complementation test between him-5 and D1086.4 to confirmed this unpublished report.

D1086.4 has been targeted by the C. elegans Gene Knockout Consortium to create a deletion allele, ok1896. I crossed D1086.4(ok1896) into sma-3(e491); him-5(e1490) and cloned Non-Sma cross progeny F1 L4s to individual plates and counted their offspring. I then compared the offspring of these trans-heterozygotes to those from sma-3(e491)/+; him-5(e1490)/+, sma-3(e491)/+; D1086.4(ok1896)/+ as well as him-5(e1490) and D1086.4(ok1896) homozygotes. As shown in Figure 2-4, both him-5(e1490) and D1086.4(ok1896) produce 35-40% males among their offspring. Heterozygotes of either allele exhibit no significant Him phenotype but e1490/ok1896 trans-heterozygotes show a Him phenotype not significantly different from him-5(e1490) or D1086.4(ok1896) homozygotes, demonstrating that him-5 and D1086.4 are allelic. For this reason, D1086.4(ok1896) will be denoted as him-5(ok1896) henceforth.

Although this experiment confirms the Meneely result and the molecular identity of him-5 it gives little clue to its molecular function. him-5 is predicted to encode a 252aa protein with a high pI of 10.75. It has no obvious domain structure nor homology to other proteins, and even searching the genomes of related nematode species with the HIM-5 sequence shows retrieves no results with standard search criteria (www.wormbase.org).
Figure 2-4: *him-5* corresponds to the ORF *D1086.4*.

*him-5(e1490)* and *D1086.4(ok1896)* homozygotes, *sma-3/++;him-5/+* and *sma-3/++;D1086.4/+* heterozygotes and *him-5/D1086.4* trans-heterozygotes were cloned as L4s and the proportions of males among their offspring were determined. *him-5(e1490)* and *D1086.4(ok1896)* both have more than 35% male offspring while heterozygotes of either gene show few if any males among their offspring. In contrast, *e1490/ok1896* generate male offspring at a frequency indistinguishable from *e1490/e1490* and *ok1896/ok1896* by Student's *t* test (*p*=0.96 for the transheterozygote compared to *e1490* and *p*=0.62 for the transheterozygote compared to *ok1896*). 5-9 whole broods were counted per condition and error bars are S.E.M.
2.4.3 *cep-1* and *him-5* Exhibit Enhanced Embryonic Inviability but *cep-1* Does Not Impact the Production of Males or XXX Dpy Progeny

This project began serendipitously, when I crossed *cep-1* worms into a *him-5* background to generate strains with a high incidence of males to facilitate examination of *cep-1* male development. I found that *cep-1; him-5* double mutants produced a high frequency of inviable embryos that was greater than the additive effect of *cep-1* and *him-5* single mutants (Figure 2-5). The Him phenotype of *him-5* is caused by a defect in meiotic chromosome stability (Hodgkin et al. 1979). Since examining mammalian p53 family proteins' role in genomic stability is made difficult by the presence of three different family members, each with multiple isoforms, and since it was conceivable that *cep-1* was enhancing *him-5* through a defect in chromosome biology, I decided to explore this interaction further (Bourdon 2007). I reasoned that a role for the sole *C. elegans* p53 family member in genome maintenance may provide insight into the more complex p53 family in mammals (Derry et al. 2001; Schumacher et al. 2001).

Figure 2-5 shows average embryonic lethality for broods generated by animals homozygous for various alleles of *cep-1* and *him-5* as single and double mutant strains. *cep-1* single mutants have very low embryonic lethality (less than 2% on average) whereas *him-5* mutants have embryonic death ranging from 9% to 20%, depending on the allele. It has been postulated that this lethality is due to nondisjunction of autosomes in the maternal germline, which is lethal to embryos in most cases (Hodgkin et al. 1979). For 3 alleles of *him-5* and 2 alleles of *cep-1*, the *cep-1; him-5* double mutants showed at least a two-fold increase in embryonic lethality compared with each corresponding *him-5* allele alone. In all cases, this increase was more than additive and statistically significant. This demonstrates that mutation of *cep-1* significantly enhances the embryonic lethality phenotype of *him-5*, suggesting the genes interact genetically and may be required in parallel for meiosis or embryonic development. Most *cep-1; him-5* worms that survive embryogenesis appeared normal, both by dissection microscopy and under Nomarski optics. Although the above result gives no evidence for or against it, it is tempting to speculate that *cep-1; him-5* worms show higher embryonic lethality due to a defect in chromosome biology during meiosis.
Figure 2-5: *cep-1; him-5* animals show enhanced embryonic lethality.

Various *cep-1* and *him-5* single and double mutants were cloned as L4s and the proportion of unhatched eggs among their offspring was quantified. *cep-1* worms show very low levels of Emb (less than 2% on average) while *him-5* worms show a higher proportion of dead eggs (from 9% to approximately 20% on average). In every case, *cep-1; him-5* doubles show at least a 2-fold increase in embryonic inviability. This increase was significant and much more than additive, suggesting that the two genes interact to promote embryonic survival. * denotes p<0.05 and ** is p<0.005 by Student's *t* test. 5-9 whole broods were counted per condition and error bars are S.E.M.
I next asked if cep-1; him-5 worms produce different percentages of males among their offspring, since this might indicate a role for cep-1 in meiotic nondisjunction. As shown in Figure 2-6, there is no consistent difference in incidences of males between him-5 single mutants and corresponding cep-1; him-5 doubles. Only the him-5(e1490) and cep-1(lg12501); him-5(e1490) pair approaches significance (p=0.05 by Student's t test) with the cep-1; him-5 double generating lower average percentage of male offspring than him-5 alone. The reason for this near-significant difference is unknown, but this combination of alleles does have the highest rate of embryonic lethality of any pair so the decreased frequency of males may not be meaningful since there are fewer progeny survive to adulthood per brood which may result in sampling error. Overall, this result demonstrates that ablation of cep-1 in a him-5 background does not significantly alter the ratio of male to hermaphrodite offspring, suggesting cep-1 does not contribute significantly to the meiotic distribution of the X chromosome in him-5 worms.

Just as meiotic nondisjunction can lead to X chromosome loss and XO male offspring among XX hermaphrodite self-progeny, the reciprocal nondisjunction event leads to offspring with three X chromosomes, which develop as Dpy hermaphrodites (Hodgkin et al. 1979). Mutations in any him gene result in some proportion of XXX Dpy offspring, though always at a lower proportion than XO males, for unknown reasons (Hodgkin et al. 1979). In order to confirm that cep-1 does not significantly contribute to nondisjunction of the X in a him-5 background, I counted the proportion of Dpy XXX offspring among the progeny of various cep-1 and him-5 single and double mutants. Figure 2-7 shows the proportion of XXX Dpy progeny for various cep-1 and him-5 singles and doubles. As I showed above for the Him phenotype in these strains, there is no significant difference between him-5 single mutants and corresponding cep-1; him-5 doubles in the proportion of XXX Dpy hermaphrodites among their offspring. In all cases, strains with the him-5(e1490) allele show substantially higher rates of XXX Dpy progeny compared with the other him-5 alleles, but there is no difference in the frequency of XXX progeny from him-5 versus cep-1; him-5. This result confirms that cep-1 does not affect the rate of X chromosome nondisjunction in him-5 backgrounds.
Figure 2-6: *cep-1* does not significantly modify the *him-5* Him phenotype.

Various *cep-1* and *him-5* single and double mutants were cloned as L4s and the proportion of males among their offspring were quantified. *cep-1* worms generate very low levels of male offspring (approximately 1.5% on average for *lg12501* while *gk138* shows even less). *him-5* mutants produce a high incidence of males, with *e1467* generating approximately 25% and the stronger alleles *e1490* and *ok1896* generate greater than 35%. Generally, *cep-1* does not modify this phenotype, since there are no significant differences between frequency of Him in any *him-5* and *cep-1; him-5* pair, although the comparison between *him-5(e1490)* and *cep-1(lg12501); him-5(e1490)* approaches significance (p=0.05 by Student's *t* test). 5-9 whole broods were counted per condition and error bars are S.E.M.
Various *cep-1* and *him-5* single and double mutants were cloned as L4s and the proportion of XXX Dpy progeny among their offspring was quantified. *cep-1* worms generate very low levels of XXX Dpy offspring. *him-5*, like other classical *him* mutants, generates a higher frequency of XXX progeny, with *e1490* showing considerably more than the other two alleles. *cep-1* does not significantly modify this phenotype, since there are no significant differences between frequency of XXX Dyps in any *him-5* allele versus the corresponding *cep-1*; *him-5* double mutant strain by Students' *t* test (for *him-5(e1467)* compared to *cep-1(gk138)*; *him-5(e1467)*, p=0.48, for *him-5(e1490)* compared to *cep-1(gk138)*; *him-5(e1490)*, p=0.06, for *him-5(e1490)* compared to *cep-1(lg12501)*; *him-5(e1490)*, p=0.62 and for *him-5(ok1896)* compared to *cep-1(gk138)*; *him-5(ok1896)*, p=0.10). 5-9 whole broods were counted per condition and error bars are S.E.M.
2.4.4 cep-1; him-5 Offspring from cep-1/+; him-5/+ Mothers Are Not Embryonic Lethal

The defect in him mutants is due to a defect in the meiotic germline (Hodgkin et al. 1979). This means that it is not clear a worm has a Him phenotype until males are seen among her offspring. During crosses using recessive him alleles (like him-5), the offspring of him/+ mothers are not Him, nor do they show embryonic lethality. I wanted to know if the defect leading to embryonic lethality in cep-1; him-5 was due to a defect in the meiotic germline, as for traditional him mutants. To do this, I used two GFP transgenes which functioned as dominant genetic markers: mgIs42, which marks two head neurons and is integrated on chromosome V, and ayls4 marks the vulva and is integrated on chromosome I. These transgenes were useful for this purpose because I could differentiate worms that contained mgIs42 alone, ayls4 alone, both, or neither using a dissecting microscope equipped with fluorescence optics. I constructed ayls4; him-5(e1490) and cep-1(lg12501); mgIs42 strains and crossed them to generate cep-1 +/- ayls4;him-5 +/- mgIs42 F1s. The F1s were cloned at the L4 stage and their offspring quantified according to which transgenes they were expressing. From a cross like this, one would expect a typically Mendelian ratio for dominant markers: 9 ayls4 and mgIs42: 3 mgIs42 only: 3 ayls4 only : 1 no GFP (Figure 2-8). Excluding the effects of recombination between the cep-1 and him-5 and their respective marker, the F2s without GFP signal would be expected to be cep-1; him-5 double homozygotes. If the excess lethality of cep-1; him-5 worms was due to a meiotic defect in the germline of double homozygotes, I would expect 1/16th of the offspring of this cross to be GFP-negative (and therefore putatively cep-1; him-5). If the excess embryonic lethality was due to a zygotic defect, there would be a reduction in the ratio of GFP-negative F2s and an increase in dead eggs in the F2 generation. As a control I also counted the offspring of + +/- ayls4; him-5(e1490) +/- mgIs42 worms. In total, I counted more than 900 worms per condition. As shown in Figure 2-8, GFP-negative worms arose at expected Mendelian ratios, and the offspring of cep-1 +/- ayls4;him-5 +/- mgIs42 worms did not show increased embryonic lethality compared to that of + +/- ayls4;him-5 +/- mgIs42 worms. I tested 58 of the GFP-negative F2s by assaying for the Him phenotype and performing PCR for the cep-1(lg12501) allele and found that 49 of them...
A
\[
\text{\underline{\text{mgls42 and ayls4:}}} \quad \text{\underline{\text{mgls42 only:}}} \quad \text{\underline{\text{ayls4 only:}}} \quad \text{\underline{\text{Neither:}}}
\]
\begin{align*}
\text{cep-1} & = \text{him-5} \\
\text{mgls42} & \Rightarrow \text{mgls42} \\
\text{cep-1} & \Rightarrow \text{him-5} \\
\text{ayls4} & \Rightarrow \text{him-5} \\
\text{cep-1} & \Rightarrow \text{mgls42} \\
\text{ayls4} & \Rightarrow \text{mgls42} \\
\text{cep-1} & \Rightarrow \text{him-5} \\
\text{ayls4} & \Rightarrow \text{him-5} \\
\text{cep-1} & \Rightarrow \text{mgls42} \\
\text{ayls4} & \Rightarrow \text{mgls42} \\
\text{cep-1} & \Rightarrow \text{mgls42} \\
\text{ayls4} & \Rightarrow \text{mgls42} \\
\end{align*}

\[
\frac{9}{(56.2\%)} : \frac{3}{(18.8\%)} : \frac{3}{(18.8\%)} : \frac{1}{(6.3\%)}
\]

B

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Figure 2-8: Embryonic lethality in cep-1; him-5 double mutants is due to a germline defect.

The offspring of cep-1(lg12501) +/+ ayIs4;him-5(e1490) +/+ mgIs42 hermaphrodites, as well as + +/+ ayIs4;him-5(e1490) +/+ mgIs42 hermaphrodites were scored for embryonic lethality, Him, larval lethality (Lvl) and transgene expression. Five broods were pooled and counted, totalling more than 900 worms scored for each condition. A. shows the expected offspring from the double heterozygote parent. Genotypes of the expected offspring are grouped in columns according to the fluorescent phenotype observed, producing the Mendelian 9:3:3:1 ratio for dihybrid crosses using dominant markers. The parent shown in A. is the experimental condition, but the control (without the cep-1 mutant allele) would be expected to give the same fluorescent offspring ratio. B. compares this expected ratio to the offspring quantified from the cep-1(lg12501) +/+ ayIs4;him-5(e1490) +/+ mgIs42 (experimental) and + +/+ ayIs4;him-5(e1490) +/+ mgIs42 (control) conditions. Non-fluorescent offspring (the putative cep-1; him-5 double mutants) arise at a frequency very close to 1/16 expected from the heterozygous parent, suggesting that ablation of cep-1 and him-5 causes lethality through a germline (not zygotic) defect.
were homozygous for both cep-1(lg12501) and him-5(e1490), demonstrating that the fact that GFP-negative worms arose in Mendelian ratios was not due to extensive recombination between the cep-1 and him-5 mutant alleles and their respective markers. This result demonstrates that the cep-1; him-5 offspring of cep-1/+;him-5/+ mothers do not show increased embryonic lethality, unlike the offspring of cep-1; him-5 homozygotes (Figure 2-5). The excess embryonic lethality of cep-1; him-5 worms is likely due to a defect in the germline of the parent, rather than a zygotic defect and is consistent with a role for cep-1 in enhancing the meiotic chromosome defects of him-5.

2.4.5 The Offspring of cep-1; him-5 Males Do Not Show Increased Embryonic Lethality

Given that cep-1; him-5 hermaphrodites show increased embryonic lethality among their offspring, I wanted to know if the offspring of cep-1; him-5 males were also embryonic lethal. To do this, I crossed cep-1; him-5 males into wild-type N2 hermaphrodites and confirmed that the mating was successful by the presence of approximately 50% males in the F1 generation. These matings resulted in less than 1% dead eggs, suggesting that the defects in the germline of cep-1; him-5 hermaphrodites that give rise to embryonic lethality are hermaphrodite-specific. him-5 males crossed to N2 hermaphrodites produced similar results.

2.4.6 cep-1 Does Not Enhance the Embryonic Lethality of him-6 Mutants

Given that I had established that the increased lethality of cep-1; him-5 worms compared to him-5 alone is a result of a germline defect and may be due to a meiotic defect, I wanted to test if cep-1 exhibited enhanced embryonic lethality interactions with other him mutants, or if this was specific to the him-5 gene. The him-6 gene encodes a protein that is homologous to the human Blm helicase and mutation of this gene in C. elegans leads to extremely high embryonic lethality, presumably due to extensive aneuploidy caused by meiotic nondisjunction in the germline (Wicky et al. 2004). To test whether cep-1 mutation modifies the embryonic lethality phenotype of him-6, I constructed cep-1(gk138); him-6(e1423) double mutants and compared their embryonic lethality to him-6(e1423) single mutants. As shown in Figure 2-9, him-6(e1423)
Figure 2-9: *cep-1* does not modify the embryonic lethality phenotype of *him-6*.

*him-6(e1423)* and *cep-1(gk138); him-6(e1423)* doubles were cloned as L4s and the proportion of unhatched eggs among their offspring were quantified. Both *him-6(e1423)* and *cep-1(gk138); him-6(e1423)* worms show approximately 90% embryonic lethality and there is no significant difference between the two strains by Students' *t* test. 5-9 whole broods were counted per condition and error bars are S.E.M.
shows very high rates of embryonic lethality (approximately 90%). The cep-1; him-6 worms showed no significant change in this frequency of embryonic lethality, suggesting that cep-1 does not significantly modify the meiotic nondisjunction phenotype of him-6. It is unlikely that cep-1 is unable to enhance the embryonic lethality phenotype of him-6 mutants since other genes, such as him-18, have been reported to have synthetically lethal interactions with him-6 mutants (Saito et al. 2009).

2.4.7 The DNA damage Checkpoint Gene hus-1 Does Not Interact With him-5

Upstream of cep-1, the hus-1 gene product functions in a complex (the 9-1-1 complex) to sense DNA damage, which is relayed to cep-1 to activate germline apoptosis in response to genotoxic stress (Hofmann et al. 2002). DNA damage-induced germline apoptosis and the cep-1-dependent activation of egl-1 expression are suppressed in hus-1 mutants, indicating that HUS-1 is required to activate known CEP-1-dependent functions (Hofmann et al. 2002). To delineate whether the enhanced embryonic lethality between him-5 and cep-1 was due to a defect in the DNA damage checkpoint, I constructed a hus-1; him-5 double mutant strain and compared embryonic lethality with him-5 single mutants. Increased embryonic lethality would suggest that hus-1 (and likely the 9-1-1 checkpoint) is required for cep-1 to maintain embryonic viability in the him-5 background. However, if hus-1; him-5 worms do not show increased embryonic lethality, it would suggest a novel role for cep-1 in the germline independent of its canonical upstream regulators.

Because hus-1 mutants become sterile after several generations, I constructed hus-1(op244)/hT2; him-5(e1490) and compared broods from hus-1; him-5 homozygous offspring with hus-1(op244) and him-(e1490) homozygotes. Like cep-1, hus-1 mutants exhibit very low embryonic lethality (Figure 2-10, compared to Figure 2-5). Compared to him-5 alone, hus-1; him-5 do not show significantly increased embryonic lethality, unlike cep-1; him-5, which does show a profound increase in embryonic lethality compared to him-5 alone (Figure 2-10). The statistically insignificant increase observed in hus-1; him-5 was due to one anomalous brood, while all others
Figure 2-10: *hus-1* and *him-5* do not show significant enhanced embryonic lethality.

*hus-1(op244)* and *hus-1(op244); him-5(e1490)* homozygotes were cloned as L4s from *hT2* balanced strains and the proportion of unhatched eggs among their offspring was quantified and compared to *him-5(e1490)* alone and *cep-1(lg12501); him-5(e1490)*. *hus-1* mutation does not significantly enhance the embryonic lethality of *him-5* alone by Students' *t* test (p=0.30), and the slight increase that is seen is a result of one anomalous brood. This is in contrast to *cep-1; him-5*, which shows a marked increase in embryonic lethality compared to *him-5* alone. ** denotes p<0.005 by Student's *t* test. 5-9 whole broods were counted per condition and error bars are S.E.M.
are within the range of him-5 single mutants. This suggests that hus-1 is not required for cep-1 to promote embryonic viability when him-5 is inactivated. This is in contrast to the role of cep-1 in DNA damage-induced cell death, which is dependent on hus-1 (Hofmann et al. 2002).

2.4.8 Enhanced lethality between cep-1 and him-5 is independent of the core apoptosis pathway

Since I ruled out a role for known upstream regulators of cep-1 in its enhancement of him-5 lethality, I wanted to determine if its downstream effectors in the apoptotic pathway played a role in this phenotype. ced-3 encodes C. elegans' only caspase family member, and ced-4 encodes an Apaf-1 homologue, and both are required for essentially all apoptotic events in the worm (Zou et al. 1997; Lettre & Hengartner 2006; Yuan et al. 1993; Xue et al. 1996). ced-3 and ced-4 are key components of the apoptotic signalling cascade that functions downstream of cep-1 to promote DNA damage-induced apoptosis in the germline (Gartner et al. 2000; Derry et al. 2001; Schumacher et al. 2001).

To ascertain whether the role of cep-1 in promoting viability in the him-5 background relies on the core apoptotic machinery, I created double mutant strains between him-5 and the ced-3(n717) and ced-4(n1162) alleles. On their own, ced-3 and ced-4 mutants have very low embryonic lethality and neither ced-3 nor ced-4 enhanced the embryonic lethality of him-5 in compound mutant strains (Figure 2-11). This indicates that the enhanced embryonic lethal interaction between cep-1 and him-5 does not involve an apoptotic mechanism.

2.4.9 Physiological Germline Apoptosis Is Unaffected in him-5 Mutants

The result outlined in Figure 2-11 showing that ced-3 and ced-4 do not enhance the embryonic lethality of him-5 suggests that, whatever cep-1 is doing in the him-5 background to promote embryonic viability, it is independent of apoptotic processes. To further test whether him-5 had any role in apoptosis, I quantified germline apoptosis in him-5 and cep-1; him-5 mutants. I hypothesized that him-5 mutants may have endogenous DNA damage arising from their meiotic defects that could activate a cep-1-dependent checkpoint to promote apoptosis of the 'damaged'
Figure 2-11: Neither ced-3 nor ced-4 enhances the embryonic lethality of him-5.

ced-3(n717) and ced-4(n1186) were crossed into him-5(e1490) and the proportion of unhatched eggs among their offspring was quantified and compared to ced-3, ced-4 and him-5 alone and cep-1(lg12501); him-5(e1490). ced-3 and ced-4 single mutants show low embryonic lethality and ced-3 and ced-4 mutations do not increase the proportion of dead eggs laid by him-5 mutants (p=0.95 for ced-3; him-5 and p=0.32 for ced-4; him-5). This is in contrast to cep-1; him-5, which shows a marked increase in embryonic lethality compared to him-5 alone. ** denotes p<0.005 by Student's t test. 5-9 whole broods were counted per condition and error bars are S.E.M.
cells. It would follow that in \textit{cep-1; him-5} double mutants (\textit{i.e.} in the absence of \textit{cep-1} activity) these damaged cells would escape the apoptotic checkpoint to become damaged zygotes, which could account for the elevated embryonic lethality observed in \textit{cep-1; him-5} animals. This hypothesis predicts that \textit{him-5} mutants would have increased levels of germline physiological (\textit{i.e.,} in the absence of exogenous stress) apoptosis compared to wild-type, which would be suppressed by \textit{cep-1} mutation. Alternatively, if the enhanced embryonic lethality between \textit{cep-1} and \textit{him-5} is independent of apoptosis (as suggested by the previous experiments), I would not expect to see increased germ cell apoptosis in \textit{him-5} mutants.

To quantify apoptosis, I made use of the \textit{bcIs39} transgene which drives expression of a CED-1::GFP fusion from the \textit{lim-7} promoter (Zhou et al. 2001). CED-1 is a transmembrane protein that surrounds apoptotic cells to be engulfed, and the \textit{bcIs39} transgene is expressed in the gonadal sheath and marks apoptotic germline corpses with a ring of fluorescence (Zhou et al. 2001). I crossed the \textit{bcIs39} transgene into \textit{cep-1}(\textit{gk138}) and \textit{him-5}(\textit{ok1896}) single and double mutants and subjected hermaphrodites 24h post-L4 to either 0 Gy or 60 Gy of IR. I then counted the number of CED-1::GFP marked cell corpses per gonad arm. As shown in Figure 2-12, \textit{bcIs39} worms in the wild-type background have an average of approximately 5 corpses per gonad arm in the absence of IR. The number of cell corpses increases significantly 24h after treatment with 60 Gy IR to an average of approximately 11 per gonad arm. In a \textit{cep-1} background, the average number of cell corpses is very similar to wild-type, and there is no increase after IR, as expected, since \textit{cep-1} is absolutely required for DNA damage-induced apoptosis (Derry et al. 2001; Schumacher et al. 2001). The levels of physiological germ cell apoptosis in \textit{him-5} mutants are not significantly different from wild-type, arguing against the hypothesis outlined above that postulates \textit{cep-1} promotes embryonic viability in \textit{him-5} mutants by inducing higher levels of apoptosis. This result is in agreement with the \textit{ced-3; him-5} and \textit{ced-4; him-5} result outlined in Section 2.4.8 and suggests that \textit{cep-1}'s role in the \textit{him-5} background is non-apoptotic. After IR, \textit{bcIs39 him-5} worms show a DNA damage-induced increase in germline apoptosis that may be higher than that seen in wild-type, though the difference is not significant (\textit{p}=0.07 by Students’ \textit{t} test). This suggests that \textit{him-5} mutants have
Figure 2-12: *him* -5 mutants have no defects in physiological germline apoptosis compared to wild-type.

The *bcIs39* transgene was crossed into *cep-1(gk138)* and *him-5(ok1896)* single and double mutants and hermaphrodites from each strain were staged at L4 and subjected to either 0 Gy or 60 Gy of IR 24h later. 24h after IR treatment, worms were mounted and the number of CED-1::GFP marked cell corpses per gonad arm was quantified. A. shows a summary of the results. At least 25 worms were scored per condition, and shown are the averages of three independent replicates. B-I. shows representative examples for each condition. A single gonad arm is shown and is outlined in red, with distal toward the top. Apoptotic nuclei are ringed with fluorescence. In almost all cases, more apoptotic nuclei were present in other focal planes in addition to the one shown. The left column (B., D., F. and H.) is the 0 Gy condition and the right column (C., E., G. and I.) shows animals treated with 60 Gy. B. and C. are *bcIs39* alone, D. and E. are *cep-1(gk138)*; *bcIs39*, F. and G. are *bcIs39 him-5(ok1896)* and H. and I. are *cep-1(gk138)*; *bcIs39 him-5(ok1896)*. * denotes p<0.05 by Student's t test.
an intact DNA damage apoptosis checkpoint. In the cep-1; him-5 background in the absence of IR, worms show similar levels of apoptosis to all the other strains tested. As expected, the cep-1; him-5 worms do not show increased apoptosis after DNA damage, since the cep-1 activity required to promote the DNA damage apoptotic checkpoint is absent.

This result, along with the ced-3; him-5 and ced-4; him-5 data from Section 2.4.8 supports a model in which cep-1 has a novel role in the germline to promote viability of embryos in cooperation with him-5. The nondisjunction defects in him-5 mutants demonstrate that HIM-5 is required for proper chromosome dynamics during meiosis, so it is reasonable to hypothesize that cep-1 may be causing embryonic lethality by enhancing this phenotype in parallel.

2.4.10 Summary

These studies began with a serendipitous finding that cep-1 enhances the embryonic lethality phenotype of the meiotic gene him-5. As had been previously reported by the Meneely lab (P. Meneely, personal communication) him-5 is allelic to D1086.4, an ORF of unknown function and no significant homology. cep-1 did not affect the proportion of males or XXX Dpy worms produced by him-5 strains. The excess embryonic lethality of cep-1; him-5 worms appeared to be a result of a hermaphrodite germline defect rather than a zygotic defect, since the cep-1; him-5 offspring of cep-1/+;him-5/+ mothers did not show significant embryonic lethality, nor did the offspring of cep-1; him-5 fathers. The enhancing effect of cep-1 is at least somewhat specific to him-5 and is not a general effect on nondisjunction mutants, since cep-1; him-6 mutants do not show increased embryonic inviability compared to him-6 alone. A hus-1 mutation did not enhance the embryonic lethality of him-5, suggesting cep-1's role in promoting viability in this background is independent of its known upstream regulators.

I then tested the hypothesis that cep-1 promotes embryonic viability in him-5 worms by promoting germline apoptosis. Two lines of evidence refute this hypothesis. First, the core apoptotic genes ced-3 and ced-4, required for nearly all apoptotic events in the worm, do not enhance the embryonic lethality of him-5 mutants, as would be expected if cep-1's role was to
promote apoptosis of 'damaged' cells. Secondly, *him-5* worms do not show increased apoptosis in the absence of DNA damage, as would be expected if *cep-1(+)*) was promoting the apoptotic clearing of damaged cells in these worms. These results suggest that *cep-1* plays a non-apoptotic role in the germline of *him-5* worms to promote embryonic viability of offspring. Since *cep-1* is mainly known for its apoptotic role in the worm, it is likely that this role is novel (Derry et al. 2001; Schumacher et al. 2001; Greiss et al. 2008). This new role for *cep-1* and an extension of our understanding of the role of *him-5* will be outlined in Chapter 3.

2.5 Discussion

2.5.1 *him-5* Corresponds with the ORF D1086.4

The complementation test shown in Figure 2-4 confirms a previous report from the Meneely lab that *him-5* corresponds to the ORF D1086.4 (P. Meneely, personal communication). The putative protein encoded by *him-5/D1086.4* lacks any recognizable domain structure and shows no significant homology to other known proteins ([www.wormbase.org](http://www.wormbase.org)). HIM-5 homologues may exist in other nematodes of the genus *Caenorhabditis*, though to date none have been annotated. The only notable feature of the predicted protein that may give a clue to its function is its relatively high isoelectric point (pI = 10.75), which may suggest it functions to bind DNA.

There have been examples in *C. elegans* of proteins that have been found to be only distantly related in sequence from their homologues in other species but are found to be similar in molecular function. One example is CEP-1, which shows only 15% identity in the DNA binding domain to p53, yet both bind to the same DNA sequence and promote apoptosis after genotoxic stress by similar molecular mechanisms (Derry et al. 2001; Schumacher et al. 2001). Another example is BRC-2, the worm homologue of the mammalian gene BRCA2 (Martin et al. 2005). Despite being 1/10th the size of mammalian BRCA2, expression of *C. elegans* BRC-2 can rescue BRCA2 deficiency in mammalian cells by binding Rad51 and promoting HR (Min et al. 2007). It is therefore possible that HIM-5 has functional homologues that are distantly related in sequence in other species, but that these relationships remain to be uncovered. In Chapter 4, I
will discuss a preliminary hypothesis concerning a putative yeast homologue of *C. elegans* HIM-5.

### 2.5.2 *cep-1* Has a Novel Role in Promoting Embryonic Survival in *him-5* Mutants That is Independent of Its Known Up- and Downstream Regulators

This project began when I noticed that *cep-1* and *him-5* double mutants show significantly increased embryonic lethality compared to either *cep-1* or *him-5* single mutants alone (Figure 2-5). This lethality is greater than additive, suggesting that *him-5* and *cep-1* function in parallel to promote some process required for embryonic survival. This significant increase in embryonic lethality was seen for multiple alleles of each gene in different combinations, suggesting it is indeed a result of defects in *cep-1* and *him-5* and not other uncharacterized mutation(s) in the genetic background.

*him-5* was characterized as one of a set of several genes whose mutations cause meiotic nondisjunction in the worm, characterized by an increased frequency of male offspring from unmated mothers (the Him phenotype), resulting from defects in X chromosome disjunction (Hodgkin et al. 1979). Most *him* mutants, including *him-5*, also show increased embryonic inviability, which is presumed to be a result of increased autosomal nondisjunction, and in most cases would yield aneuploid inviable offspring.

Aneuploidy and p53 mutation often coincide in human cancers, and there has been much research and speculation concerning the degree to which p53 inactivation causes or permits cells to become aneuploid and the extent to which this contributes to cancer progression (Duensing & Duensing 2005). I reasoned that *cep-1* mutation may be exacerbating the meiotic chromosome defects of *him-5* and that the study of this process could shed light on the role of p53 homologues in promoting faithful chromosome segregation.

Since *cep-1* does not modify either the frequency of males or the frequency of XXX Dpy hermaphrodites of *him-5* single mutants (Figures 2-6 and 2-7), *cep-1* function does not appear to
influence the frequency of X chromosome nondisjunction in him-5 mutants but may act on another process in the germline. I also showed that the embryonic lethality defect is a result of a defect in the germline of cep-1; him-5 homozygotes rather than a zygotic effect (see Section 2.5.4 for further discussion).

It has been postulated but not proven that the embryonic lethality seen in him-5 mutants is due to a defect in chromosome autosomal segregation during meiosis (Hodgkin et al. 1979). Since there are five pairs of autosomes and only one set of sex chromosomes, it would be expected that mutations with equal likelihood of missgregation of all chromosomes would show embryonic inviability several fold higher than the frequency of males among their offspring (Hodgkin 2005). him-6 mutants, for example, show a phenotypic pattern like this (for e1423, 89% Emb, 11% Him among surviving adults, my results and Wicky et al. 2004). Strong him-5 mutants, on the other hand show higher frequency of males than dead eggs (for e1490, 20% Emb, 38% Him among surviving adults, my results and Hodgkin et al. 1979). If the embryonic lethality of him-5 mutants is caused by autosomal nondisjunction, it must be the case that nondisjunction of the autosomes is much less frequent than nondisjunction of the X chromosome in these worms. Or, it is possible that HIM-5 function is required for efficient segregation of the X chromosome and some other function important for embryonic survival that is unrelated to autosomal chromosome segregation. These possibilities will be further addressed in Chapter 3.

cep-1(lg12501) and cep-1(RNAi) lead to a low-penetrance Him phenotype, suggesting this gene may have a role in nondisjunction of the X chromosome (Derry et al., 2001 and Figure 2-5). cep-1 mutants also show slightly increased embryonic lethality compared to wild-type, and this may be a result of increased autosomal nondisjunction at meiosis or some other defect (Derry et al, 2001). Though these low-penetrance Him and embryonic inviability phenotypes may suggest a role for cep-1 in genomic stability, this has not been extensively studied. The enhanced embryonic lethality phenotype with him-5 (a gene with a known role in genomic stability) allowed me to ask if cep-1 has a role in genomic stability in this process or whether the embryonic inviability is a result of a defect in some other process.
Cep-1 has primarily been studied for its role in promoting apoptosis in response to genotoxic stress, which has been postulated to be its main role and the role most ancestral to the p53 family (Greiss et al. 2008; Derry et al. 2001; Schumacher et al. 2001). More recently, other roles for CEP-1 are emerging as well, including in regulation of germline mitotic arrest in response to particular stressors, regulation of innate immunity and a putative role in aging in response to different levels of mitochondrial function (Derry et al. 2007; Fuhrman et al. 2009; Ventura et al. 2009). Given that new roles of cep-1 in nematode development are being recognized, I wanted to know if enhancement by cep-1 of the embryonic lethality phenotype in the him-5 background was the result of a defect in stress-induced apoptosis or was due to a novel process.

Mammalian cells have a meiotic checkpoint that causes the apoptotic clearance of meiotic cells that fail chromosome synapsis, which, in mammals, is dependent on the completion of crossover recombination (Odorisio et al. 1998). C. elegans has been found to have a meiotic checkpoint that is activated when homologous pairing fails to occur, well before the onset of recombination (Bhalla & Dernburg 2005). Even though this process was found to be p53/cep-1 independent in both mammals and nematodes, I hypothesized that CEP-1 may be functioning in another meiotic checkpoint in him-5 mutants. If this were the case, the meiotic defects of him-5 mutants may activate the checkpoint, giving increased apoptosis in the him-5 background, clearing the 'damaged' cells before they go on to form zygotes. I showed that ced mutants do not enhance the embryonic lethality phenotype of him-5 animals and that him-5 animals do not show increased levels of apoptosis in the absence of DNA damage (Figures 2-10 and 2-11). These two results suggest that the survival-promoting function of cep-1(+) in him-5 mutants is unrelated to its ability to induce apoptosis. Rather, it is likely that the him-5 mutation has uncovered a novel role for CEP-1.

In addition to the downstream apoptotic pathway, known CEP-1 apoptotic functions also require several upstream regulators to sense damage and activate CEP-1. Important among these is the 9-1-1 complex, a conserved complex involved in recognition and coordination of the cellular response to DNA damage (Parrilla-Castellar et al. 2004). Originally described in yeast, this
complex is also found in nematodes, and is involved in maintenance of telomeres and the recognition and response to DNA damage (Ahmed & Hodgkin 2000; Hofmann et al. 2002; Boulton et al. 2002). One component of this complex, HUS-1, is genetically upstream of cep-1 in DNA damage-induced apoptosis, such that hus-1 function is required for CEP-1-induced apoptosis after DNA damage (Hofmann et al. 2002). Even though I had shown that the embryonic lethality seen in cep-1; him-5 worms was not a result of faulty apoptosis it was still possible that hus-1 function was required for CEP-1 to rescue embryonic inviability in him-5 animals. In support of this idea, it has been found previously that animals with mutations in the 9-1-1 complex genes, like cep-1, have a modest Him phenotype, suggesting that these genes may play a direct role in genomic stability (Ahmed & Hodgkin 2000; Hofmann et al. 2002). However, I found that hus-1 mutation does not enhance the embryonic lethality phenotype of him-5 animals (Figure 2-9), suggesting that CEP-1’s role in promoting embryonic survival in him-5 mutants is likely independent of its normal upstream activator, HUS-1.

Together, the results outlined in Figures 2-9, 2-10 and 2-11 show that CEP-1’s function in him-5 mutants is novel. cep-1 mutant worms show no gross defects and are indistinguishable from wild-type in the absence of stress (Derry et al. 2001; Schumacher et al. 2001). Despite this, cep-1 appears to have other cellular roles that are only revealed in response to stress, either from the environment or from defective genetic backgrounds (Derry et al. 2007; Fuhrman et al. 2009; Ventura et al. 2009, this work).

2.5.3 cep-1 Enhances the Embryonic Lethality of him-5 But Not him-6

Before I began this project, there were some reasons to believe that cep-1(+) may have a role in genomic stability. First, cep-1(lg12501) and cep-1(RNAi) worms showed modest increases in the frequency of males amongst their offspring (Figure 2-5 and Derry et al., 2001). cep-1 mutant alleles and RNAi also show increased embryonic lethality, and this could be a result of slight increases in genomic instability, though other explanations are also possible (Figure 2-4 and Derry et al., 2001). In addition, loss of mammalian p53 is highly correlated with genomic instability, especially aneuploidy (Duensing & Duensing 2005). Certainly some of p53’s activity
in promoting genomic stability is a result of its role in promoting apoptosis or cell cycle arrest, but more direct roles have also been suspected (Chumakov 2007).

Given that we might expect *cep-1* to play a role in genomic stability, and that I uncovered a role for *cep-1* in promoting embryonic survival in the *him-5* nondisjunction mutant, I wanted to ask if *cep-1* has a similar role in the background of other *him* nondisjunction mutants. *him-6* mutants have decreased recombination and a high level of achiasmate univalents in their diakinesis oocytes, suggesting that the elevated embryonic inviability is a result of nondisjunction leading to aneuploid offspring (Wicky et al. 2004). *cep-1* mutation did not affect the frequency of embryonic lethality of *him-6* mutants (Figure 2-9), suggesting that the enhancement of *him-5* by *cep-1* is not the result of a general role for *cep-1* in promoting survival in meiotic mutants, but is at least somewhat specific to *him-5*.

### 2.5.4 *cep-1; him-5* Double Mutants Show Increased Embryonic Lethality as a Result of a Germline Defect

Embryonic lethality in *C. elegans* can be caused by failure of a wide range of cellular and morphological processes, and can be caused by defects in either the maternal germline or in the zygote itself (Isnenghi et al. 1983). Since *him-5* mutants have defects in meiosis and *cep-1* is known to function in the germline, I hypothesized that the increased embryonic inviability of *cep-1; him-5* double mutants was a result of a maternal germline defect (Derry et al. 2001; Hodgkin et al. 1979; Schumacher et al. 2001). To test this hypothesis, I generated marked *cep-1/+; him-5/+* heterozygotes and showed that *cep-1; him-5* homozygotes arose at the expected ratio of 1/16, suggesting that *cep-1; him-5* offspring of *cep-1/+; him-5/+* mothers do not show increased embryonic inviability (Figure 2-8). Coupled with my result that showed *cep-1; him-5* fathers mated to wild-type mothers do not show increased frequency of lethality in their offspring it is clear that the defect in *cep-1; him-5* is a result of a defect in the maternal germline and not solely in the zygote. The role being played by *cep-1* and *him-5* in the maternal germline will be further explored in Chapter 3.
2.5.5 Summary

I have shown that *cep-1* and *him-5* mutations show enhanced embryonic lethality as a result of a defect in the maternal germline. Despite its effects on embryonic survival, *cep-1* mutation does not modify the high X chromosome nondisjunction seen in *him-5* mutants. HIM-5, as has been shown by the Meneely lab, corresponds to the ORF *D1086.4*, which show no obvious domain structure nor clear homology with other known genes. *cep-1* does not modify the phenotype of a severely embryonic lethal meiotic mutant, *him-6*, suggesting its role in promoting embryonic survival is at least somewhat specific to *him-5*. I have shown that *cep-1*’s role in the germline of *him-5* mutants is not a result of its ability to promote apoptosis, nor does it require activation by the 9-1-1 complex, a known upstream activator of *cep-1* after genotoxic stress. An examination of the germline defect seen in *cep-1; him-5* double mutants will be outlined in Chapter 3.
3 cep-1 and him-5 Are Required in Parallel for Meiotic Recombination

3.1 Abstract

As shown in Chapter 2, cep-1 mutation enhances the embryonic lethality of him-5 mutations. CEP-1's role in this process appears to be independent of its role in promoting germline apoptosis and of its known up- and downstream regulators. Since him-5 is a meiotic mutant that disrupts chromosome stability, I hypothesized that cep-1; him-5 double mutants may die as embryos due to meiotic chromosome stability defects in the maternal germline. In support of this hypothesis, I found that cep-1; him-5 double mutants had abnormal numbers of DAPI-staining objects in their diakinesis-arrested oocytes. In addition, the DAPI-stained objects in cep-1; him-5 double mutants were abnormal in shape with an aggregated, stringy appearance and DNA bridges between objects, reminiscent of the phenotype seen in rad-51 mutants. RAD-51 is the C. elegans recombinase required for meiotic HR, and rad-51 mutants show aggregated diakinesis chromatin along with very high frequencies of embryonic lethality. I hypothesized that cep-1; him-5 mutants had a defect in meiotic recombination, leading to increased frequency of embryonic lethality in their offspring. This was supported by epistasis analysis with spo-11, which encodes the C. elegans nuclease required for induction of the DSBs which serve to initiate meiotic recombination. Mutation of spo-11 rescues the chromosome aggregation defects in cep-1; him-5 diakinesis oocytes, suggesting the aggregation is a result of an inability to accurately repair SPO-11-induced DSBs by HR.

A report from the Yanowitz and Meneely labs suggested that him-5 had a role in promoting SPO-11-induced DSBs since its defects could be rescued by a moderate dose of IR during meiosis. I showed that while the Him phenotype of him-5 mutants is efficiently rescued by IR, embryonic lethality was not. This suggests that the increased frequency of male offspring in him-5 animals is a result of reduced programmed DSBs on the X chromosome but embryonic lethality caused by him-5 mutations is not solely a result of reduced recombination on the autosomes. As in him-5 single mutants, cep-1; him-5 double mutants showed efficient rescue of
the Him phenotype after a moderate dose of IR. However, the embryonic lethality showed significant rescue only with \textit{cep-1}(gk138) and not with \textit{cep-1}(lg12501), raising the possibility that these two alleles have differences in residual function. The lack of clear rescue of embryonic lethality of \textit{him-5} and \textit{cep-1; him-5} animals suggests these worms have defects downstream or in parallel to SPO-11-induced DSBs that impair embryonic survival. These defects may be a result of inefficient repair of DSBs (either programmed or exogenous) as suggested by the \textit{rad-51}-like diakinesis chromosome phenotype. In support of this, I found that \textit{cep-1; spo-11; him-5} triple mutants do not show significant rescue after a moderate dose of IR, unlike \textit{spo-11} single mutants which show significant rescue of embryonic lethality under the same conditions. This supports the hypothesis that \textit{cep-1; him-5} double mutants have defects that impede embryonic survival apart from, or in addition to, a reduction of SPO-11-induced DSBs on autosomes.

Taken together, these experiments suggest a role for \textit{him-5} and \textit{cep-1} in promoting meiotic recombinational repair in \textit{C. elegans}. HIM-5 also has a clear role in promoting SPO-11-induced DSBs, at least on the X chromosome. A role for HIM-5 in recombinational repair would mean it has a role at two different points in the recombination process, similar to \textit{S. cerivisae} Mre11 and its \textit{C. elegans} homologue. A role for CEP-1 in recombinational repair provides a direct link between this p53 family member and genomic stability in the worm, and also supports some studies from mammals which show p53 interacts with or alters the transcription of various HR proteins to modify their function. These studies deepen our understanding of the roles of both CEP-1 and HIM-5 during normal germline development.

3.2 Introduction

3.2.1 \textit{C. elegans} Recombination Mutants and Their Phenotypes

In this chapter, I outline evidence for \textit{cep-1; him-5} mutants having defects in meiotic recombination. An overview of recombination in \textit{C. elegans} was given in Chapter 1. Here, I will detail the mutant phenotypes of the recombination mutants referred to in this study.
In eukaryotes, recombination is initiated by a programmed DSB created by a homologue of the Spo11 nuclease family (Keeney 2001). *C. elegans* spo-11 mutants are morphologically normal and produce near-normal numbers of eggs, but more than 95% of progeny die before adulthood, and of the few escapers that survive, half are male (Dernburg et al. 1998). These phenotypes suggest a defect in meiotic chromosome segregation (Dernburg et al. 1998; Hodgkin et al. 1979). During meiosis I in *C. elegans*, homologous chromosomes pair and are joined by the SC. This is followed by meiotic recombination during the pachytene stage of meiosis I (Dernburg et al. 1998). By diakinesis, the SC has disappeared. Bivalents are maximally condensed and are held together solely by crossovers between homologues, the physical result of meiotic recombination. Cytological analysis of *spo-11* mutants revealed that their diakinesis arrested oocytes contained 12 univalents instead of the six bivalents seen in wild-type worms, suggesting *spo-11* mutants have an absence of crossing over during meiosis (Dernburg et al. 1998). This result was verified genetically by showing complete absence of recombination between genetic markers over a large interval in *spo-11* mutants (Dernburg et al. 1998). These phenotypes, along with the molecular identity of SPO-11 as a homologue of the yeast Spo11 nuclease, led Dernburg et al. (1998) to suggest that *spo-11* is required to initiate meiotic recombination in *C. elegans*. Supporting the hypothesis that SPO-11 is required to induce the programmed DSBs that initiate recombination is the fact that recombination and survival in *spo-11* mutants can be significantly rescued by exogenous DSBs provided by IR during meiosis (Dernburg et al. 1998). No defects in chromosome morphology are seen prior to diakinesis in *spo-11* mutants, and homologous pairing and SC formation appear normal in these animals (Dernburg et al. 1998).

The genes *com-1*, *brc-2* and *rad-51* all have very similar phenotypes when mutated, including complete embryonic inviability of offspring and diakinesis chromosomes that appear ragged, stringy, diffuse and aggregated (Penkner et al. 2007; Martin et al. 2005; Takanami et al. 1998). These diakinesis phenotypes are particularly striking since mitotic proliferation, entry into meiosis, homologue pairing and SC formation all appear normal in these mutants. For *com-1*, *rad-51* and *brc-2* mutants, diakinesis aggregation is suppressed by mutation of *spo-11*, suggesting these genes operate downstream of *spo-11* and are involved in processing and repair
of the SPO-11-induced DSBs (Penkner et al. 2007; Martin et al. 2005; Rinaldo et al. 2002). COM-1 is the homologue of the *S cerevisiae* nuclease Com1/Sae2 and human CtIP (Chinnadurai 2006; McKee & Kleckner 1997; Prinz et al. 1997; Penkner et al. 2007). It is thought that, like its yeast homologue, COM-1 is required to resect SPO-11-induced meiotic DSBs to produce the 3' ssDNA ends required for HR to occur (Penkner et al. 2007). Like *spo-11* mutants, *com-1* mutants show no somatic defects, and homozygous offspring from heterozygous mothers develop into normal-looking adults (Penkner et al. 2007). Mitotic bridges are often seen in embryos of *com-1* homozygotes, which may be a consequence of defects in meiosis or may suggest COM-1 has a role in mitosis (Penkner et al. 2007). Unlike wild-type, but similar to *spo-11* mutants, *com-1* mutants show no RAD-51 foci in late zygotene and early pachytene nuclei, suggesting COM-1 function is required for loading the RAD-51 recombinase onto meiotic DSBs (Dernburg et al. 1998; Penkner et al. 2007).

*rad-51* encodes the main *C. elegans* homologue of the Rad51/RecA recombinase family (Shinohara & Ogawa 1999; Rinaldo et al. 1998). Like other members of its family, RAD-51 is required to catalyze the strand invasion step of meiotic recombination (Rinaldo et al. 2002). In addition to the diakinesis chromosome aggregation seen in *rad-51* mutants, these mutants are hypersensitive to IR in late embryogenesis, suggesting RAD-51 plays a somatic role in DSB repair (Rinaldo et al. 2002). As with *com-1*, offspring of *rad-51* homozygous mothers die during early embryogenesis with evidence of abnormal chromosome fragments and bridges (Takanami, Mori et al. 2003). Pachytene nuclei of *rad-51* mutants are more sensitive to DNA damage-induced apoptosis than are wild-type nuclei (Takanami, Zhang et al. 2003).

*brc-2* is the *C. elegans* homologue of the mammalian BRCA2 breast cancer susceptibility locus (Martin et al. 2005; Thorslund & West 2007). Although absent from yeast, BRCA2 homologues in *C. elegans*, *Drosophila* and mammals are thought to bind to the Rad51 recombinase homologues, directing them to the meiotic DSB and modifying their function (Bennett & Noor 2009; Martin et al. 2005; Thorslund & West 2007). Like *com-1* and *rad-51*, *brc-2* mutants show complete embryonic lethality of offspring, and diffuse, aggregated chromatin during diakinesis.
(Martin et al. 2005). Depletion of lig-4, a gene required for the NHEJ pathway, suppresses the aggregated chromatin defect in brc-2 mutants but not that of rad-51 mutants (Clejan et al. 2006; Martin et al. 2005). This suggests that in the absence of proteins required for HR (such as in com-1, brc-2 and rad-51 mutants) other, more error-prone repair systems such as NHEJ are used, causing the aggregation phenotype (Martin et al. 2005). BRC-2 is thought to function in single-strand annealing (SSA), another rad-51-independent, error-prone pathway used in the absence of HR, explaining the suppression of aggregation in brc-2; lig-4 double mutants but not in rad-51; lig-4 worms (Martin et al. 2005; Petalcorin et al. 2006). brc-2 mutants have defects in RAD-51 focus formation both during normal meiotic recombination and in recombinational repair after IR, showing far fewer of these foci than observed in wild-type. This suggests that BRC-2 has a role in directing RAD-51 to the sites of DSBs (Martin et al. 2005). In contrast, the number of RPA-1 foci are increased in brc-2 mutants, suggesting BRC-2 normally functions to restrict accumulation of RPA-1 at meiotic DSBs (Martin et al. 2005). Like rad-51 mutants, but unlike com-1 mutants, brc-2 animals have increased levels of germ cell apoptosis (Takanami, Zhang et al. 2003; Penkner et al. 2007; Martin et al. 2005). The increased PCD of rad-51 and brc-2 mutants is thought to be a result of activation of a DNA damage checkpoint that functions to remove oocytes with DSBs that remain unrepaired (Martin et al. 2005). DSBs also persist in com-1 mutants and it is not clear why this unrepaired damage does not result in activation of an apoptotic checkpoint as is seen in brc-2 and rad-51 mutants. It has been suggested that the meiotic DSB checkpoint may be activated by the ssDNA generated by activity of COM-1, explaining why the checkpoint is not activated in com-1 mutant animals (Penkner et al. 2007).

The spo-11, com-1, rad-51 and brc-2 genes are all strictly required for meiotic recombination in C. elegans (Dernburg et al. 1998; Penkner et al. 2007; Rinaldo et al. 2002; Martin et al. 2005). Their striking diakinesis chromosome phenotypes (12 individual univalents seen in spo-11 mutants and aggregated, stringy decondensed chromatin seen in com-1, rad-51 and brc-2 mutants) have provided clear examples of the phenotypic effects of recombination defects in C. elegans, allowing for more recent identification of other recombination genes with more subtle, pleiotropic or synthetic effects (Dernburg et al. 1998; Penkner et al. 2007; Takanami et al. 1998;
Ward et al. 2010; Tang et al. 2010). An understanding of the phenotypes of recombination mutants lead me to hypothesize that the increased embryonic lethality I saw in the offspring of cep-1; him-5 mutants is due to a defect in meiotic recombination repair, based on the diakinesis phenotypes of these double mutants presented in this chapter.

3.2.2 Meiotic Recombination Initiation

Meiotic recombination is required in eukaryotes for efficient separation of homologues during meiosis to form genetically stable gametes (Keeney 2001). The importance of meiotic recombination to the genomic stability of eukaryotes during sexual reproduction has lead to detailed study of this process in many organisms. A general overview of meiotic recombination and a detailed examination of the process in C. elegans is given in Chapter 1. This section will focus on the initiation of meiotic recombination by programmed DSBs, since a role for HIM-5 in this process has been postulated and is corroborated by results presented in this chapter (J. Yanowitz and P. Meneely, personal communication).

The greatest amount of work on meiotic recombination initiation has been done in S. cerevisiae. Programmed DSBs appear during meiosis in budding yeast when meiotic recombination was expected to occur, and the location and frequency of these breaks correlates well with recombination frequency (Zenvirth et al. 1992; Fan et al. 1995). Formation of meiotic DSBs depends on the topoisomerase-like nuclease Spo11 (Cao et al. 1990; Bergerat et al. 1997). In addition to the Spo11 nuclease, several other proteins are required for efficient formation of meiotic DSBs and can be thought of as falling into three groups of interacting protein partners: Mre11-Rad50-Xrs2, Mer2- Mei4-Rec114 and Rec102-Rec104-Ski8 (Keeney 2001). Each complex is required for meiotic DSB formation but an understanding of the molecular role of each complex is still emerging. Mre11, Rad50 and Xrs2, form the MRX complex and are required for DSB formation by Spo11 as well as subsequent resection of the break (Usui et al. 1998; Malone & Esposito 1981; Ajimura et al. 1993; Ivanov et al. 1992). One of the main
functions of Xrs2 in this complex appears to be to target Mre11 to the nucleus (Tsukamoto et al. 2005). The nuclease function of Mre11 is critical for resection of the meiotic break, but not for its formation (Moreau et al. 1999).

*MER2/REC107* has no clear homologues in other species, but is required for DSB formation in budding yeast (Engebrecht et al. 1990; Nakagawa & Ogawa 1997; Malone et al. 1991). It appears to form a link between replication and DSB formation since it is phosphorylated by a cyclin-dependent kinase to allow loading of other DSB machinery onto chromatin (Sasanuma et al. 2008). Mer2 interacts with Mei4 and Rec114, other proteins required for induction of meiotic DSBs (Jiao et al. 1999; Maleki et al. 2007). Rec114 is specifically required for Spo11's association with meiotic DSB hotspots (Prieler et al. 2005).

*REC103/SKI8* is required for meiotic DSB formation and has been shown to bind directly to Spo11 (Malone et al. 1991; Uetz et al. 2000). It is also required to recruit the Rec102-Rec104 complex to meiotic chromosomes (Maleki et al. 2007). Rec102 and Rec104 are also required for meiotic DSB initiation (Malone et al. 1991). In addition to Rec104, Rec102 binds Spo11 and associates with meiotic chromosomes in a Spo11-dependent manner. Spo11 multimerization and binding to meiotic chromosomes is Rec102/Rec104 dependent as well (Kee & Keeney 2002; Malone et al. 1991; Sasanuma et al. 2007).

In addition to the MRX, Mer2-Mei4-Rec114, and Rec102-Rec104-Ski8 complexes there are other *S. cerevisiae* proteins required for meiotic recombination initiation. One of these is Rec112, which appears be homologous to the meiosis-specific Rec7 from *Schizosaccharomyces pombe* (Malone et al. 1991; Fox & Smith 1998; Nau et al. 1997). The genes *RED1*, *HOP1* and *MEK1* are related in function and are required for efficient meiotic DSB formation (Hollingsworth & Byers 1989; Rockmill & Roeder 1990; Rockmill & Roeder 1991). Red1 and Hop1 are structural components of the meiotic SC and its associated structures, and Mek1 is a kinase required to modify their function. The fact they are involved in programmed DSB formation suggests proper chromosomal structure is required for efficient Spo11 function. Other genes are involved in DSB formation in more subtle ways. Rec8, for instance, has a role in targeting Spo11 to
specific chromosome regions and mutations in the Rec8 cohesin change the distribution and frequency of DSBs without abolishing them (Kugou et al. 2009).

Although recombination sites vary from cell to cell, they are not distributed randomly. There are known 'hotspots' which show increased frequencies of meiotic DSB formation as well as 'coldspots' which show decreased frequencies. The presence of one meiotic DSB can also inhibit another one occurring nearby, whereas open chromatin states and chromatin loops promote increased frequencies of programmed DSB formation (Kirkpatrick et al. 1999; Wu & Lichten 1994; Blat et al. 2002; Fan et al. 1997). Most DSB hotspots are in promoter regions in *S. cerevisiae*, though this may not be true in other organisms (Nicolas 1998; Cromie et al. 2007). Some sequence specificity has been found in meiotic recombination hotspots, but sequence is clearly only one factor that determines the frequency of recombination at any particular site (Blumental-Perry et al. 2000). It is becoming clear that altered chromatin structure through changes in histone modifications can affect DSB distribution and frequency both locally and across the genome in various species (Mieczkowski et al. 2007; Merker et al. 2008; Buard et al. 2009).

In the fission yeast *S. pombe*, transient meiotic breaks also occur and are probably catalysed by the Spo11 homologue Rec12, which is required for meiotic recombination (Lin & Smith 1994). Unlike in budding yeast, however, the fission yeast Mre11 homologue Rad32 is not required in DSB formation, though the frequency of breaks is reduced in *rad32* mutants (Young et al. 2004). Although Rec32 is not required for DSB formation, this Mre11 homologue retains its critical role in DSB processing (Fox & Smith 1998). This illustrates the fact that even highly conserved processes such as meiotic recombination can have substantial differences in the specific roles of even highly conserved genes. *S. pombe* also has homologues of Ski8, Mei4 and Rec114, termed Rec7, Rec24 and Rec14, respectively, and all are required for Rec12's association with DSB hotspots and Rec12-dependent DSB formation (Evans et al. 1997; Fox & Smith 1998; Ludin et al. 2008; Martín-Castellanos et al. 2005). Rec7 and Rec24 have also been shown to interact
during meiosis (Steiner et al. 2010). In addition to these conserved factors, meiotic recombination initiation in *S. pombe* also requires Rec6, a novel protein (Cervantes et al. 2000).

Meiotic recombination initiation by DSB induction also appears to be conserved in other fungi. *Coprinus cinereus* spo11 mutants arrest during meiosis and undergo apoptosis, likely due to a failure of recombination (Celerin et al. 2000). mre11 mutants have a somewhat weaker phenotype than spo11 mutants in this species, suggesting that the Mre11 nuclease is not required for DSB induction, as it is in budding yeast (Gerecke & Zolan 2000). *Neurospora crassa* also has a Spo11 homologue that is required to initiate meiotic recombination (Bowring et al. 2006). *Sordaria macrospora* has a Ski8 homologue that retains its role in meiotic initiation and its localization to meiotic nuclei is mutually interdependent with that of Spo11 in this species (Tessé et al. 2003).

*Drosophila* also contains a Spo11 homologue, mei-W68, that is required for meiotic recombination (McKim & Hayashi-Hagihara 1998). In addition to this, the gene *mei-P22* encodes a small, basic protein of unknown molecular function and is required for meiotic DSB formation in these animals (McKim et al. 1998; Liu et al. 2002). It localizes to sites of DSBs before their formation (Mehrotra & McKim 2006).

In the plant model *Arabidopsis thaliana* there are three Spo11 homologues. Only two of them, SPO11-1 and SPO11-2, have roles in meiotic recombination initiation (Hartung & Puchta 2000; Grelon et al. 2001; Stacey et al. 2006). AtPRD1 is related to mouse Mei1 and both are required for DSB formation during meiosis (Muyt et al. 2007). A recent screen shows that two other genes, AtPRD2 and AtPRD3, are also required for this process (Muyt et al. 2009). Neither of these genes have any obvious homologues, though AtPRD3 contains motifs characteristic of DNA binding proteins.

Mouse Spo11 mutant germ cells fail to initiate meiotic recombination due to a failure of DSB induction (Baudat et al. 2000; Mahadevaiah et al. 2001). In addition to the AtPRD1-related Mei1, mammals contain highly diverged homologues of the *S. cerevisiae* Mei4 and Rec114.
proteins which retain their role in promoting meiotic recombination initiation (Libby et al. 2003; Kumar et al. 2010).

An overview of meiotic recombination in *C. elegans*, including the initiation step, was presented in Chapter 1. Briefly, *C. elegans* SPO-11 is required for meiotic recombination initiation, probably for creation of programmed meiotic DSBs. (Dernburg et al. 1998). As was found in *S. cerevisiae*, *C. elegans* MRE-11 is required for both formation and resection of SPO-11-induced DNA breaks (Chin & Villeneuve 2001). Other proteins required for wild-type levels of meiotic DSB formation include the SC component HTP-1 and chromatin modification proteins HIM-17 and DPY-28 (Mets & Meyer, 2009; Reddy & Villeneuve, 2004; Goodyer et al. 2008).

The production of meiotic DNA breaks is a complex interplay between chromatin structure and recombination initiation proteins. Some components of meiotic initiation, like Spo11 and its homologues, are highly conserved and maintain their function throughout evolution (Cao et al. 1990; Dernburg et al. 1998; Mahadevaiah et al. 2001). Others, like Mre11 and Rad50 are also highly conserved and play important roles in recombination in all species, but in some species they are required for the initiation step of meiotic recombination, and in others they are not (Usui et al. 1998; Gerecke & Zolan 2000; Dernburg et al. 1998; Young et al. 2004). Other components play important roles in DSB formation in particular species but don't appear to be conserved in other models (Cervantes et al. 2000; McKim et al. 1998). Still other meiotic initiation components were thought to be unique to their species but more recent searches have revealed highly diverged homologues which retain their meiotic initiation function in other species (Kumar et al. 2010). Meiotic recombination initiation is a critical process in sexual reproduction and much remains to be elucidated on how this process works. Work presented here will aid understanding of both meiotic DSB induction and subsequent recombinational repair in *C. elegans* and extends unpublished results from the Yanowitz and Meneely labs.
3.3 Materials and Methods

3.3.1 Nematode Strains and Culture

All strains used were derivatives of the N2 wild-type strain and were cultured on NGM plates at 20ºC as described (Brenner 1974). Listed below are the mutant alleles used. Sources for and information on all mutations listed can be found at Wormbase (www.wormbase.org).

LG I: cep-1(gk138), cep-1(lg12501)

LG IV: him-6(e1423), rad-51(lg8701), spo-11(ok79), dpy-9(e12), unc-17(e245)

LG V: him-5(e1490), him-5(ok1896)

LG X: lon-2(e678), unc-7(e139)

Rearrangement: nT1[qIs51] (IV;V)

Transgenic arrays: mlsII[myo-2::gfp] IV

3.3.2 Genotyping by Single-Worm PCR

Single-worm PCRs for genotyping were done as described in Section 2.3.2. Like other him-5 alleles, him-5(ok1896) shows a Him phenotype and in most cases this phenotype was used to confirm homozygosity of the Him mutation (Hodgkin et al. 1979). However, I was unable to use the Him phenotype of ok1896 when making strains containing spo-11(ok79) and him-5(ok1896) since the high embryonic lethality phenotype of spo-11 masks the Him phenotype of him-5(ok1896) (Dernburg et al. 1998). Primers used are as follows: him-5(ok)OF: 5'TGACGTCAAACGTTTTCAGC 3' and him-5(ok)OR: 5' GATGACGTTCGAGACCAAT 3' (primary PCR) and him-5(ok)IF: 5' AGCAATCAGGTGCCATTTGT 3' and him-5(ok)IR: 5' AAGGCCCTGAGAGCGTTTAT 3' (secondary PCR). Since him-5 is covered by the nT1 balancer that I used to maintain spo-11(ok79) (www.wormbase.org), no PCR check for
homozygosity was required. A representative example of this him-5(ok1896) genotyping PCR is shown in Figure 3-1.

3.3.3 DAPI Staining of Whole Worms

For rad-51(lg8701)/mIs11 and all strains containing spo-11(ok79)/nT1, gravid homozygous hermaphrodites were identified among the progeny of balanced heterozygotes by lack of pharyngeal GFP staining and picked individually into 1mL phosphate-buffered saline 0.2% Tween-20 (PBS-T). For all other strains, animals were washed off crowded plates in 1mL PBS-T. Animals were then fixed in cold methanol for at least 5min at -20ºC, washed once in PBS-T and then stained with 1µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 30min in the dark. Samples were washed twice in PBS-T, then mounted in 0.02g/mL 1,4-diazabicyclo[2.2.2]octane (DABCO), 90% glycerol in PBS.

For chromosome counting, animals were visualized on a Leica DMR HC fluorescence microscope. The number of DAPI staining objects in diakinesis oocytes was counted and scored as either '6 objects' or 'not 6 objects'. At least 20 oocytes were counted per trial and shown is the average of three independent experimental trials. Error bars are S.E.M. and in all cases I asked others to arbitrarily label the slides so I would be blind to the strain I was scoring until after counts were completed.

For diakinesis chromosome pictures and chromosome scoring, animals were visualized using a Zeiss LSM510 Multiphoton Laser Scanning Confocal Microscope. All oocytes pictured were either the first or second most mature oocyte in the gonad arm, ensuring they were arrested in diakinesis and had achieved maximal DNA condensation. Pictures shown are representative of the range of defects and effort was made to show oocytes where all DAPI-staining objects were visible on a single focal plane, though for the strains carrying spo-11(ok79) this was rarely possible. Pictures were captured using the LSM510 software package. For chromosome scoring, only the most mature oocyte in a given gonad arm was scored. DAPI staining in this oocyte was examined and assigned to one of the following three categories: 1) At least 5 DAPI-
**Figure 3-1: ok1896 was genotyped by PCR.**

A. shows a schematic outlining the primers used for genotyping *him-5(ok1896)*. Primary single-worm PCRs were done using primers *him-5(ok)OF* and *him-5(ok)OR*. 1µL of this primary reaction was used as template for a secondary reaction using primers *him-5(ok)IF* and *him-5(ok)IR*. In B., wild-type N2 (lane N) and *him-5(ok1896)* homozygotes (lane H) were done as positive and negative controls, respectively and show bands of differing sizes, corresponding to the wild-type and *ok1896* alleles. Lanes labelled 1 through 7 are examples of animals tested for their genotype. Animals 6 and 7 have the deletion band, demonstrating they are *him-5(ok1896)* heterozygotes while 1 through 5 do not. Note that sometimes the wild-type band is absent, as in 1 through 5. Since animals 1 through 5 were GFP-positive and were therefore *nT1[qIs51] (IV;V)* heterozygotes, no homozygosity PCR test was needed.
staining objects are present, and no more than 2 of them show any visible abnormality (eg. diffuse edges, 'lumpy' morphology, bridges with other chromosomes); 2) At least 5 DAPI-staining objects are present, but at least 3 of them look abnormal; or 3) only 4 or fewer aggregated DAPI-staining objects are present. Approximately 20 oocytes were scored for each strain.

3.3.4 Recombination Frequency Experiments

The proportion of recombinant offspring was calculated for linked heterozygotes over the intervals dpy-9 unc-17(IV) and unc-7 lon-2(X) in the wild type, cep-1(gk138), him-5(e1490) and cep-1(gk138); him-5(e1490) backgrounds. For the autosomal interval, dpy-9(e12) unc-17(e139)/++ heterozygotes were constructed in wild type and the cep-1 and him-5 single and double mutant backgrounds and their offspring counted as outlined in Section 2.3.3. In addition to categorizing offspring as male or hermaphrodite their phenotypes were also scored as wild-type, Unc, Dpy or Dpy Unc. No attempt was made to score XXX Dpy offspring due to difficulties in differentiating these from worms expressing the Dpy-9 phenotype. 5-9 entire broods were counted for each condition. Similar analysis was performed for the lon-2(e678) unc-7(e139) interval.

Since X-linked markers are hemizygous in males, only hermaphrodite offspring were analyzed to provide consistency between Him and non-Him strains and between the autosomal and X linked markers. Hermaphrodite offspring from all broods counted for each condition were pooled and differences in phenotypic distribution were analyzed between wild-type and cep-1 and him-5 single and double mutant backgrounds by a χ² test, calculated using the online tool available at http://www.physics.csbsju.edu/stats/ (Kirkman, 1996). For the lon-2 unc-7 interval, only the Lon recombinants are compared to total hermaphrodites counted due to reduced viability of the Unc animals, as was done in Hodgkin et al. (1979).
3.3.5 Radiation Rescue Experiments

Radiation rescue experiments were done essentially as described (Dernburg et al. 1998). $P_0$s were synchronized as very early L4s and then treated with either 0 Gy or 15 Gy IR 4h later using a Gammarcell 1000 Elite Cesium-137 radiation source. Approximately 22h hours later, the worms began to lay eggs. Offspring were then collected in three 12h batches by moving $P_0$s to new plates. Offspring counts were then done as described in Section 2.3.3. For all experiments $spo-11(ok79)$ controls were dosed with 15Gy IR in parallel to confirm they were rescued to <50% embryonic lethality in the first 12h of laying. The progeny of 5-9 animals were scored for each condition and error bars are S.E.M.

3.3.6 RAD-51 Immunofluorescence

Gravid hermaphrodites were washed off plates in PBS onto poly-lysine coated slides and immobilized with 60µM tetramisole. Gonads were extracted using two needles and fixed in 2% paraformaldehyde for 10min. Samples were then freeze cracked on dry ice and immersed in 1:1 methanol:acetone for 1min at -20ºC followed by incubation in PBS 1% Triton X-100 for 10min. Gonads were washed three times in PBS 0.1% Tween 20 for 10min and incubated in 1 drop Image-iT FX Signal Enhancer (Invitrogen I36933) for 20min. Samples were then blocked for 30min in PBS 0.1% Tween 20 1% BSA and incubated overnight with a 1:100 dilution of rabbit $\alpha$-RAD-51 (SDIX 2948.00.02) at room temperature in a humidified chamber. After washing three times in PBS 0.1% Tween 20 for 10min the gonads were incubated in a 1:1000 dilution of Alexa Fluor 488 conjugated goat $\alpha$-rabbit secondary antibody (Invitrogen A11008) in PBS 0.1% Tween 20 1% BSA for 1h in a humidified chamber in the dark. Samples were then washed three times in PBS 0.1% Tween 20 for 10min, mounted in Prolong Gold (Invitrogen P36930) and visualized with a Leica DMR HC fluorescence microscope. Images were captured using the Openlab software and processed using GIMP.
3.4 Results

3.4.1 *cep-1; him-5* Double Mutants Have Abnormal Numbers of DAPI-Staining Bodies in Their Diakinesis-Arrested Oocytes

I have shown in Chapter 2 that *cep-1; him-5* worms show increased embryonic lethality compared to *him-5* mutants alone, and that this appears to be due to a defect in the hermaphrodite germline, rather than in the zygote. In addition, I have shown that *cep-1(+)* does not promote embryonic viability in *him-5* mutants by increasing physiological germline apoptosis, suggesting that *cep-1; him-5* mutants uncover a novel, non-apoptotic role for *cep-1*. Since *him-5* is known to cause nondisjunction of chromosomes at meiosis, it was reasonable to hypothesize that *cep-1* mutation may decrease embryonic viability by enhancing *him-5*'s defects in meiotic chromosome behaviour (Hodgkin et al. 1979). I decided to examine the meiotic chromosomes directly to test for defects in chromosome morphology in *cep-1; him-5* double mutants.

The *C. elegans* germline has a convenient spatio-temporal organization wherein nuclei in the mitotic region of the proximal gonad enter meiosis, and then progress through meiotic prophase and along the gonad until they reach the proximal arm and arrest in diakinesis as oocytes. This creates a direct relationship between nuclear position in the gonad arm and stage of meiosis (Greenstein 2005). Diakinesis-arrested oocytes accumulate in the proximal gonad and DAPI staining of these cells in wild-type worms shows six diakinesis-arrested bivalents that are easily distinct from one another by fluorescence microscopy. I decided to DAPI stain *cep-1* and *him-5* single and double mutants and visualize their diakinesis bivalents to look for chromosome defects that might explain the *cep-1; him-5* embryonic lethality phenotype.

I stained whole animals of N2, *cep-1(gk138), him-5(e1490), cep-1(gk138); him-5(e1490)* and *him-6(e1423)* genotypes with DAPI and counted the number of DAPI staining bodies in their diakinesis-arrested oocytes by fluorescence microscopy. The results shown in Figure 3-2 are the averages of three different experimental replicates, and in all cases I was blinded to the condition I was scoring until the experiment had been completed. Both N2 and *cep-1(gk138)* had very high proportions of oocytes with six DAPI staining bodies. Since N2 worms have very low
Figure 3-2: *cep-1; him-5* double mutants have fewer oocytes with exactly six DAPI staining bodies than *him-5* alone:

A. Confocal image of a normal wild-type DAPI-stained gonad. Diakinesis-arrested oocytes with well-condensed, easily differentiated bivalents are marked with white arrows. N2, *cep-1(gk138), him-5(e1490), cep-1(gk138); him-5(e1490)* and *him-6(e1423)* worms were fixed and stained with DAPI. B. Average proportion of oocytes with exactly six DAPI staining objects for each strain. At least 20 oocytes were scored per strain and the graph shows the average of three independent experiments. N2 and *cep-1* animals both show a high proportion of oocytes with exactly six DAPI staining bivalents, as expected since these strains show very low levels of male offspring and embryonic lethality. As expected, approximately half of *him-5* oocytes contain six DAPI staining objects. *cep-1; him-5* worms have a significantly increased fraction of oocytes with abnormal numbers of DAPI-staining objects. *cep-1; him-5* worms have a similar proportion of oocytes with abnormal numbers of objects as *him-6* mutants. * denotes p < 0.05 by Student's t-test and error bars are S.E.M.
embryonic lethality and give rise to very few male offspring, the majority of the cells that I scored as not having exactly six DAPI staining bodies likely had a normal chromosome complement, but may have had two bivalents in very close proximity such that I scored them as one DAPI staining body. Even with these scoring imperfections, however, him-5 worms had significantly fewer oocytes with exactly six DAPI staining bodies than wild-type (p < 0.05 by Student's t test). cep-1; him-5 double mutants possessed even fewer oocytes with normal numbers of DAPI staining bodies than him-5 alone. This difference is significant, and cep-1; him-5 worms have similar proportions of oocytes with abnormal numbers of DAPI staining bodies as him-6 mutants, which have been previously shown to be extensively aneuploid (Wicky et al. 2004). This demonstrates that cep-1; him-5 double mutants have defects in meiotic chromosome biology that are more extensive than him-5 and cep-1 single mutants, which likely accounts for the increased embryonic lethality observed in the offspring of cep-1; him-5 double mutants.

3.4.2 cep-1; him-5 Double Mutants Show an Aggregated Chromosome Phenotype During Diakinesis Reminiscent of rad-51 Recombination Mutants

In the course of doing these counts I noticed a related and potentially more important phenotype in the cep-1; him-5 double mutants which I explored further using confocal microscopy. In wild-type animals the six bivalents are compact with clear distinct edges and are easily distinguished from one another by DAPI staining (Figure 3-3). However, in cep-1; him-5 double mutants I found that their diakinesis oocytes contained DNA objects that not only occurred in abnormal numbers, but were abnormal in morphology as well. Instead of appearing compact and distinct, cep-1; him-5 bivalents are diffuse, aggregated and stringy-looking. Frequently, it was difficult to distinguish how many objects were present due to their indistinct morphology. The abnormalities were extensive enough that it was possible to differentiate cep-1; him-5 animals from the other strains even when blinded to their identities. I reasoned that this abnormal chromosome morphology may lead to difficulties in chromosome disjunction or chromosome integrity once these oocytes are fertilized and meiosis resumes, and thus may be the cause of the
Figure 3-3: *cep-1; him-5* double mutants have diakinesis chromosome phenotypes reminiscent of those in *rad-51* mutants.

Whole animals were fixed and DAPI stained and diakinesis oocytes were examined by confocal microscopy. Pictured are three representative diakinesis oocyte nuclei from each indicated strain. In every case, the pictured oocyte was either the first or the second most mature oocyte in the gonad arm, ensuring they were arrested in diakinesis. Scale bar is 5µm.
increased embryonic lethality I see in cep-1; him-5 worms.

I was unfamiliar with this phenotype, and so I went to the *C. elegans* literature to see if something similar had been noted previously for other mutants. I found that this aggregated chromosome phenotype was a hallmark of animals with mutations in *rad-51*, *brc-2* and *com-1*, all genes required for meiotic homologous recombinational DSB repair (Penkner et al. 2007; Rinaldo et al. 2002; Martin et al. 2005). These three genes all encode conserved components required for HR during meiosis. The chromosome aggregates seen in these strains have been attributed to a failure of DSB repair leading to the use of less efficient, back-up repair systems such as SSA and NHEJ, resulting in fragmented and poorly repaired DNA (Martin et al. 2005). All mutations in these three genes lead to extensive embryonic lethality and, the only other gene reported to cause this phenotype is the germline-specific Dicer related helicase *drh-3*.

Interestingly, this phenotype in *drh-3* mutants has been suggested to be related to a defect in recombinational repair, though this has not been examined extensively (Nakamura et al. 2007).

A comparison between N2, *rad-51*, and *cep-1* and *him-5* single mutants and double mutants is shown in Figure 3-3. In all strains shown, as has been previously reported for *rad-51* mutants, no defects were apparent in DAPI stained gonads from any strain prior to diakinesis (Rinaldo et al. 2002). While N2 worms show the expected six compact and easily differentiated diakinesis bivalents, *rad-51* mutants show aggregation phenotypes with a range of severity, from multiple misshapen objects to severely abnormal nuclei where only one DAPI-staining diffuse mass can be seen. *cep-1* single mutants almost always show six largely normal looking bivalents, with some nuclei showing one or more bivalents that are somewhat abnormal in shape. These largely normal-looking nuclei correlate with a low level of embryonic lethality and frequency of male progeny seen in *cep-1* mutant offspring (see Figure 2-5 and Derry et al., 2001). *him-5* mutant diakinesis nuclei show little evidence of aggregation but *him-5* oocytes frequently contain more than six DAPI staining objects, most frequently seven. These phenotypes correlate with the increased embryonic lethality and proportion of male progeny seen in *him-5* mutants (see Figure 2-5 and Hodgkin et al., 1979). *cep-1*; *him-5* mutants, however, showed much more severe
phenotypes than *him-5* singles. In these strains, chromosome aggregation is extensive with most DAPI-staining objects showing irregular shape, frequent bridges between objects and extensive decondensation of DNA. Aggregation is apparent in all *cep-1; him-5* strains tested. Given the similarity in phenotype between the published accounts and my examination of *rad-51* and other recombination pathway mutants I hypothesized that *cep-1; him-5* worms are defective in recombinational DSB repair during meiosis, and that this defect is the basis for the increased embryonic lethality in these strains.

In an effort to quantify this phenotype I scored N2, *cep-1*(gk138), *him-5*(e1490), *cep-1*(gk138); *him-5*(e1490), *rad-51*(lg8701), and *him-6*(e1423) animals using the system outlined in Section 3.3.3. *him-6* was included as a strain that exhibits a high degree of aneuploidy, but no apparent aggregation and could therefore act as a negative control. *rad-51* mutants were included as a positive control for chromosome aggregation phenotype.

The results of the aggregation score analysis are shown in Table 3-1. Almost all N2 worms showed nuclei with little or no evidence of aggregation, while the bulk of *rad-51* oocytes (80%) fall into the category where more than half of the DAPI staining objects present in the nucleus appear abnormal. Despite extensive aneuploidy, *him-6* worms have no aggregation on this scale, with scores very close to those of wild-type. *cep-1* worms show moderately more aggregation than wild-type, but the majority of their nuclei still fall in the least-aggregated category. *him-5* single mutants have aggregation scores very similar to wild-type and *him-6*. Contrary to that seen for *cep-1* and *him-5* single mutants, *cep-1; him-5* double mutants exhibit substantially increased aggregation, with the majority of oocytes showing 3 or more abnormal looking DAPI-stained objects. The aggregation in *cep-1; him-5* as scored by this method is not as extensive as that of *rad-51* mutants, but is substantially more than any other strain tested. These results suggest that *cep-1; him-5* worms show *rad-51*-like chromosome aggregation defects during diakinesis, but these defects are not as severe as that seen in *rad-51* mutants. This corresponds to the high but not completely penetrant embryonic lethality phenotype seen in *cep-1; him-5* worms compared to the essentially 100% penetrant embryonic lethality phenotype among offspring of
Table 3-1: Proportions of Diakinesis Oocytes Showing Different Chromosome Aggregation Scores

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Oocytes with 2 or Fewer Abnormal Chromosomes (at least 5 objects present)</th>
<th>% Oocytes with 3 or More Abnormal Chromosomes (at least 5 objects present)</th>
<th>% Oocytes with 4 or Fewer Aggregated Objects</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>97</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>rad-51(lg8701)</td>
<td>10</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>cep-1(gk138)</td>
<td>76</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>him-5(e1490)</td>
<td>95</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>cep-1(gk138); him-5(e1490)</td>
<td>41</td>
<td>52</td>
<td>7</td>
</tr>
<tr>
<td>him-6(e1423)</td>
<td>97</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Animals were fixed, DAPI-stained and examined by confocal microscopy. For all strains, only the most mature oocyte in the gonad arm was examined and approximately 20 nuclei were scored per strain.
rad-51 mutants.

The chromosome number scores for these strains are shown in Table 3-2. As expected, the vast majority of wild-type animals have nuclei with the expected number of chromosomes. rad-51 mutants displayed a wide range of numbers of DAPI staining objects, with the majority containing more than six objects. No him-6 nuclei have fewer than 5 objects and the majority contain more than six, in agreement with previous reports (Wicky et al. 2004). The majority of cep-1 nuclei contain the expected number of DAPI-stained objects while the majority of him-5 mutants possess more than six, corresponding to the high frequency of X nondisjunction in this strain (see Figure 2-6 and Hodgkin et al., 1979). Like rad-51 mutants, cep-1; him-5 double mutants contain a wide range of numbers of DAPI-staining objects, with the majority showing more than six. Taken together, the data from Tables 3-1 and 3-2 show that cep-1; him-5 double mutants have a diakinesis oocyte chromosome profile that is very similar to rad-51 (though the number of oocytes showing extensive aggregation is somewhat less) but distinct from him-6, which generates a high fraction of inviable embryos due to defects in meiotic chromosome biology. Despite having similar numbers of nuclei that show discrepancies in the number of oocytes with normal numbers of DAPI-staining objects (Figure 3-1 and Table 3-2), cep-1; him-5 worms also exhibit extensive rad-51-like chromosome morphology defects, where chromosomes have abnormal shapes and an absence of condensation that are not observed in him-6. This lead me to hypothesize that cep-1; him-5 offspring were dying due to defects in recombination.

3.4.3 spo-11 Mutation Suppresses the Chromosome Aggregation Phenotype of cep-1; him-5 Doubles Mutants

The spo-11 nuclease is required to initiate meiotic recombination and functions upstream of the rad-51 recombinase, which is involved in the strand exchange interaction of recombination repair (Dernburg et al. 1998; Alpi et al. 2003; Rinaldo et al. 2002). The chromosome aggregation phenotype of rad-51, brc-2 and com-1 have all been shown to be suppressed by mutation in spo-11, suggesting this phenotype is dependent on the formation of meiotic DSBs
Table 3-2: Proportion of Diakinesis Oocytes With Different Numbers of DAPI-staining Objects

<table>
<thead>
<tr>
<th>Strain</th>
<th>% With Fewer than 5 DAPI Staining Objects</th>
<th>% With 5 or 6 DAPI Staining Objects</th>
<th>% With More than 6 DAPI Staining Objects</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>0</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>rad-51(lg8701)</td>
<td>10</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>cep-1(gk138)</td>
<td>16</td>
<td>76</td>
<td>8</td>
</tr>
<tr>
<td>him-5(e1490)</td>
<td>0</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>cep-1(gk138); him-5(e1490)</td>
<td>7</td>
<td>19</td>
<td>74</td>
</tr>
<tr>
<td>him-6(e1423)</td>
<td>0</td>
<td>19</td>
<td>81</td>
</tr>
</tbody>
</table>

Animals were fixed, DAPI-stained and examined by confocal microscopy. For all strains, only the most mature oocyte in the gonad arm was examined and approximately 20 nuclei were scored per strain. The same nuclei were scored for Table 3-1 and 3-2.
and that the aggregation phenotype seen in these mutants is a failure of DSB repair and not a result of other defective meiotic processes, such as chromosome condensation (Penkner et al. 2007; Alpi et al. 2003; Martin et al. 2005). In an effort to test whether the cep-1; him-5 chromosome aggregation phenotype is due to a failure of recombination or is a result of some other defect, I crossed cep-1; him-5 double mutants into a spo-11 background.

I constructed the strain cep-1(gk138); spo-11(ok79)/nT1; him-5(ok1896)/nT1 as well as cep-1(gk138); spo-11(ok79)/nT1 and spo-11(ok79)/nT1; him-5(ok1896)/nT1. The near-fully penetrant embryonic lethality phenotype of spo-11 worms requires they be maintained as balanced strains (Dernburg et al. 1998). I fixed, DAPI-stained and examined homozygous offspring from these strains, as well as spo-11(ok79), by confocal microscopy. As shown in Figure 3-4, instead of six well-condensed bivalents, spo-11 worms show 12 well-condensed univalents (Dernburg et al. 1998). This is due to the lack of programmed DSBs, which require spo-11 activity, leading to a complete failure of meiotic recombination. In the absence of recombination, homologous chromosomes fail to remain associated even though earlier homologous pairing events occur normally (Dernburg et al. 1998). In practice, although I could easily detect 12 individual univalents in most spo-11 cells by focusing up and down, it was very rare to see all 12 in one focal plane. All nuclei pictured in Figure 3-4 show at least eight univalents in the photographed focal plane, and I ensured the remaining univalents were visible in other focal planes.

As shown in Figure 3-4, I found that the cep-1; spo-11; him-5 worms had a diakinesis chromosome phenotype indistinguishable from spo-11. There was no sign of aggregation and all univalent chromosomes looked well-condensed with clear, distinct edges. cep-1; spo-11 and spo-11; him-5 worms also exhibited no differences in diakinesis chromosome phenotype. This experiment suggests that the cep-1; him-5 aggregation phenotype is dependent on spo-11 induced DSBs and may be a result of faulty repair of these breaks, as suggested for rad-51, brc-2 and com-1 (Alpi et al. 2003; Penkner et al. 2007; Martin et al. 2005).
Figure 3-4: The aggregation phenotype of *cep-1; him-5* is completely suppressed by mutation of *spo-11*.

Animals of genotype *spo-11(ok79), cep-1(gk138); spo-11(ok79), spo-11(ok79); him-5(ok1896)* and *cep-1(gk138); spo-11(ok79); him-5(ok1896)* were fixed, DAPI-stained and examined by confocal microscopy. All nuclei shown are from diakinesis-arrested oocytes that are either the first or second most mature in their gonad arm. Although 12 univalents were apparent by focusing up and down for all nuclei pictured it was difficult to find nuclei in which all univalents existed on the same focal plane. Pictured are focal planes that show at least eight univalents. Scale bar is 5μm.
3.4.4 Surviving *cep-1; him-5* Animals Show Reduced Frequency of Recombination Between Autosomal Markers

The chromosome phenotypes of *cep-1; him-5* double mutants and their epistatic relationship with *spo-11* suggests that the increased embryonic lethality seen in *cep-1; him-5* animals may be a result of a defect in meiotic recombination. I decided to explore whether surviving *cep-1; him-5* offspring have changes in recombination frequency between linked visible markers compared to wild-type and *him-5* mutants alone.

I constructed *dpy-9(e12) unc-17(e245)/++ IV* linked heterozygotes in wild-type, *cep-1(gk138), him-5(e1490)* and *cep-1(gk138); him-5(e1490)* genetic backgrounds and scored the phenotypes of their offspring (Table 3-3). In the wild-type background, recombinant hermaphrodites (Dpy or Unc) make up 24.9% of the offspring of *dpy-9(e12) unc-17/++* animals, a value which agrees quite well with the predicted 23.7 map unit separation between the two genes (*www.wormbase.org*). *him-5* animals show a significant decrease in the proportion of recombinant offspring using these two markers, as was also shown in Hodgkin et al. (1979). *cep-1* single mutants also show a small but significant decrease in recombinant offspring for this interval. *cep-1; him-5* double mutants, however, show a further decrease in recombination for this interval, with Dpy or Unc (uncoordinated) offspring arising only about half as often as seen in the wild-type background. This decrease is significant both compared to wild-type and *him-5* mutants alone, suggesting that, at least for this interval, *cep-1; him-5* animals have defects in establishing the normal numbers of crossovers and supporting a role for these genes in meiotic recombination.

A similar analysis was performed using the X linked interval *unc-7(e139) lon-2(e678)* (Table 3-4). As was done in Hodgkin et al. (1979) the analysis in Table 3-4 is performed only on the proportion of Lon (long) recombinants arising from *unc-7 lon-2/++* heterozygotes. I found that *cep-1; him-5* animals showed significantly fewer Lon recombinants compared to wild-type but
not compared to him-5 alone. This agrees well with the observation that cep-1; him-5 animals do not give rise to increased numbers of male offspring in their progeny compared to him-5 alone.

Table 3-3: Phenotypes of Offspring from dpy-9 unc-17/++ Heterozygotes in Wild-type, cep-1, him-5 and cep-1; him-5 Genetic Backgrounds.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%WT</th>
<th>%Dpy</th>
<th>%Unc</th>
<th>%DpyUnc</th>
<th>χ² compared to N2</th>
<th>χ² compared to him-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>2956</td>
<td>65.9</td>
<td>12.7</td>
<td>12.2</td>
<td>9.2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>cep-1 (gk138)</td>
<td>1566</td>
<td>64.9</td>
<td>10.3</td>
<td>12.2</td>
<td>12.6</td>
<td>16.3**</td>
<td>--</td>
</tr>
<tr>
<td>him-5(e1490)</td>
<td>756</td>
<td>68.4</td>
<td>7.1</td>
<td>10.2</td>
<td>14.3</td>
<td>33.7***</td>
<td>--</td>
</tr>
<tr>
<td>cep-1 (gk138); him-5(e1490)</td>
<td>590</td>
<td>72.0</td>
<td>6.8</td>
<td>5.4</td>
<td>15.9</td>
<td>60.4***</td>
<td>11.3*</td>
</tr>
</tbody>
</table>

Hermaphrodite offspring of dpy-9(e12) unc-17(e245)/++ offspring of the genotypes listed in the first column were scored by phenotype. Strains were compared using a 4X2 χ² contingency table (Kirkman, 1996). * denotes p < 0.05, ** is p < 0.01 and *** is p < 0.001.
Table 3-4: Phenotypes of Offspring from *unc-7 lon-2/++* Heterozygotes in Wild-type, *cep-1, him-5* and *cep-1; him-5* Genetic Backgrounds.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>% Lon</th>
<th>$\chi^2$ compared to N2</th>
<th>$\chi^2$ compared to him-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>1605</td>
<td>19.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><em>cep-1 (gk138)</em></td>
<td>1199</td>
<td>22.4</td>
<td>2.35</td>
<td>--</td>
</tr>
<tr>
<td><em>him-5(e1490)</em></td>
<td>505</td>
<td>15.8</td>
<td>2.37</td>
<td>--</td>
</tr>
<tr>
<td><em>cep-1 (gk138); him-5(e1490)</em></td>
<td>365</td>
<td>13.4</td>
<td>5.22*</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Hermaphrodite offspring of *unc-7(e245) lon-2(e678)/++* offspring of the genotypes listed in the first column were scored by phenotype. Strains were compared using a 2X2 $\chi^2$ contingency table (Kirkman, 1996). * denotes p < 0.05.
3.4.5 A Moderate Dose of IR Rescues the Him Phenotype of *him-5* and *cep-1; him-5* Mutants But The Embryonic Lethality Phenotype is Resistant to Rescue by DSBs

Meiotic recombination is a complex process that occurs in several steps. First, DSBs are created by SPO-11, initiating and providing the substrate for recombination. These breaks are then processed to form single stranded tails, and then strands are exchanged to produce crossover products (Longhese et al. 2009). Each of these steps is dependent on the one previous, but defects in all of these processes give similar phenotypes in the worms: very highly penetrant embryonic lethality (Dernburg et al. 1998; Takanami et al. 1998). It is therefore difficult to tell genetically at what step a given recombination gene acts since the phenotype is identical at the organism level. In several species exogenous double DSBs caused by moderate doses of IR can rescue mutants with defects in SPO11-induced meiotic break formation, as long as the IR dose is administered during early meiotic prophase (Celerin et al. 2000; Dernburg et al. 1998; Bowring et al. 2006; Thorne & Byers 1993). Therefore, this test can be used to prove that a recombination gene is involved in the initiation phase of meiotic recombination, rather than at a later step.

In 2009, the Yanowitz and Meneely labs reported at the International Worm Meeting that the Him and embryonic lethality phenotypes of *him-5* could be rescued by a moderate dose of IR during the L4 larval stage, suggesting that these phenotypes are caused by a defect in meiotic recombination initiation (J. Yanowitz and P. Meneely, personal communication). I decided to repeat this experiment in *cep-1; him-5* double mutants to ask if the more severe embryonic lethality in these worms is a result of a defect in meiotic recombination initiation.

N2 and *cep-1* worms showed very similar increased rates of embryonic lethality and Him phenotype after moderate doses of IR during the mid-L4 larval stage. As shown previously, IR-treated N2 worms generate an increased frequency of males compared with untreated worms, with the frequency of males increasing to 0.3% 12-24h after the onset of egg laying and 0.6% 24-36h after the onset of egg laying (Dernburg et al. 1998). *cep-1* worms generate increased
frequencies of males from irradiated mothers as well (for \textit{gk138}, 1.4% and 2.2% Him for \textit{lg12501} 12-24h and 24-36h post-laying respectively and 3.3% and 3.9% Him 12-24h and 24-36h post-laying respectively). Embryonic death increases to 15-20% 12h-36h after the onset of egg laying for N2 and \textit{cep-1} mutants. This increased embryonic lethality in wild-type irradiated animals has been shown previously (Dernburg et al. 1998). In the first 12h of egg laying there is very little difference between irradiated and unirradiated worms in frequency of dead eggs or male progeny from wild-type or \textit{cep-1} mutants.

Figure 3-5 shows the frequency of male progeny for irradiated and unirradiated \textit{him-5} mutants. For both the \textit{him-5}(\textit{e1490}) and \textit{him-5}(\textit{ok1896}) alleles the frequency of males decreases dramatically within the first 12h of egg laying after a moderate dose of IR compared to unirradiated controls. After the first 12h of egg laying, the frequency of males increases and approaches that of unirradiated worms. Presumably this is because the nuclei that are likely to benefit from exogenous DSBs to rescue recombination have moved through meiosis, and nuclei being laid as embryos during the 12-24h and 24-36h time points were at a stage of development too early for irradiation to rescue recombination on the X chromosome. These results confirm the Yanowitz and Meneely lab reports and suggest that the Him phenotype of \textit{him-5} is wholly a result of a defect in meiotic initiation by SPO-11-induced double strand breaks on the X chromosome.

Figure 3-6 shows the severity of the embryonic lethality phenotype in irradiated and unirradiated \textit{him-5} worms. Unlike the results for the Him phenotype during the first 12h of laying, neither allele shows a significant decrease in embryonic lethality after IR compared to unirradiated controls (p = 0.15 for \textit{e1490} and p = 0.61 for \textit{ok1896} by Student's \textit{t} test). This is not in agreement with the results reported by the Yanowitz and Meneely labs (J. Yanowitz and P. Meneely, personal communication). It is possible that this difference is due to a slight difference in IR dosage. I used 15Gy as reported in Dernburg (1998) while Yanowitz and Meneely used 20Gy. Some researchers also report differences in outcome due to differences in the rate at which different IR sources administer equivalent dosages (J. Yanowitz, personal communication).
Figure 3-5: A moderate dose of IR during L4 rescues the Him phenotype of *him-5* mutants.

Early L4 larvae were cloned, given either 0 Gy or 15 Gy IR and their offspring collected in three 12h batches and the frequency of males quantified. A. shows the results for *him-5(e1490)* and B. is *him-5(ok1896)*. At least 5 broods were counted per condition and error bars are S.E.M. * denotes p < 0.0005 by Student's *t* test.
Figure 3-6: A moderate dose of IR during L4 fails to rescue the embryonic lethality phenotype of him-5 mutants.

Embryonic lethality phenotype of him-5 mutants following treatment with IR. Early L4 larvae were cloned and given either 0 Gy or 15 Gy IR 4h later. A. shows the results for him-5(e1490) and B. is him-5(ok1896). At least 5 broods were counted per condition and error bars are S.E.M.
communication). These data were generated from the same individual worms as the data shown in Figure 3-5. The rescue of the him-5 Him phenotype was near complete and the rescue of spo-11 embryonic lethality was efficient (see below), suggesting that the IR dose was administered at the correct time to rescue recombination. Given that no significant rescue of him-5 embryonic inviability was seen it suggests that a lack of DSBs on autosomes is not the cause, or at least not the only cause, of unhatched progeny in these mutants.

I next wanted to test whether the Him and embryonic inviability phenotypes of cep-1; him-5 double mutants were a result of a defect in SPO-11-induced DSBs. I administered a moderate dose of IR to cep-1; him-5 mutants, collected their offspring in 12h batches and quantified the proportion of males. The results are shown in Figure 3-7. I found that cep-1; him-5 double mutants produced a dramatic and statistically significant reduction in frequency of male offspring in the first 12h of laying after a moderate dose of IR, compared to unirradiated controls. These results are very similar to those for him-5 mutants alone (Figure 3-5). This suggests that the Him phenotype of cep-1; him-5 mutants is solely a result of the defect in him-5, which results from a defect in meiotic recombination initiation on the X chromosome. These results, along with the fact that cep-1 mutation does not modify the Him phenotype of him-5 (Figure 2-6), suggest that cep-1 function has no effect on the Him phenotype of him-5.

I next wanted to ask whether the increased embryonic lethality of cep-1; him-5 double mutants compared to him-5 alone is a result of a defect in meiotic DSB formation on the autosomes. I administered a moderate dose of IR to cep-1; him-5 mid-L4s, collected their offspring in 12h batches and quantified the embryonic lethality of their offspring compared to unirradiated controls. The results for this experiment are shown in Figure 3-8. Unlike the data for these strains in terms of Him phenotype, the results of this experiment are mixed for different cep-1; him-5 strains. There was a significant reduction in embryonic lethality after IR for cep-1(gk138); him-5(e1490) and cep-1(gk138); him-5(ok1896) but no significant reduction in cep-1(lg12501); him-5(e1490). In addition to these mixed results, I also noticed that cep-1(lg12501); him-5(e1490) animals showed decreased embryonic lethality in the absence of IR than they had
Figure 3-7: A moderate dose of IR during L4 rescues the Him phenotype of *cep-1; him-5* mutants.

Him phenotype of *him-5* mutants following treatment with IR. Early L4 larvae were cloned and given either 0 Gy or 15 Gy IR 4h later. A. shows the results for *cep-1(gk138); him-5(e1490)*, B. is *cep-1(lg12501); him-5(e1490)* and C. is *cep-1(gk138); him-5(ok1896)*. At least 5 broods were counted per condition and error bars are S.E.M. * denotes p < 0.005 and * is p < 0.0005 by Student's *t* test.
Figure 3-8: A moderate dose of IR during L4 shows mixed results in rescuing the embryonic lethality phenotype of *cep-1; him-5* double mutants.

Embryonic lethality phenotype of *cep-1; him-5* mutants following treatment with IR. Early L4 larvae were cloned and given either 0 Gy or 15 Gy IR 4h later. A. shows the results for *cep-1*(gk138); *him-5*(e1490), B. is *cep-1*(lg12501); *him-5*(e1490) and C. is *cep-1*(gk138); *him-5*(ok1896). At least 5 broods were counted per condition and error bars are S.E.M. * denotes p < 0.05 and ** is p < 0.005 by Student's *t* test.
when I scored whole broods (Figure 2-5). I reasoned that this discrepancy may be due to an increase in the lethality of progeny with maternal age, since the IR experiments only score progeny for 36h, while the animals continue producing eggs for at least twice that long. I examined the lethality of *cep-1(lg12501); him-5(e1490)* and *cep-1(gk138); him-5(e1490)* animals in 12h batches over the first 48h of laying. These results are shown in Figure 3-9. While lethality among the progeny of *cep-1(gk138); him-5(e1490)* remains relatively constant over the first 48h of laying, the frequency of lethality of the progeny of *cep-1(lg12501); him-5(e1490)* increases over time, such that the proportion of dead embryos laid in the first 12h of laying is significantly lower than that seen between 36 and 48h of laying. The proportion of dead embryos produced by *cep-1(lg12501); him-5(e1490)* during the 36-48h time period is similar to the proportion shown in the whole brood counts shown in Figure 2-5. Since these animals continue laying for 1-2 days past the time points shown in Figure 3-9 this could account for the discrepancy in embryonic lethality shown between Figure 2-5 and 3-8. The reason for increase in *cep-1(lg12501); him-5(e1490)* and not in *cep-1(gk138); him-5(e1490)* is not clear, but some possibilities will be addressed in Section 3.5.4.

It is also not clear why *gk138* and *lg12501* show a difference in the ability of moderate doses of IR to rescue embryonic lethality. Potential reasons for this discrepancy will be discussed in Section 3.5.4.

Since *cep-1(lg12501); him-5* embryonic lethality phenotype, unlike its Him phenotype, is not efficiently rescued by endogenous DNA breaks, it suggests these mutants have another defect downstream or in parallel to a defect in meiotic recombination initiation on the autosomes that also contributes to the embryonic lethality in this strain. The aggregated chromosome phenotype of *cep-1; him-5* worms during diakinesis (Figure 3-3) and its rescue by mutation of *spo-11* (Figure 3-4) suggests that this additional defect may be inefficient recombinational repair of SPO-11-induced DSBs.
The bar chart shows the proportion of embryonic lethality over different time periods (0-12h, 12-24h, 24-36h, 36-48h). The chart compares two conditions:

- **cep-1(gk138); him-5(e1490)** (gray bars)
- **cep-1(lg12501); him-5(e1490)** (black bars)

A significant difference is indicated by an asterisk (*) at the 24-36h mark.
Figure 3-9: The frequency of embryonic lethality among progeny of cep-1(lg12501); him-5(e1490), but not cep-1(gk138); him-5(e1490), increases with maternal age.

Progeny of cep-1(gk138); him-5(e1490) and cep-1(lg12501); him-5(e1490) were collected in four 12h batches from the beginning of laying and the embryonic lethality was calculated. * denotes p < 0.05 by Student's t test and error bars are S.E.M.
It has been shown previously that spo-11 mutants, which show almost complete embryonic lethality of progeny due to a failure of meiotic initiation, show significant rescue when given a moderate dose of IR, presumably because it provides sufficient DSB substrate to rescue recombination (Dernburg et al. 1998). I reasoned that if cep-1; him-5 worms had a defect in recombinational repair downstream of SPO-11 meiotic initiation, there would be a decrease in the degree of rescue seen in cep-1; spo-11; him-5 triple mutants compared to spo-11 alone when given a moderate dose of IR during meiosis.

I cloned spo-11, cep-1; spo-11, spo-11; him-5 and cep-1; spo-11; him-5 homozygote L4s from balanced strains, subjected them to 15 Gy IR and collected and quantified the degree of embryonic lethality among their offspring in three 12h batches. The results of this experiment are shown in Figure 3-10. spo-11 worms show almost complete embryonic inviability in the absence of IR. As reported previously, spo-11 mutants show very significant rescue after 15 Gy IR in the first 12h of egg laying, and the majority of embryos produced during this time period (59.6% on average) develop to adulthood (Dernburg et al. 1998). As was seen for the Him phenotype of him-5 single mutants and cep-1; him-5 double mutants, this embryonic lethality in irradiated spo-11 mutants then increases in penetrance over the course of the next 24h, approaching the level observed in unirradiated controls. These results suggest that the high embryonic lethality in spo-11 mutants is due to an absence of endogenous meiotic DSBs that serve as the substrate for recombination, and that this defect can be rescued by providing exogenous DSBs during meiosis (Dernburg et al. 1998). Embryonic viability in cep-1; spo-11 and spo-11; him-5 worms is very similar to spo-11 alone, showing a significant rescue after IR treatment in the first 12h of egg laying. However, cep-1; spo-11; him-5 triple mutants exhibit reduced rescue of embryonic lethality after treatment with IR. In contrast to the other spo-11 mutant strains examined, the difference between the irradiated and unirradiated controls for cep-1; spo-11; him-5 mutants is not significant (p = 0.11). This suggests that cep-1; spo-11; him-5 triple mutants have an additional defect not observed in spo-11, cep-1; spo-11 or spo-11; him-5 animals that cannot be efficiently rescued by exogenous DSBs during meiosis.
Figure 3-10: A moderate dose of IR fails to efficiently rescue the embryonic lethality phenotype of *cep-1; spo-11; him-5* triple mutants.

Early L4 larvae were cloned, given either 0 Gy or 15 Gy IR 4h later and the embryonic lethality of among their offspring was quantified. A. shows the results for *spo-11(ok79)* B. is *cep-1(gk138); spo-11(ok79)*, C. is *spo-11(ok79); him-5(ok1896)* and D. is *cep-1(gk138); spo-11(ok79); him-5(ok1896)*. At least 5 broods were counted per condition and error bars are S.E.M. * denotes p < 0.005, ** is p < 0.0005 and *** is p < 0.000005 by Student's t test.
Again, these results are somewhat puzzling. If the progeny of *cep-1*(gk138); *him-5*(e1490) are significantly rescued by IR it isn't necessarily clear why adding a mutation in *spo-11* should change this. However, it is the case that the embryonic viability of *spo-11* progeny is rescued from near 100% to 35% by moderate IR (Figure 3-10). This rescue, though significant, is not complete, probably for technical reasons. In order to rescue meiotic recombination, and therefore the embryonic lethality that results from nondisjunction in the progeny of *spo-11*, each chromosome must receive at least one IR-induced DSB to create a crossover, but not so many that the recombination machinery is swamped and embryos die due to persistent damage or IR-induced mutation. The residual 35% embryonic lethality seen in irradiated *spo-11* alone is likely a result of the failure of this balance between too many and too few IR-induced DSBs. If the *cep-1* and *him-5* mutations add any stress to the recombination machinery they may decrease the efficiency of IR rescue enough such that the differences between *cep-1*(gk138); *spo-11*; *him-5*(e1490) is no longer significantly different between irradiated and unirradiated controls.

Taken together with the chromosome aggregation phenotype seen in *cep-1*; *him-5* double mutants (Figure 3-3) and its similarity to that seen in recombination mutants, and the fact that *cep-1*; *spo-11*; *him-5* triple mutants cannot be efficiently rescued by exogenous DNA breaks suggests that *cep-1* and *him-5* mutation leads to a defect in DSB repair of SPO-11-induced DSBs (Penkner et al. 2007; Rinaldo et al. 2002; Martin et al. 2005).

### 3.4.6 *cep-1*; *him-5* Animals Show Intense and Persistent RAD-51 Foci During Oogenesis

The chromosome aggregation defects of *cep-1*; *him-5* diakinesis oocytes and the reduced recombination frequency seen in these animals suggest that these genes play a role in meiotic recombination. The epistatic relationship of the *cep-1*; *him-5* aggregation phenotype with *spo-11* and the resistance of the embryonic lethality of these worms to rescue by modest doses of IR suggests that the main defect in *cep-1*; *him-5* animals occurs at the repair step of meiotic recombination. To test this further I performed RAD-51 immunofluorescence on *cep-1*; *him-5* germlines.
RAD-51 is the *C. elegans* recombinase required for all HR events in the worms, including meiotic recombination. During oogenesis, RAD-51 localizes to distinct foci in the zygotene/early pachytene region, corresponding to the sites of meiotic recombinational repair (Alpi et al., 2003 and Figure 3-11). In wild-type animals, RAD-51 foci disappear by the end of pachytene as meiotic DSBs are repaired and are absent from maturing oocytes. RAD-51 foci that persist beyond pachytene are hallmarks of defects in meiotic DSB repair (Ward et al, 2010).

Figure 3-11 shows dissected gonads from wild-type, *cep-1*, *him-5* and *cep-1; him-5* animals immunostained for RAD-51. All of these strains show RAD-51 zygotene/early pachytene foci, suggesting that meiotic DSBs are present and are processed in each of these strains (Penkner, 2007). In wild-type animals, multiple RAD-51 foci are uniformly present in zygotene/early pachytene nuclei but disappear from oocyte nuclei as they round the bend of the gonad, cellularize and enter diplotene and diakinesis. *cep-1*(gk138) and *him-5*(e1490) animals show essentially the same pattern of RAD-51 immunostaining, though occasionally RAD-51 foci persist beyond the gonad bend. *cep-1;him-5* animals also show RAD-51 zygotene/early pachytene foci, suggesting these developing oocytes contain meiotic DSBs that are properly processed to allow RAD-51 binding. Compared to wild-type and *cep-1* and *him-5* single mutants however, *cep-1; him-5* double mutant gonads contain RAD-51 foci that are greater in intensity and persist well into the gonad bend. These persistent and intense RAD-51 foci seen in *cep-1; him-5* double mutants suggests these animals have a defect in repair of meiotic DSBs.

### 3.4.7 Summary

In Chapter 2, I outlined an enhanced embryonic lethality phenotype I had identified in *cep-1; him-5* worms that revealed a novel role for *cep-1* that is independent of its known up- and downstream regulators, and independent of its characterized role in promoting DNA damage-induced apoptosis in the germline. In this chapter I have shown that the defects in *cep-1; him-5* double mutants are a result of defects in meiotic recombination. First, I noted that *cep-1; him-5* worms have abnormal numbers of DAPI-staining objects in their diakinesis-arrested oocytes at a higher frequency than *him-5* single mutants. Even more importantly, *cep-1; him-5* diakinesis
Figure 3-11: *cep-1; him-5* double mutants show intense and persistent meiotic DSBs.

Gonads of wild-type, *cep-1(gk138)*, *him-5(e1490)*, and *cep-1(gk138); him-5(e1490)* were extruded, fixed and immunostained for RAD-51. At least 20 gonads were examined per strain, and representative examples are shown. The early pachytene region is at the bottom of each image and nuclei progress through meiosis to form cellularizing oocytes at the top of each image. RAD-51 foci are seen as brighter spots against the general background staining that is seen in each nucleus. Arrows show intense and persistent RAD-51 foci in *cep-1; him-5* animals and scale bar is 20μm.
oocytes show DAPI stained chromatin that is diffuse, aggregated and abnormal in shape, much different than the six well-condensed and easily differentiated bivalents present in wild-type animals. These defects were much more severe in the cep-1; him-5 double mutants than in either cep-1 or him-5 single mutants. A literature search revealed that this phenotype was very similar to rad-51 mutants, as well as other members of the meiotic recombinational DSB repair pathway (Penkner et al. 2007; Rinaldo et al. 2002; Martin et al. 2005). I hypothesized that the increase in embryonic lethality observed in cep-1; him-5 double mutants may be a result of a defect in repair of meiotic DSBs.

The diakinesis chromosome aggregation defects reported in rad-51, brc-2 and com-1 are suppressed by mutation of spo-11, which encodes the nuclease responsible for creation of meiotic DSBs (Dernburg et al. 1998; Alpi et al. 2003; Penkner et al. 2007; Martin et al. 2005). I found also that the aggregation phenotype was absent in cep-1; spo-11; him-5 triple mutants, suggesting that the aggregation defect depended on the presence of meiotic DSBs, consistent with a role for cep-1 and him-5 in repair of meiotic DNA breaks. This hypothesis was corroborated by the fact that surviving cep-1; him-5 offspring show reduced frequency of crossovers between linked markers compared to that seen in the wild-type background.

An abstract from the Yanowitz and Meneely labs reported that the him-5 Him and embryonic lethality phenotypes could be rescued by a moderate dose of IR during meiosis, suggesting that these phenotypes arise as a result of a defect in meiotic SPO-11-induced DSBs (J. Yanowitz and P. Meneely, personal communication). I found that the Him phenotype of both him-5 and cep-1; him-5 animals was almost completely rescued by a moderate dose of IR, suggesting the reason for this Him phenotype in both strains is a lack of SPO-11-induced meiotic DSBs on the X chromosome. In contrast to this, and to the results reported from the Yanowitz and Meneely labs, the embryonic lethal phenotype of both him-5 and cep-1; him-5 double mutants were at least somewhat resistant to rescue by IR. This suggests there is a defect downstream or in parallel to meiotic initiation that causes at least some of the embryonic lethality in these strains. This hypothesis is supported by the fact that cep-1; him-5 germlines show RAD-51 foci in their 136
pachytene nuclei, suggesting that they contain meiotic DSBs that are properly processed in preparation for repair. However, these foci are increased in intensity compared to wild-type frequently persist beyond the gonad bend, a phenotype associated with defects in meiotic DSB repair (Ward et al., 2010).

Taken together, the diakinesis aggregated chromatin phenotype, reduction in crossover frequency, IR rescue data and RAD-51 immunostaining phenotype suggest that cep-1 and him-5 have a role in meiotic recombinational repair.

3.5 Discussion

3.5.1 cep-1; him-5 Double Mutants Have Defects in Meiotic Chromosome Biology

In Chapter 2, I began to explore the basis of the enhanced embryonic lethality phenotype I observed in cep-1; him-5 double mutants and found that cep-1(+)’s role in promoting embryonic survival in him-5 mutants is unrelated to its ability to induce apoptosis in the germline and of its known upstream regulatory pathway. Since this cep-1; him-5 phenotype was due to a defect in the maternal germline and the fact that him-5 is a known meiotic nondisjunction mutant I hypothesized that cep-1; him-5 double mutants were dying as embryos due to a defect in meiotic chromosome biology (Hodgkin et al. 1979). In support of this hypothesis I showed that cep-1; him-5 animals have abnormalities in the number and morphology of DAPI staining objects in their diakinesis-arrested oocytes (Figure 3-3).

3.5.2 cep-1; him-5 Double Mutants Show a Similar Aggregated Chromosome Phenotype to rad-51 Mutants

I suspected the aberrant chromosome morphology in cep-1; him-5 double mutants was the basis for the high embryonic inviability I saw in these strains. A literature search revealed that a similar phenotype had been previously described and found to be characteristic of several genes in the rad-51 HR pathway (Penkner et al. 2007; Rinaldo et al. 2002; Martin et al. 2005). Very similar to what I see in cep-1; him-5 double mutants, in rad-51, com-1 and brc-2 mutants the
diakinesis chromatin is stringy, decondensed and aggregated. Also similar to cep-1; him-5 double mutants, the chromosome defects of rad-51, com-1 and brc-2 single mutants are apparent at diakinesis, while germ cells at earlier meiotic stages exhibit normal DAPI-staining patterns.

com-1 is thought to be required for processing programmed meiotic DSBs whereas brc-2 and rad-51 function in strand invasion that leads to repair of the DSB, creating crossovers between homologous chromosomes (Penkner et al. 2007; Rinaldo et al. 2002; Martin et al. 2005). All of these genes and their functions are conserved in a wide variety of organisms (Shinohara & Ogawa 1999; Takeda et al. 2007; Thorslund & West 2007). It is thought that the aggregated chromosome phenotype seen in these mutants is a result of chromosome fragmentation and repair by inefficient and error-prone pathways such as NHEJ and SSA in the absence of the more accurate HR pathway (Martin et al. 2005). As a result of these defects, the offspring of com-1, brc-2 and rad-51 mutants all show very high frequency of embryonic inviability (Takanami et al. 1998; Martin et al. 2005; Penkner et al. 2007). The similarity in phenotype between cep-1; him-5 worms and mutants of the rad-51 meiotic HR pathway led me to hypothesize that cep-1; him-5 double mutants showed high embryonic death as a result of defects in meiotic HR.

There is evidence previous to this work that him-5 may play a role in recombination. him-5 mutation leads to altered patterns of recombination frequency on the X chromosome and in some autosomal regions (Broverman & Meneely 1994; Hodgkin et al. 1979). Broverman & Meneely argue that this altered recombination frequency on the X chromosome results from frequent failure of crossovers on this chromosome which would also explain the high frequency of males in these mutant strains.

There is also some evidence that p53 homologues may have roles in HR. p53 binds both RAD51 and BRCA2 in human cells and decreases their function, hindering promiscuous recombination and promoting more faithful repair of DSBs (Marmorstein et al. 1998; Buchhop et al. 1997; Stürzbecher et al. 1996). For this role, p53 appears to function directly at the site of the DNA damage, binding specifically to Holliday junction recombination intermediates, a role for which its transcriptional activity is not required (Yoon et al. 2004; Lee et al. 1997). p53 abrogation
leads to dramatically increased rates of spontaneous and damage-induced recombination (Meyn et al. 1994; Ishizaki et al. 1994). The transcriptional activity of p53 also seems to play a role in regulation of HR, since p53 has been shown to transcriptionally downregulate RAD51, reducing the number of Rad51 functional foci (Arias-Lopez et al. 2006). More recently, Lin et al. (2009) reported that the HR genes BRCA2, RAD51 and MRE11 are all transcriptional targets of the p53 family members p63 and p73 and that p63- and p73-deficient cells show defects in DNA repair. Most of this evidence of a role for the p53 family in HR, was obtained from studies in somatic cells. Less work has been done on the putative role for p53 family members in meiotic recombination, but since the same repair machinery (including Rad51 and Brca2) is involved in both processes, a role for p53, p63 or p73 in meiotic homologous recombination is certainly possible. The little work that has been done shows that murine p53 binds to the mouse meiosis-specific RecA homologue Dmc1, where it may play a similar role in HR as it plays in somatic cells by binding Rad51 (Habu et al. 2004). In addition, p53 is present at the right time and place in mouse testes to be influencing recombination (Sjöblom & Lähdetie 1996). However, p53 deficient mice were still found to undergo meiotic recombination at normal frequencies (Gersten & Kemp 1997). Recent work has shown that p53 becomes activated by Spo11-induced DSBs in both Drosophila and mice, suggesting p53 may play an evolutionarily conserved role in recombination (Lu et al. 2010). It is possible that p53 may be playing a redundant role in recombination with its other family members or with other genes. These redundant factors may also include its homologues, p63 and p73, which have also been hypothesized to play a role in gametogenesis (Hu 2009; Tomasini et al., 2009).

The similarity of the cep-1; him-5 diakinesis phenotype to rad-51 and other recombination pathway mutants, along with the reduced viability in the offspring of cep-1; him-5 double mutants is consistent with cep-1 and him-5 acting in parallel to promote accurate meiotic recombination. Previous work on him-5 in C. elegans and p53 family members in mammalian cells has hinted at a role for these genes in HR. The suggestion that cep-1 has a role in HR during meiosis challenges the notion that the sole ancestral role for p53 family members is in promoting apoptosis in response to DNA damage and suggests that cep-1 has roles in C. elegans
that may only be required in the presence of particular genetic defects or environmental stressors (Greiss et al. 2008). These roles may be conserved among p53 homologues in other species.

3.5.3 spo-11 Acts Upstream of the Aggregated Chromatin Phenotype of cep-1; him-5 Double Mutants

Having formulated the hypothesis that cep-1 and him-5 have a redundant role in promoting accurate meiotic recombination I performed experiments to test this hypothesis. In a wide variety of organisms, meiotic recombination is initiated by DSBs created by a topoisomerase-like Spo11 homologue (Keeney 2001). These DSBs form the substrate upon which the rad-51 pathway works to create the meiotic crossovers required for accurate homologue segregation in meiosis I. In the absence of Spo11 crossing over does not occur and meiotic chromosome segregation usually fails, with disastrous consequences for the sexual reproduction of the organism.

When it was first noticed, the chromosome aggregation phenotype of C. elegans rad-51 mutants was thought to be a result of a defect of chromosome condensation (Takanami et al. 1998). Later it was found that the aggregation defect could be rescued by mutation of spo-11. This result demonstrates that the chromatin aggregation defects seen in rad-51 require spo-11 induced DSBs and suggests the chromatin defects in rad-51 are a result of inefficient repair of these breaks and not due to condensation defects (Alpi et al. 2003). Subsequent to this, spo-11 epistasis analysis with com-1 and brc-2 mutants provided evidence that the aggregation phenotype in these mutants was also due to defects in meiotic DSB repair rather than in chromosome condensation (Penkner et al. 2007; Martin et al. 2005). Similar to rad-51, com-1 and brc-2, I found that the diakinesis morphology defect in cep-1; him-5 mutants was downstream of spo-11 (Alpi et al. 2003; Penkner et al. 2007; Martin et al. 2005). This experiment strongly suggests that the aggregation defect in cep-1; him-5 double mutants is due to a defect in repair of meiotic DSBs.
3.5.4 *cep-1; him-5* Survivors Show Decreased Crossover Frequency Compared to Wild-type

The aggregated chromatin defects and the epistatic relationship with *spo-11* seen in *cep-1; him-5* double mutants suggests these animals have defects in meiotic recombination and that these defects likely underlie the enhanced embryonic inviability seen in these worms. To assess this hypothesis further, I decided to ask whether *cep-1; him-5* double mutants that survive to adulthood show changes in the frequency of crossing over between linked visible markers. I chose two intervals that have been previously explored in *him* mutants: the autosomal interval *dpy-9 unc-17 IV* and the X-linked interval *unc-7 lon-2*. Hodgkin et al. (1979) showed that *him-5* single mutants show decreased frequency of recombination between *dpy-9* and *unc-17*, a result which I confirm here (Table 3-3). *cep-1; him-5* double mutants show a significant further reduction in crossing over in this interval, such that linked heterozygotes from this strain give rise to approximately half as many recombinant offspring as wild-type linked heterozygotes. The enhanced crossing over defect in *cep-1; him-5* animals compared to *him-5* alone corresponds to similarly enhanced embryonic lethality and diakinesis chromosome aggregation defects in these strains, and suggests these phenotypes are a result of an enhanced defect in meiotic recombinational repair in *cep-1; him-5* animals. When meiotic HR repair is defective it has been shown that less accurate, non-crossover DSB repair pathways, such as NHEJ and SSA attempt to repair the break (Martin et al., 2005). These pathways frequently fail, leading to the increased embryonic lethality seen in mutants with defects in HR. The surviving *cep-1; him-5* animals may represent animals where pathways such as NHEJ or SSA functioned to repair the breaks, rescuing the animal but with reduced crossover events.

For the *unc-7 lon-2* X-linked interval *cep-1; him-5* double mutants show significantly reduced crossover frequency compared to wild-type, but not compared to *him-5* single mutants alone (Table 3-4). Reductions of crossing over on the X leads to increased X chromosome nondisjunction and a Him phenotype (Broverman and Meneely, 1994). The result that *cep-1(If)* does not significantly reduce crossover frequency in the *him-5* background makes sense in light of the fact it also doesn't enhance the Him phenotype of him-5 animals (Figure 2-6).
3.5.5 him-5 Promotes Meiotic DSBs to Initiate Recombination on the X Chromosome, but Failure of DSBs on the Autosomes Do Not Account for All of the Defects in cep-1; him-5 Double Mutants

Spo11 mutants fail to initiate meiotic recombination, causing massive nondisjunction of homologous chromosomes during meiosis I, leading either to failure of meiosis or death of offspring, depending on the organism (Keeney 2001). Once it was realized that the role of Spo11 was to create programmed DSBs to initiate recombination, it was shown in several species that meiotic recombination and offspring survival could be rescued by providing exogenous DSBs during prophase I using a moderate dose of IR (Celerin et al. 2000; Dernburg et al. 1998; Bowring et al. 2006; Thorne & Byers 1993). These DSBs provide the substrate for meiotic recombination and rescue the meiotic nondisjunction defects and viability of offspring of spo11 mutants. This has provided a simple test to identify mutants that have defects of meiotic initiation rather than those that exhibit meiotic failure or embryonic inviability for other reasons. If a moderate dose of IR can rescue embryonic inviability in offspring of a meiotic mutant, then the mutant likely has a defect in programmed meiotic DSBs rather than defects in other meiotic processes.

At the International C. elegans meeting in 2009, the Yanowitz and Meneely labs reported that the embryonic lethality and high incidence of male phenotypes of him-5 could be rescued by a moderate dose of IR during the early stages of meiosis (J. Yanowitz and P. Meneely, personal communication). This result suggests that the Him phenotype of him-5 is due to a failure of programmed DSBs on the X chromosome and the embryonic inviability phenotype is due to a failure of programmed DSBs on one or more autosomes. When I repeated this experiment, I replicated the results for the Him phenotype, but not for the embryonic lethality phenotype (Figures 3-5 and 3-6). I saw a no significant decrease in embryonic inviability for e1490 or for ok1896. The reasons for this discrepancy may be a difference in the experimental methods, since the Yanowitz and Meneely groups used 20 Gy of IR (J. Yanowitz, personal communication), while I used 15 Gy as was done by Dernburg et al. (1998). Rescue of the Him phenotype by IR in him-5 mutants suggests the Him phenotype in these strains is due to a failure to initiate
meiotic DSBs on the X chromosome. Because I was able to rescue Him phenotype so efficiently I concluded that the IR was administered at a dose and timing sufficient to rescue the recombination defect of him-5 mutants. The inability of IR to rescue embryonic lethality suggests that him-5 mutants may have another defect rather than or in addition to defective meiotic recombination initiation on autosomes.

As has been shown for wild-type animals, cep-1 single mutants show an increased frequency of male progeny after the first 12h of laying in mothers irradiated during L4. 24-36h after the onset of egg laying irradiated wild-type mothers have an average frequency of male offspring of 0.6%, while the frequencies for cep-1(gk138) and (lg12501) are 2.2% and 3.9% respectively. The higher percentage of males among irradiated cep-1 worms compared to wild-type may be a result of higher sensitivity to DNA damage and a higher tendency to produce male offspring in the absence of IR. Whether this higher sensitivity is a result of a partial defect in recombinational repair or the absence of a DNA damage-induced apoptotic is not clear.

I also wished to assess the effects of a moderate dose of IR on the Him and embryonic lethality phenotypes of cep-1; him-5 double mutants. As with him-5 alone, the Him phenotype of double mutants was efficiently rescued by IR administered during late mid-L4, suggesting that the Him phenotype in these animals is solely attributable to the mutation in him-5 and is not affected by cep-1 (Figure 3-7). This result is also supported by the fact cep-1 mutations do not significantly alter the frequency of males nor the frequency of recombination between X-linked markers in the offspring of him-5 mutants (Figure 2-6 and Table 3-4). The results were mixed, however, in terms of rescue of the embryonic lethality phenotype, with significant rescue seen in in double mutants with him-5 mutation and the cep-1(gk138) but not with the lg12501 allele (Figure 3-8).

The reason for this difference between strains is unclear, but may stem from a difference in function between these alleles. Although both show identical apoptotic responses to DNA damage signals in the germline (ie. no induction is seen above background physiological apoptosis) there is some indication that lg12501 may be the stronger allele for other phenotypes (Deng et al. 2004). For instance, lg12501, like cep-1(RNAi) gives a modest Him phenotype,
while *gk138* does not give an appreciably increased frequency of males compared to wild-type (Figure 2-6). In addition, *cep-1* mutants develop more quickly than wild-type and this effect is much more pronounced in *lg12501* than in *gk138* (data not shown). But this does not explain the progressively greater phenotype in *cep-1(lg12501); him-5(e1490)* over time, especially when such a progression in penetrance is lacking in *cep-1(gk138); him-5(e1490)* (Figure 3-9).

*lg12501* and *gk138* are both deletion alleles of *cep-1* that leave the 5' end of the gene intact, perhaps allowing some mutant protein expression (Figure 2-1). A recent analysis showed some RNA expression from the 5' region of *cep-1* is present in *lg12501* animals and truncated protein products have been seen in lysates from *gk138* worms using antibodies raised against the C-terminus of CEP-1 (Torgovnick et al., 2010; M. Gao and B. Derry, personal communication). A careful analysis of the RNAs produced from *cep-1* in animals carrying this alleles has not been performed and so all of these ideas remain speculative, but it possible that the differences in residual function in protein expressed from these mutant alleles may account for the differences in the ability of IR to rescue viability in different *cep-1; him-5* mutant strains. For instance, it is possible that CEP-1(*gk138*), predicted to be an internal in-frame deletion, contains some residual function in DSB repair that is lost from CEP-1 encoded by *lg12501*, which is predicted to contain a frameshift mutation beginning near the end of the DBD (Figure 2-1). The putative increased function of *gk138* may allow IR rescue in animals containing *gk138* but not those with *lg12501*.

Given the differing rescue results for *him-5* mutants between my work and the Yanowitz and Meneely labs, and between different combinations of alleles in *cep-1; him-5* double mutant strains, it appears that rescue of embryonic lethality is more complex than the rescue of the Him phenotype. It should be noted that even in *cep-1; him-5* strains where I see rescue, it is not as efficient as that seen for the Him phenotype (Figures 3-7 and 3-8). It is clear that the Him phenotype of *him-5* and *cep-1; him-5* mutants is solely attributable to a failure of programmed DSB formation on the X chromosome (and therefore failure of meiotic recombination). The mixed results and inefficient rescue of the embryonic inviability phenotype may be a result of
him-5 and cep-1; him-5 mutants showing a defect in meiotic initiation on one or more autosomes, but also having a defect downstream or in parallel that contributes to the embryonic lethality. My results showing that cep-1; him-5 double mutants have a rad-51-like chromatin aggregation phenotype that is suppressed by mutation of spo-11 suggest that this downstream defect may be inefficient DSB repair. If cep-1; him-5 double mutants have defects both in spo-11-induced DSBs on the autosomes and, repair of these breaks later, it may explain the mixed results and inefficient rescue of cep-1; him-5 mutants, especially if cep-1(gk138) shows partial function with respect to repair of DSBs while cep-1(lg12501) does not.

him-5 single mutants do not show appreciable aggregation defects, but do show some embryonic lethality (Figures 2-5 and 3-3, Table 3-1). This embryonic inviability has been attributed to a defect in autosomal segregation, but this assertion has not been proven (Hodgkin et al. 1979). cep-1; him-5 worms show aggregation defects that are suppressed by mutation of spo-11, suggesting that him-5 plays a role in recombinational repair, in parallel with cep-1. Given this, it is possible that the embryonic lethality associated with him-5 mutation alone is due to impenetrant defects in recombinational repair. This does not preclude him-5 also having an impenetrant effect on meiotic initiation on one or more autosomes, which could also cause embryonic lethality. A downstream role in recombinational repair would explain why I don't see significant rescue of the embryonic viability of him-5 mutants after a moderate dose of IR (Figure 3-6).

There are examples in the literature of genes required both for spo-11-induced DSB induction and for subsequent repair of these breaks. For instance, Mre11 is a conserved nuclease that functions as part of the MRX/MRN complex and is required for meiotic recombination in a variety of organisms (Borde & Cobb 2009). It has been shown by separation of function mutants and phenotypic analysis that Mre11 homologues have dual roles in some organisms, including S. cerevisiae and C. elegans, where it is required for both meiotic DSB formation and the subsequent repair of these breaks (Johzuka & Ogawa 1995; Chin & Villeneuve 2001). It has been suggested that the MRX/MRN complex may have evolved to target Spo11 to chromatin in
order to initiate DSBs such that MRX/MRN is in close proximity to immediately process the breaks and initiate repair. So, it is not without precedent to hypothesize that him-5 may be required both for DSB formation and for their subsequent repair. Interestingly, Mre11 homologues retain their recombinational repair role in all organisms where they have been identified, but they are not required with Spo11 to initiate meiotic DSBs in *C. cinereus, A. thaliana* or *S. pombe* (Gerecke & Zolan 2000; Puizina et al. 2004; Young et al. 2004)

I found that *cep-1(gk138); spo-11; him-5* triple mutants exhibited less efficient rescue of embryonic lethality after a moderate dose of IR than *spo-11* alone, *cep-1; spo-11* or *spo-11; him-5*. This suggests that *cep-1* and *him-5* act redundantly to decrease the efficiency of IR rescue of *spo-11* mutants. This would be consistent with a role for *cep-1* and *him-5* in DSB repair downstream of *spo-11*.

### 3.5.6 *cep-1; him-5* Animals Show Intense and Persistent RAD-51 Foci, Suggesting a Defect in Meiotic DSB Repair

RAD-51 is localized to nuclear foci during pachytene in the germlines of wild-type animals, and these foci are thought to correspond to the locations of meiotic DSB repair events. In mutants where meiotic DSBs are not formed (*eg. spo-11*) or where the DSBs are formed but not processed (*eg. com-1*) RAD-51 foci fail to form at pachytene (Alpi et al, 2003, Penkner et al., 2007). On the other hand, RAD-51 foci that persist beyond the end of pachytene into maturing oocytes are seen in mutant strains that have defects in meiotic DSB repair (Ward et al., 2010, Saito et al., 2009).

The diakinesis chromosome aggregation defects and inefficient rescue of *cep-1; him-5* double mutants by exogenous DSBs suggest these animals have defects in meiotic DSB repair. To test this hypothesis, I immunostained wild-type, *cep-1, him-5* and *cep-1; him-5* germlines for RAD-51 (Figure 3-11). All four strains show RAD-51 foci in the zygotene/early pachytene region, demonstrating that all of these strains contain at least some properly processed meiotic DSBs. However, most *cep-1; him-5* animals show a progressive intensification of RAD-51 foci as meiosis progresses, and many nuclei in these animals (as well as rare nuclei in *cep-1* and *him-5*...
single mutants) show persistence of RAD-51 foci into the gonad bend, beyond that seen in wild-type. This result, together with the spo-11-dependent chromatin aggregation phenotypes seen in these animals, suggests that cep-1; him-5 animals have defects in meiotic DSB repair.

### 3.5.7 Partially Redundant Roles for cep-1 and him-5 in Meiotic Recombination

In this chapter, I have provided experimental evidence that cep-1 and him-5 mutation contribute to failure of meiotic recombination in a high proportion of oocytes. This recombination failure, leading to missegregated or poorly repaired and fragmented chromosomes may be the basis for the enhanced embryonic lethality in cep-1; him-5 double mutants that I characterized in Chapter 2. The data presented here contribute and extend our understanding of both genes. him-5 was previously hypothesized to function in recombination. My work, alongside that of the Yanowitz and Meneely labs, refines this hypothesis to suggest that it has roles both in meiotic recombination initiation through programmed DSBs, and also in the subsequent repair of these breaks (Broverman & Meneely 1994). Previous to this work, the only evidence that might suggest the cep-1 p53 family member homologue would have a role in meiotic recombination in the worm was a modest Him phenotype, but there is a body of evidence from mammalian p53, p63 and p73 that they have both transcriptional and non-transcriptional roles in HR (Marmorstein et al. 1998; Buchhop et al. 1997; Stürzbecher et al. 1996; Yoon et al. 2004; Lee et al. 1997; Arias-Lopez et al. 2006; Lin et al. 2009; Habu et al. 2004; Sjöblom & Lähdetie 1996). This suggests that the ancestral form of the p53 family, which cep-1 has been suggested to represent, may have had other roles in genome maintenance besides its ability to induce cell cycle arrest and apoptosis in DNA damaged germlines (Derry et al. 2007; Greiss et al. 2008). Since p53 family members frequently perform protective functions it is possible that other roles for cep-1 may be uncovered in genetic backgrounds or environmental conditions that compromise genomic integrity or cellular function (Vousden & Lane 2007).

The work I have outlined here raises many questions about the roles cep-1 and him-5 may be playing in recombination. What is the molecular function of him-5 and and how does one
protein play roles in both DSB initiation and repair? Is cep-1's role in this process transcriptional or non-transcriptional, or a combination of the two? Since him-5 appears to play a role in promoting programmed DSBs either exclusively or largely on the X chromosome, what factors are performing analogous roles on the autosomes? Which isoforms of cep-1 are involved in this process? Some of these unanswered questions will be examined in Chapter 4.
4 Conclusion

4.1 p53 Family Genes May Have an Ancestral Role in HR

p53 has been called the 'guardian of the genome' for its role in promoting genomic stability (Lane 1992). The most widely understood ways in which p53 protects genomic integrity are by promoting apoptosis of damaged cells or halting the cell cycle until the damage can be repaired (Das et al. 2008). There is also evidence that the other mammalian members of the p53 family have roles in protecting tissue by activating apoptotic and cell cycle arrest programs (Pietsch et al. 2008). p53 also has other, more direct effects on DNA repair (Helton & Chen 2007). For instance, p53 activates NER after UV-IR by transcriptionally upregulating the NER gene XPC (Adimoolam & Ford 2002). p53 plays a non-transcriptional role in promoting BER by interacting with DNA polymerase β and stabilizing its interaction with damaged DNA (Zhou et al. 2001).

4.1.1 Future Directions: A Transcriptional or Non-transcriptional Role for CEP-1?

Mammalian p53 has been suggested to have transcriptional and non-transcriptional roles in DNA repair by HR and these were summarized in Chapter 3. Briefly, p53 reportedly alters the function of HR proteins such as Rad51 and Brca2 both by binding them directly and by altering the expression of the genes that encode them (Buchhop et al. 1997; Marmorstein et al. 1998). The overall effect of p53 function in cells undergoing HR appears to be to decrease the function of the Rad51 recombinase, thereby decreasing spurious recombination reactions (Stürzbecher et al. 1996). The other p53 family members, p63 and p73, also appear to modify the expression of HR genes (Lin et al. 2009). p53’s role in meiotic recombination has been less studied than its function in HR after DNA damage in mammalian somatic cells, but recent studies reported that p53 is activated after Spo11-induced DSBs in both Drosophila and mice (Lu et al. 2010).

The work I have presented here showing that the C. elegans p53 family member cep-1 is involved in meiotic recombination adds to this body of work and suggests a role for p53-like
genes in HR that may be highly conserved. A microarray study by Greiss et al. (2008) provided evidence that CEP-1 transcriptionally regulates very few genes and only in the presence of DNA damage, leading to speculation that the main, ancestral function of p53-like genes is to induce apoptosis in damaged cells. The data presented here calls this hypothesis into question and instead suggests that p53 family members have an evolutionarily conserved and ancient role in promoting effective meiotic recombination. The work shown here also emphasizes that this role may be largely redundant with other recombination factors, a difficulty that may only be exacerbated in mammals where three p53-related genes are present, in addition to their multiple isoforms with unique functions (Bourdon 2007).

The role I have shown for CEP-1 in meiotic recombination serves to pose more questions. For instance, is CEP-1’s role in meiotic recombination dependent on its transcriptional activity? For mammalian p53 homologues, there is evidence for a role in modulating HR by affecting transcription of recombination genes (Arias-Lopez et al. 2006; Lin et al. 2009). There is also some evidence that the p53 protein interacts with meiotic proteins and substrates and so may modulate recombination directly (Buchhop et al. 1997; Marmorstein et al. 1998). Examination of CEP-1’s molecular role in recombination would be aided by mutant versions of CEP-1 that are unable to bind DNA to affect transcription, but where the remainder of the protein is largely intact. Such mutations are known in mammalian p53 and have proven useful for studying the degree to which p53 functions transcription-independently in various processes (Haupt et al. 1995; Suzuki et al. 2009). Transactivation-dead CEP-1 mutations could either be genomic mutations or transgenes expressed in strong cep-1(lf) mutants, provided the transgenes are expressed sufficiently well in the germline. Construction or recognition of these mutants would require greater understanding of the residues in CEP-1 that are required for DNA binding and transactivation. Immunofluorescence of CEP-1 would also be useful in evaluating whether CEP-1 plays a transcriptional or more direct role in meiotic recombination. If CEP-1 showed a similar staining pattern to other meiotic recombination proteins, such as RAD-51, it would suggest that CEP-1 was modulating HR at the level of the DNA break (Alpi et al. 2003). Demonstration of direct binding between CEP-1 and RAD-51, BRC-2 or other components of the HR machinery.
in vitro and in vivo would also be evidence for involvement of CEP-1 directly at the level of DNA repair.

Data supporting a transactivating role for CEP-1 in meiotic HR would entail finding a gene or genes whose expression or repression is both dependent on CEP-1 and required for accurate recombination. The promoters of meiotic genes could be searched for the consensus CEP-1 binding site, which is very similar to the p53 binding site in mammals (Schumacher et al. 2001; Huyen et al. 2004). Studies done previously using microarrays could also be used to search for candidate DNA damage genes under the control of CEP-1. For instance, Derry et al. (2007) found that several DNA repair genes including brc-1, brc-2 and mre-11 are repressed after UV irradiation in a cep-1-dependent manner. Expression of candidate CEP-1-controlled genes would then have to be examined in wild-type and cep-1(λf) worms to prove CEP-1 is required for changes in expression. Mutation of the putative CEP-1 binding site could be used to show that this DNA element is required for modulation of expression.

The evidence presented in this thesis is insufficient to suggest CEP-1 is playing a transcriptional or non-transcriptional role in meiotic recombination. Evidence from mammals implicates p53 family members in both direct action at the site of DNA recombinational repair as well as in modulating transcription of recombination-related genes (Arias-Lopez et al. 2006; Buchhop et al. 1997; Lin et al. 2009; Marmorstein et al. 1998). It is important to remember that evidence for a transcriptional role does not preclude a direct role at the break as well, and vice versa.

4.1.2 Future Directions: Do CEP-1 and HIM-5 Function with RFS-1 and HELQ-1 in RAD-51 Removal?

Chromosome aggregation defects like those seen in cep-1; him-5 double mutants can be caused by defects at various points the meiotic recombinational repair downstream of SPO-11 induced DSBs. For instance, com-1 mutants have defects in double DSB processing to reveal ssDNA (Penkner et al. 2007). HELQ-1 (also known as HEL-308) and RFS-1 appear to act redundantly later in the recombination process to promote disassemblly of RAD-51 filaments after strand exchange has occurred to allow recombination to be completed (Ward et al. 2010). Both com-1
and helq-1 rfs-1 double mutants show very similar phenotypes – aggregated chromosomes during diakinesis – despite the very different steps they function in during recombination. Since cep-1; him-5 double mutants show persistent accumulation of RAD-51 foci it suggests that at least part of the recombinational repair defect is downstream of DSB processing, unlike com-1 mutants, and instead may be more similar to that seen in helq-1 rfs-1 mutants (Penkner et al., 2007, Ward et al, 2010).

One interesting note that may shed light on these hypotheses was communicated in the Worm Breeder's Gazette, a C. elegans community newsletter, in 1985, where M. Finney and H.R. Horvitz discuss a genetic interaction they found between various alleles of what was later shown to be unc-86 rfs-1 and him-5 (Finney et al. 1988; Yanowitz 2008). As I have found for cep-1, Finney and Horvitz (1985, personal communication) report that unc-86 rfs-1; him-5 animals show greatly increased embryonic lethality compared to either single mutant alone. They found that unc-86 alone did not have this effect, so we can assume that the gene responsible for this effect is actually rfs-1 and not unc-86. As with cep-1; him-5 double mutants, the excess embryonic lethality of unc-86 rfs-1; him-5 animals is a result of a defect in the maternal germline. No cytological characterization of the germline of unc-86 rfs-1; him-5 double mutants was reported, but due to the similarities of phenotype of cep-1; him-5 and unc-86 rfs-1; him-5 animals I hypothesize that RFS-1 may play a redundant role in meiotic recombination with HIM-5, just as I have found for CEP-1.

Interestingly, RFS-1 is a RAD-51 paralogue homologous to the mammalian RAD51D (Yanowitz 2008; Pittman et al. 1998). Although C. elegans has only two members of the RecA/Rad51 gene family, rad-51 and rfs-1, mammals have seven: Rad51, Dmc1, XRCC2, XRCC3, Rad51B, Rad51C and Rad51D (Ward et al. 2007; Lin et al. 2006). In mammals, extensive protein interactions between Rad51 and its paralogues have been found (Schild et al. 2000; Masson et al. 2001). RAD51D appears to play major roles in promoting HR-mediated repair of endogenous and exogenous damage during replication and in the maintenance of telomeres in somatic cells (Tarsounas et al. 2004; Hinz et al. 2006). In C. elegans, RFS-1 is critical for repair at blocked
replication forks, but is dispensable for HR during meiosis or at IR-induced DSBs (Ward et al. 2007). *rfs-1* mutants have a mortal germline phenotype and defects in telomere maintenance (Yanowitz 2008). Like its mammalian counterpart, RFS-1 appears to act mainly during recombination processes in somatic cells, but more recently a redundant role for RFS-1 with HELQ-1 has been recognized in promoting disassembly of RAD-51 filaments from DNA after the strand exchange step of meiotic recombination. *helq-1 rfs-1* double mutants show persistence of RAD-51 foci into late meiotic prophase I and the same diakinesis aggregated chromatin phenotype characteristic of *rad-51* mutants and that I see in *cep-1; him-5*, suggesting these animals have defects in meiotic recombination.

This role for RFS-1 with HELQ-1 in meiotic recombination repair and the enhanced embryonic inviability of both *rfs-1; him-5* and *cep-1; him-5* suggests new directions of inquiry. Since both *rfs-1* and *cep-1* are enhance the embryonic lethality of *him-5*, and both have similar recombination defects in specific mutant backgrounds, do *rfs-1* and *cep-1* function in the same pathway to promote accurate recombination repair? If so, does CEP-1 transcriptionally regulate *rfs-1*? Alternatively, do CEP-1 and RFS-1 interact at the point of the break? Since RFS-1 shows major roles in promoting HR in somatic cells, is CEP-1 required for that process as well? Do HELQ-1 and HIM-5 function in the same pathway, or are their effects additive?

Some of these questions can be answered by building all combinations of *cep-1*, *him-5*, *rfs-1* and *helq-1* mutants and assessing the extent of their chromosome aggregation and embryonic lethality defects. If CEP-1 and RFS-1 function in the same pathway to promote accurate recombination *cep-1; rfs-1; him-5* triple mutants will not show a more severe phenotype than either *cep-1; him-5* or *rfs-1; him-5* alone. Similar arguments can be made about HELQ-1 and HIM-5. Phenotypic analysis of all these strains would tell us whether CEP-1, RFS-1, HIM-5 and HELQ-1 define two, three or four pathways which contribute to meiotic recombinational repair. If CEP-1 and RFS-1 are found to function in the same pathway, immunostaining for RFS-1 in *cep-1* mutants would show whether CEP-1 is required for RFS-1 expression or localization. The reciprocal experiment could also be done to assess RFS-1's role in localization of CEP-1.
If genetic experiments suggest they function in the same pathway, immunofluorescence analysis of HELQ-1 and HIM-5 could examine whether the localization of one is dependent on the function of the other. The protein-protein interaction profiles of CEP-1, RFS-1, HIM-5 and HELQ-1 with respect to one another and other recombination proteins would aid in building hypotheses about the molecular function of these factors.

Like \textit{rfs-1} mutants, \textit{cep-1} mutants are only weakly sensitive to IR in survival assays (Ward et al. 2007; Derry et al. 2001; Schumacher et al. 2001). It would be interesting to know if \textit{cep-1} animals were sensitive to DNA interstrand crosslinking agents, as has been shown for \textit{rfs-1} mutants (Ward et al. 2007). If \textit{cep-1} mutants are sensitive to these agents and CEP-1 and RFS-1 function in the same pathway for meiotic recombination it would suggest that these genes show extensive functional relationship to promote HR in different contexts.

The data presented here will lead to new questions concerning the role of p53 family members in meiotic recombination and recombinational repair. Future work should focus on the specific role of these homologues in these processes. Particularly important will be elucidating whether p53 family members function at the site of recombination or modulate the transcription of recombination machinery or both, and at which specific step in recombination p53 family members are functioning. \textit{C. elegans} provides an invaluable system for answering these questions, with its single p53 family member, convenient spatio-temporal germline conformation and well-defined meiotic recombination phenotypes. This work and future studies examining the role of CEP-1, perhaps in conjunction with RFS-1, will shed light on the role of p53 homologues in genomic stability by HR.

4.2 HIM-5 Has Roles in Meiotic Recombination Initiation and Repair

Defects in recombination result in missegregation of homologous chromosomes at meiosis, and the missegregation defects of several nondisjunction mutants in \textit{C. elegans} are due to a failure of crossing over (Lamb et al. 2005; Phillips et al. 2005; Wicky et al. 2004). Broverman and
Meneely (1994) found evidence that him-5 played a role in recombination on the X chromosome and the data presented in this thesis and by the work of the Yanowitz and Meneely labs suggests that HIM-5 plays a role in initiation of recombination and in meiotic recombinational repair (J. Yanowitz and P. Meneely, personal communication).

It is not without precedent that HIM-5 may play a role at two different steps during meiotic recombination. In S. cerevisae and in C. elegans Mre11 and Rad50 are required both for meiotic recombination initiation and for recombinational repair of the break after it has occurred (Johzuka & Ogawa 1995; Chin & Villeneuve 2001). Though the repair role appears to have been maintained in all eukaryotes, the recombination initiation role has not, illustrating the significant variation seen throughout evolution in this generally well conserved process (Gerecke & Zolan 2000; Puizina et al. 2004; Young et al. 2004; Borde 2007).

Many meiotic recombination genes, despite having highly conserved roles in the process of crossing over, are poorly conserved at the sequence level. For instance, it was only recently discovered that humans have highly diverged homologues of the budding yeast genes Mei4 and Rec114 which retain their role in meiotic recombination initiation (Kumar et al. 2010). Also, C. elegans BRC-2 functions in a manner very similar to its mammalian homologue Brca2, despite being approximately 1/10th the size (Petalcorin et al. 2006). No homologues of HIM-5 are reported in the C. elegans database Wormbase, and it contains no clear domain structure that could give a clue to its function (www.wormbase.org). No HIM-5 homologues have been recognized in related nematode species either. It is possible that homologues exist outside nematodes and retain their role in recombination but remain unrecognized because they have diverged so extensively. Conversations with Charly Chahwan led us to examine whether we could find any sequence similarities between HIM-5 and any of the meiotic recombination initiation genes of S. cerevisae and its homologues. After searching for homology between HIM-5 and several S. cerevisae meiosis genes we found some motifs that appeared conserved between HIM-5 and Rec104. C. Chahwan and I found the putative Rec104 homologues from Vanderwaltozyma polyspora, Zygosaccharomyces rouxii, Candida glabrata, Kluyveromyces
lactis, Kluyveromyces thermotolerans, Ashbya gossypii, and aligned these along with Rec104 from S. cerevisiae and HIM-5 from C. elegans using the MAFFT algorithm (JALVIEW software) followed by some minor modifications (Waterhouse et al. 2009; Katoh et al. 2005). It should be noted that these putative Rec104 homologues have been recognized by synteny and/or homology to Rec104 from S. cerevisiae and have not been shown experimentally to play roles in meiotic recombination initiation. The result of this alignment is shown in Figure 4-1. The Rec104 homologues from the various other yeast species are not well conserved with Rec104 from S. cerevisiae or with each other, though blocks of similarity are evident. This is consistent with the idea that some meiotic initiation proteins are evolving quickly, as has been found for Mei4 and Rec114 (Kumar et al. 2010). It also gives more weight to the suggestion that Rec104 homologues may exist outside yeast but remain unrecognized due to extensive divergence. The alignment shown in Figure 4-1 also shows that HIM-5 from C. elegans shares some limited blocks of similarity with the S. cerevisiae Rec104 and the various putative Rec104 homologues from other yeast. We reasoned that HIM-5 may be a highly diverged homologue of S. cerevisiae Rec104. It is interesting to note that CEP-1 is also a very divergent member of its protein family, with very low sequency similarity to other known p53 family members (Derry et al. 2001; Schumacher et al. 2001).

Rec104 is required in S. cerevisiae for initiation of meiotic recombination by Spo11 (Malone et al. 1991). Mutations in Rec104 drastically reduce the frequency of meiotic recombination and show no defects in mitosis (Malone et al. 1991). Rec104 is only transcribed in meiosis, confirming it is specifically required during the formation of gametes and not during vegetative growth (Galbraith & Malone 1992). This protein was also found to be a high-copy suppressor of mutations in Hop1, a component of the SC that is required for efficient meiotic DSB formation (Hollingsworth & Johnson 1993). A genetic interaction was found between REC102 and REC104 where high copies of REC102 can suppress a hypomorphic (but not a null) allele of rec104 (Salem et al. 1999). Later, it was found that the Rec102, Rec104 and Spo11 proteins all interact during meiotic recombination initiation (Jiao et al. 2003). Rec104 and Rec102 are mutually dependent for one another's subcellular localization and require the presence of both
Figure 4-1: HIM-5 may be a highly diverged Rec104 homologue.

From top to bottom, Rec104 from *S. cerevisiae* (NCBI Accession number 6321951), putative Rec104 homologues from *Vanderwaltozyma polyspora* (NCBI Accession number 156848372), *Zygosaccharomyces rouxii* (254578644), *Candida glabrata* (50289819), *Kluyveromyces lactis* (50309289), *Kluyveromyces thermotolerans* (255712023), and *Ashbya gossypii* (299788940) and HIM-5 from *C. elegans* (NCBI Accession number 17559136) were aligned with using JALVIEW software and the MAFFT algorithm followed by minor manipulations (Waterhouse et al. 2009; Katoh et al. 2005). The colours highlighting the base pairs conform to the default Clustal colour scheme, described at [http://ekhidna.biocenter.helsinki.fi/pfam2/clustal_colours](http://ekhidna.biocenter.helsinki.fi/pfam2/clustal_colours).
Spo11 and Ski8 for targeting to chromatin (Kee et al. 2004). Conversely, Spo11's association with chromatin and its dimerization both require Rec102, Rec104 and Rec114 (Sasanuma et al. 2007; Prieler et al. 2005). It appears that an essential function of Rec102-Rec104 is to connect the Mei4-Rec114 complex to Spo11 (Maleki et al. 2007). Despite the fact the only known role for the Rec102-Rec104 complex is is promoting Spo11-induced DSBs, it remains on chromatin until pachytene, well after DSB formation (Kee et al. 2004). Homologues of Rec104 are found in other Saccharomyces species and can complement the defect in S. cerevisiae rec104 mutants, but no homologues of this protein have been reported outside the yeasts (Nau et al. 1997).

The similarity between Rec104 and HIM-5 is not nearly extensive enough to confirm their homology. Although they are both involved in meiotic initiation, only a similarity in specific molecular function would give greater weight to the hypothesis that HIM-5 is a nematode Rec104 homologue. The only known molecular function of Rec104 centres on its binding partners, particularly Rec102 and Spo11. If HIM-5 were found to bind SPO-11 or an (as yet unrecognized) C. elegans Rec102 homologue, it would lend strength to the hypothesis it is homologous to Rec104.

Irrespective of the question of Rec104 homology, protein-protein interaction studies with HIM-5 may shed light on its function. For instance does HIM-5 bind SPO-11 directly? Such an interaction would make sense, since HIM-5 is required for SPO-11-induced DSBs, at least on the X chromosome (P. Meneely and J. Yanowitz, personal communication and Figure 3-5). A yeast-two-hybrid system may be useful in finding HIM-5 binding partners, both using a candidate protein approach (such as asking if HIM-5 binds SPO-11) and using a C. elegans cDNA expression library to look for novel interacting proteins. There are probably other factors necessary for this process that remain unrecognized. In particular, HIM-5 appears to be required almost exclusively for recombination initiation on the X chromosome, suggesting that there may be other factors required to fulfill this function for the autosomes. Novel HIM-5 binding proteins would be candidates for a role in meiotic recombination initiation. Using a yeast-two-hybrid
system with SPO-11 as bait may also reveal novel *C. elegans* proteins required for recombination initiation during meiosis.

It would also be interesting to assess if HIM-5 affects the localization of SPO-11. For instance, is SPO-11 localization to chromatin disrupted in a *him-5* mutant? Asking this question would require the creation of either tagged SPO-11 transgenes that are adequately expressed in the germline or SPO-11 antibodies useful for immunofluorescence. In *S. cerevisae* functional Spo11 is a dimer, and Rec104 is required for its dimerization (Sasanuma et al. 2007). It would be interesting to ask whether HIM-5 is required for a similar process. There is currently no published evidence suggesting whether or not dimerization is required for function in *C. elegans* SPO-11. Co-immunoprecipitation of two differently tagged SPO-11 transgenes would provide evidence for SPO-11 multimerization, and retesting the interaction in a *him-5* mutant background would reveal whether HIM-5 is required for this process.

Also of interest is ascertaining the molecular function of HIM-5 in repair of DSBs. Rec104 has no known function in meiotic recombinational repair, but it is possible that it (like HIM-5) plays a redundant role in DSB repair that is yet unrecognized in *S. cerevisae*. It is interesting that Rec104 persists at the site of the meiotic DSB well after it has been formed and into pachytene, when DSB repair is occurring (Kee et al. 2004). Binding partners of HIM-5 may lend understanding to its role in repair, for instance if HIM-5 is found to bind proteins required for HR such as RAD-51 or BRC-2. As was noted above, the persistence of RAD-51 foci in *cep-1; him-5* mutants may suggest that CEP-1 and HIM-5 are involved in removing RAD-51 from the DSB after strand invasion has occurred, as has been shown for *helq-1 rfs-1* (Ward et al. 2010).

The work presented here demonstrates that HIM-5 has a role both in meiotic recombination initiation (at least on the X chromosome) and meiotic recombinational repair. This sets HIM-5 with the MRX/MRN complex in *S. cerevisae* and *C. elegans* as the only genes known to play roles in both processes (Ajimura et al. 1993; Chin & Villeneuve 2001). It is significant to note that the role of HIM-5 in meiotic recombination initiation is only uncovered in the background of mutation in *cep-1*. This suggests there may be significant redundancy in the process of
recombination, and other genes may be found to have redundant roles at various points in the recombination process. Searching for redundant pathways is always somewhat difficult using genetic methods, though some screens may prove useful. For instance, it would be interesting to ask whether Rec104 in budding yeast plays a redundant role in recombinational repair. This could be done by screening for mutants that can still undergo vegetative growth but, fail to be rescued by moderate doses of IR during meiosis when in the background of rec104. This might most easily be done using a Rec104 rescuing plasmid. The clones of interest would be those that can undergo meiosis in the presence of the Rec104 plasmid but not in the absence of it after IR. In addition, it would also be interesting to do enhancement screens in C. elegans for other genes that enhance the embryonic lethality of him-5. Since both cep-1 and rfs-1 enhance embryonic inviability in this background there may be other genes that do as well (Yanowitz 2008, Finney and Horvitz, 1985, personal communication). him-5/+ balanced strains could be mutagenized, and those mutants that produce significantly greater embryonic lethality could be crossed into a him-5(+) strain. him-5/him-5 animals could be isolated and assessed for high embryonic inviability, and the offspring of him-5/+ sisters of potentially interesting mutants could be examined. Mutants of interest would be those that show increased embryonic lethality when mothers are him-5/him-5 than when they are him-5/+. Similar experiments could be done with other genes with known roles at particular steps in the recombination pathway to look for redundant roles at other points in the pathway. Priority should be given to proteins that appear or are retained at the recombinational break well before or after they are required during recombination, or those that already have some known redundancy. For instance, perhaps the MRN complex retains a role in DSB formation in species such as S. pombe, where no such role has been described, but this role has now become redundant (Young et al. 2004). Rec104 is also a candidate for redundancy since it remains at the meiotic DNA break well after DSB formation has occurred (Kee et al. 2004).

Given the importance of meiotic recombination to the survival and genomic integrity of sexually reproducing organisms, a significant amount of built-in redundancy would make sense, and I expect layers of redundancy exist that are currently unknown. Simple genetic screens for single
mutants that are defective in meiotic recombination have given a sketch of the crossing over process in several organisms, but other novel recombination genes as well as known genes with novel roles almost certainly play redundant but important roles in efficient meiotic recombination. The work presented here and in other recent reports highlights the roles of redundant and dual-function genes in the highly conserved process of meiotic recombination (Ward et al. 2010).

4.3 Conclusions and Overall Themes

4.3.1 Non-apoptotic Functions of p53 Family Members are Evolutionarily Ancient

CEP-1, the sole *C. elegans* p53 family member, has been heralded since its discovery for the insight it can lend to the early evolution of the p53 protein family (Frantz 2001; Derry et al. 2001; Schumacher et al. 2001). It was found that *cep-1* mutant animals have no gross developmental defects but were defective for apoptosis and (in some cases) cell cycle arrest after DNA damage in the mitotically active germline, functions conserved in mammalian p53 (Derry et al. 2001; Schumacher et al. 2001; Zuckerman et al. 2009). Later, a microrarray report showed that CEP-1 transcriptionally controlled only three genes after IR (Greiss et al. 2008). Two of these, *egl-1* and *ced-13*, are known apoptotic transducers. The same report showed that, in the absence of IR, *cep-1* mutation did not significantly affect the mRNA levels of any gene. This, along with the normal development and somatic DNA repair capacity of *cep-1* mutants lead to the assertion that the ancestral role of p53 was in promoting apoptosis, and that all other functions of p53 and its homologues in mammals, such as in DNA repair and normal development, were added more recently (Greiss et al. 2008; Derry et al. 2001; Schumacher et al. 2001; Helton & Chen 2007; Tomkova et al. 2008). This hypothesis is at odds with another microarray study which found that more than one thousand genes show significant changes in expression in a *cep-1*-dependent manner (Derry et al. 2007). Nevertheless, it is clear that the best characterized function of CEP-1 is in transcriptionally promoting apoptosis after DNA damage and that this is predicted to be one of the main ancestral functions of the p53 family.
When the Him phenotype of *cep-1* mutants was first noted, it was still possible that CEP-1 was promoting accurate meiotic chromosome segregation by causing apoptosis of cells that were in danger of creating aneuploid gametes (Derry et al. 2001). For instance, meiotic cells containing unsynapsed chromosomes in *C. elegans* are subject to an apoptosis checkpoint, and it remained possible that the Him phenotype of *cep-1* could have been caused by a failure of a similar checkpoint (Bhalla & Dernburg 2005). Likewise, when I found the enhanced embryonic inviability interaction between *cep-1* and *him-5* mutants I hypothesized that CEP-1's role in promoting embryonic survival in *him-5* mutants was by promoting apoptosis of defective *him-5* mutant gametes. However, I showed in two different ways that CEP-1's role in this process is not apoptotic in nature. First, *ced-3* or *ced-4* mutations did not enhance the embryonic inviability of *him-5* mutants. *ced-3* and *ced-4* are required for essentially all apoptosis in *C. elegans*. The absence of increased embryonic lethality in *ced-3; him-5* and *ced-4; him-5* double mutants suggested that *him-5* animals do not show reduced survival in the absence of germline apoptosis (Ellis & Horvitz 1986; Gartner et al. 2008). I also tested this hypothesis by assaying germline apoptosis directly. If CEP-1 rescues *him-5* mutants by promoting apoptosis, *him-5* mutants should show elevated germline apoptosis compared to wild-type. Contrary to this hypothesis, I found that *him-5* mutant worms did not show elevated physiological germline apoptosis (Figure 2-12). This corroborates the *ced; him-5* lethality data and suggests that whatever function CEP-1 has in promoting survival in the *him-5* mutant background, it is not to promote apoptosis. The work presented in this thesis calls into question the hypothesis that the sole role for ancestral p53 was in promoting PCD (Greiss et al. 2008).

Evidence has been presented for CEP-1 having other roles outside apoptosis and cell cycle arrest, including in hypoxic survival, lifespan and innate immunity (Derry et al. 2001; Arum & Johnson 2007; Fuhrman et al. 2009). The work presented in this thesis joins the reports of mammalian p53 homologues to suggest that p53 family members have a role in promoting efficient HR (Buchhop et al. 1997; Marmorstein et al. 1998; Stürzbecher et al. 1996; Lu et al. 2010). It also argues that this role in recombination is evolutionarily ancient, though it may be hidden behind layers of redundancy. This redundancy may explain why the mammalian p53 network is
activated during meiotic recombination, but p53 deficient mice undergo recombination with no clear defects (Lu et al. 2010; Gersten & Kemp 1997). The role I have demonstrated for CEP-1 in meiotic recombination may prompt further examination of the role of p53 family proteins in HR in mammalian cells.

4.3.2 Meiotic Recombination Pathways Have Significant Redundancy

Accurate meiotic recombination is critical for genomic stability of sexual reproducing organisms (Longhese et al. 2009). Because of this, it makes sense that redundant pathways may have evolved to ensure meiotic recombination occurs in a timely and accurate fashion. Indeed, several redundant pathways are known to be required for different aspects of meiotic recombination in several species. During resection of the meiotic break, the Exo1 and Dna2 nucleases act redundantly to lengthen the ssDNA tract after Spo11 removal (Manfrini et al. 2010). In *A. thaliana*, two Spo11 homologues, AtSPO11-1 and AtSPO11-2, function partially redundantly to initiate meiotic recombination (Stacey et al. 2006). Also, a recent report in *C. elegans* shows that HELQ-1 and RFS-1 act redundantly to remove RAD-51 from DNA recombination intermediates after strand invasion has occurred to allow resolution of the crossovers (Ward et al. 2010). The work reported here shows that CEP-1 and HIM-5 have redundant roles in meiotic recombination in *C. elegans* and adds to our knowledge of the functional redundancy present in meiotic recombination.

There are several ways to envision the mechanistic basis of redundancy between CEP-1 and HIM-5. They could be viewed as defining two equivalent parallel pathways required for recombinational repair. Or, CEP-1 could be acting as a fail-safe, allowing recombination to proceed when the normal pathways are abrogated. In this way, the p53 family protein CEP-1 may be fulfilling its function as 'guardian of the genome' in a novel fashion, by promoting accurate recombination rather than cell fate decisions such as apoptosis or cell cycle arrest (Lane 1992).
Redundant pathways are more difficult than non-redundant ones to find by genetic screens though once they are recognized genetic tests exist that can be used to characterize them (Thomas 1993). I expect that other layers of redundancy, complete with novel genes and gene functions, remain to be discovered as important in the process of meiotic recombination. Genetic screens designed to uncover redundant pathways in *C. elegans* and other organisms looking for defects in meiosis may be fruitful avenues of discovery to increase the depth of our understanding of meiotic recombination.

4.3.3 Proteins May Have Dual Functions within the Meiotic Recombination Process

In all eukaryotes examined, the MRX/MRN complex proteins (Mre11, Rad50 and either Nbs1 or Xrs2) are required for efficient meiotic recombination, probably by processing the programmed DSB and removing the covalently attached Spo11 protein from the break (Borde 2007). In *S. cerevisae* and *C. elegans*, however, Mre11 is also required for formation of the DSB, causing *mre11* mutants to resemble *spo11* mutants rather than *rad51* mutants as they do in other organisms (Malone & Esposito 1981; Chin & Villeneuve 2001). The reason for this difference in MRN/MRX function is not known, but it is possible that MRN/MRX retains its role in DSB formation organisms other than *C. elegans* and budding yeast, but that this role has now become redundant with other factors. It does make some sense that proteins needed for repair of the DSB be required to be in place before the break occurs. DSBs are dangerous for genomes and must be repaired accurately, and if repair factors are already in place before the programmed break occurs, repair may be more timely and efficient (Hochwagen & Amon 2006).

HIM-5 now joins the proteins of the MRX/MRN complex as known to be required both for meiotic DSB formation (at least on the X) as well as for later repair of the break. Since the role of HIM-5 in recombinational repair is largely redundant with CEP-1 (and with RFS-1) other recombination initiation proteins may also be required later in the recombination process but remain unrecognized due to functional redundancy. Rec104, for instance, persists at the DNA break site long after the DSB has been formed, and though no repair role for Rec104 has been
described it could be playing a role redundantly with other factors (Kee et al. 2004). Surprisingly, the *S. pombe* Spo11 homologue Rec12 appears to have roles in accurate chromosome segregation during meiosis I and meiosis II that are independent of and occur later than its roles in initiating meiotic crossovers (Sharif et al. 2002). Other recombination proteins likely also have dual roles in meiotic processes, but these may be hidden under layers of redundancy that ensure genomic stability is maintained during crossing over and subsequent rounds of chromosome segregation.

Overall, the work shown here demonstrating a largely redundant role for the p53 homologue CEP-1 and the meiotic segregation protein HIM-5 in promoting embryonic survival via efficient meiotic recombinational repair raises new questions in this complex field. It suggests that p53 family proteins have an evolutionarily ancient role in HR and should motivate a careful examination of the roles of p53, p63 and p73 in recombinational processes in mammals. It also emphasizes the role for redundancy in meiotic recombination as well as the possibility that genes may have multiple functions in this complex process. These insights should prompt new examination of known and novel proteins to deepen our understanding of meiotic recombination, a multifaceted process critical for genomic stability in sexually reproducing eukaryotes.
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