DOUBLE-STRAND BREAK REPAIR AND G4 DNA STABILITY IN *CAENORHABDITIS ELEGANS*

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Cover image: Endogenous double-strand breaks marked by RAD-51 (red) in *C. elegans* germ cell nuclei (blue)

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Reparatie van dubbel-strengs breuken
en G4 DNA stabiliteit in *Caenorhabditis elegans*
(met een samenvatting in het Nederlands)

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CHAPTER 1

GENERAL INTRODUCTION

Genome instability

Our DNA is under constant challenge from a wide range of DNA damaging agents and endogenous mutagenic processes. Whereas low levels of sequence variety are desirable to drive evolution, sequence changes, called mutations, are intrinsically much more likely to disrupt than to improve gene function. It is therefore of vital importance that sequence information is accurately stored and faithfully inherited by subsequent generations, keeping the number of mutations to a minimum. Paradoxically, besides driving evolution, mutations are also the driving force behind a common disease associated with high mortality and morbidity; cancer. This illustrates the need for an equilibrium between allowing only a very limited number of mutations for the benefit of the species, while removal of most mutations is required for the benefit of the individual.

Mutations can arise from rare errors during DNA replication, or following DNA damage from a wide variety of sources. First, bases or backbone bonds in the DNA can be hydrolyzed spontaneously under physiological conditions (Lindahl, 1993). Second, chemical reactions involved in normal cell metabolism expose DNA to reactive molecules such as reactive oxygen species, which can damage the DNA (Mena et al., 2009). Finally, a wide range of exogenous sources, including ionizing radiation, UV light from the sun and certain chemicals, can cause DNA damage and destabilize the genome (Hoeijmakers, 2001). Damaged DNA is target of myriad highly specialized repair pathways that are active in a cell to prevent excessive mutagenesis.

How can genome instability be the driving force behind the two seemingly paradoxical processes cancer and evolution? The answer lies in a common denominator between the two; survival of the fittest. Evolution is driven by random mutation followed by natural selection: while random mutations will most often have detrimental consequences and fail to be passed on to the progeny, occasional beneficial mutations will thrive and expand in subsequent generations. Evolution is slow because of this trial-and-error, and because many generations need to pass for a novel trait to spread. Cancer can also be regarded as an evolutionary process, but here, a single cell is the subject of random mutation and natural selection. A cell that has, through random mutation, acquired the ability to divide and expand faster than its non-mutated counterparts, will by definition soon outnumber its neighboring cells, forming a potential tumor-precursor. However, expansion alone is not sufficient to form a malicious, invasive tumor. For this to occur, many other intrinsic tumor-defense mechanisms of the body need to be bypassed, and additional mutations are required that allow for e.g. immune system evasion, insensitivity to growth-inhibition signals from neighboring cells, and activation of angiogenesis to support the expanding tumor (See Text Box 1 for more detail). All these mutations need to be acquired and accumulated in subsequent rounds of mutation and selection (Nowell, 1976).

Considering the low intrinsic mutation frequency in humans (5.0×10^{-11} per base per replication round (Drake et al., 1998)), it is highly unlikely that a cell spontaneously

Text Box 1: Examples of pathways commonly mutated in cancer

(1) Insensitivity to growth signals. A process called contact inhibition ensures that cells stop dividing when they come in close contact with their neighbors; these neighboring cells send a "stop" signal to the dividing cell to prevent it from expanding further. Tumor cells have generally become insensitive to contact inhibition, and they keep on dividing, ignoring the stop signals.

(2) Escape of immune system detection. The immune system can recognize early tumors, and there is evidence that many early-stage tumors are killed by the immune system before they even reach the stage of detection. Late-stage tumors have obviously escaped this detection, often through loss of expression of proteins that are a target for immune system detection (Seliger, 2005; Zitvogel et al., 2006).

(3) The ability to metastasize. Normal cells have limited capacities to migrate and invade into tissues where they do not belong. Tumor cells have acquired these capacities, allowing them to metastasize e.g. by expression of matrix metalloproteinases that degrade the extracellular matrix and by stimulating vessel growth to supply the tumor with vital nutrients.

(4) Evasion of apoptosis. p53 is the most frequently mutated gene in human cancer and a master switch gene in the initiation of apoptosis (http://p53.free.fr/). Apoptosis is a cell-suicide program that can be activated by the cell itself in the presence of excessive DNA damage (intrinsic activation) or by signals from its neighbors in the case of abnormal cell growth (extrinsic activation). Tumor cells, despite their usually extensive DNA damage, generally lack the ability to initiate apoptosis through mutations in the p53 signaling pathway (Lowe and Lin, 2000).

(5) Limit senescence. The ends of chromosomes pose a dual problem to replicating cells: they may be recognized as DSBs by the DSB repair machinery, leading to chromosome fusions, and the DNA replication machinery has trouble replicating them properly, and consequently, chromosomes get shorter each generation. Expression of an enzyme called telomerase is required to maintain telomere length (Cheung and Deng, 2008). The seemingly unlimited replication potential of tumor cells requires circumvention of senescence, and tumor cells often acquire mutations that lead to high levels of telomerase expression to prevent telomere shortening, thereby extending their growth potential.

acquires the wide spectrum of mutations required for tumorigenesis. The frequency is low because of the actions of a highly efficient DNA damage response (DDR), which ensures DNA repair or elimination of damaged cells through apoptosis. Thereby, the number of DNA damage-induced mutations is strongly limited. It has therefore been suggested that the high mutation frequency required for tumor development can be established only in the context of a so-called mutator phenotype: an increased intrinsic mutation rate as a result of dysfunctional DNA repair (Bielas et al., 2006; Loeb, 1991). If repair is compromised, through inherited or acquired mutations in key repair or DDR genes, DNA damage and thus, mutations, can accumulate. Indeed, mutations in DDR or repair pathways, both congenital and sporadic, are often the first step in and the underlying source of cancer.

Familial cancers

A well known example to illustrate the complex process of tumor formation is familial breast cancer. It has been found that mutations in two genes, called BRCA (for BReast CAncer related) 1 and -2, are mutated in breast cancers in approximately 20% of the familial cases (Easton et al., 1993; Fackenthal and Olopade, 2007; Hall et al., 1990; Miki et al., 1994; Wooster et al., 1994). The cancer predisposition arises in individuals that inherited a single dysfunctional BRCA1 or -2 copy from one of their parents. Besides the dysfunctional copy, an intact copy of the BRCA gene is in most cases inherited from the other parent, as homozygosity for these mutations generally leads to early embryonic lethality. Through random mutation, the remaining functional copy can be lost during life, resulting in complete absence of functional BRCA gene in a subset of cells. BRCA1 and BRCA2 both play a role in the repair of DNA double-strand breaks (DSBs, see below), which can occur both spontaneously and as a result of certain chemicals. Without the BRCA genes, DSBs cannot be accurately repaired, conferring a permanent mutator phenotype to cells that have lost the intact copy. This way, 40-80% of women who inherit one mutated, dysfunctional version of one of these genes develop cancer early in their lives (Fackenthal and Olopade, 2007). Unfortunately, it remains largely unknown why BRCA1 and BRCA2 mutations predispose specifically to breast and ovarian –and not other- cancers, although hormonal causes are likely influencing the process (Liu et al., 2008).

Another example to illustrate the role of a mutator phenotype in cancer predisposition is Fanconi Anemia, which is caused by inherited mutations in either one of thirteen genes involved in the crosslink repair pathway (see below). Patients suffering from this disease are congenitally compromised in the repair pathway that resolves crosslinks between DNA strands when these are encountered during DNA replication. Cells from these patients display high sensitivity to crosslinking agents, and patients suffer from bone marrow failure and cancer predisposition, in particular acute leukemia and squamous cell carcinomas (Alter et al., 2003).

Many other genetic predispositions to cancers can be traced back to inherited mutations in the above and other DNA repair pathways. However, mutations in DNA repair pathways can also occur spontaneously in individual cells and thereby account for many sporadic tumors as well. Naturally, these initiating mutations occur more frequently in tissues that are particularly exposed to DNA damaging agents because these have the highest mutation frequency. This explains frequent lung cancer in smokers and high incidence of skin cancer in countries where the sun is very intense, such as Australia. Altogether, the accumulation of the wide spectrum of mutations to initiate and further develop cancer remains a matter of chance, making time, thus age, one of the most important determinants of cancer risk in addition to genetic make-up.

Caenorhabditis elegans as a model for DNA repair studies

Because impaired DNA repair is often an initiating step in tumor formation, a better understanding of DNA repair processes is required to comprehend tumorigenesis. DNA repair pathways have been predominantly studied in unicellular organisms or in cell-based assays. Besides the limited genetics of cell culture and the lack of conservation of some important DDR genes in e.g. yeast, another drawback of these systems is that their roles and regulation within a developing animal remain unclear. We have used the nematode *Caenorhabditis elegans* to study DNA repair. *C. elegans* has many advantages as a model organism: Animals are small and translucent, and can be kept on small Petri dishes seeded with *E. coli* bacteria as a food source (Brenner, 1974). Adults are composed of only 959 cells that have all been mapped to a cell lineage. The genome has been completely sequenced (*C. elegans* Sequencing Consortium, 1998) and mutant alleles are now available for many genes thanks to the efforts of an international knock-out consortium. In addition, gene knockdown can be achieved by feeding the animals transformed *E. coli* that express double-stranded RNA against the gene of interest (Timmons and Fire, 1998). Also, animals can be easily genetically manipulated by injection of a DNA sequence of interest into the germline. Although *C. elegans* does not get cancer, the molecular mechanisms for DNA repair are well-conserved. For example, p53 (mutated in >50% of human cancers (http://p53.free.fr/)) and BRCA2 (mutated in ~5 % of familial breast cancers (Fackenthal and Olopade, 2007)), and other Fanconi anemia genes are not conserved in yeast, but have functional orthologues in *C. elegans* (Collis et al., 2006; Lee et al., 2010; Martin et al., 2005; Schumacher et al., 2001).

In this thesis, we describe novel findings in two important DNA repair pathways: The closely related double-strand break repair (**Chapters 2, 3 and 4**) and Fanconi Anemia pathways (**Chapter 5 and 6**). A summarizing review on these pathways follows below.

DNA double strand break repair

DNA double strand breaks (DSBs) are amongst the most toxic DNA lesions that can occur in the genome and their repair is essential for proper gene and cell function. Inadequate or absence of repair can lead to mutations, deletions and genomic rearrangements, events that are typical for the formation and development of cancer. Currently, three main highly conserved DSB repair pathways have been described (Figure 1). During homologous recombination (HR), the sister chromatid or homologous chromosome is used as a template for error-free repair of the break (Lee et al., 1999). In contrast, the non-homologous end-joining (NHEJ) pathway joins the broken ends using no or only small stretches of sequence homology, usually resulting in the loss of sequence information (for review, see Daley et al., 2005; Hefferin and Tomkinson, 2005). Finally, the single-strand annealing (SSA) pathway combines features of both the above pathways; here, a repetitive sequence within the same chromosome is used for homology-based repair, resulting in deletion of the sequence between the repeats (Lee et al., 1999).

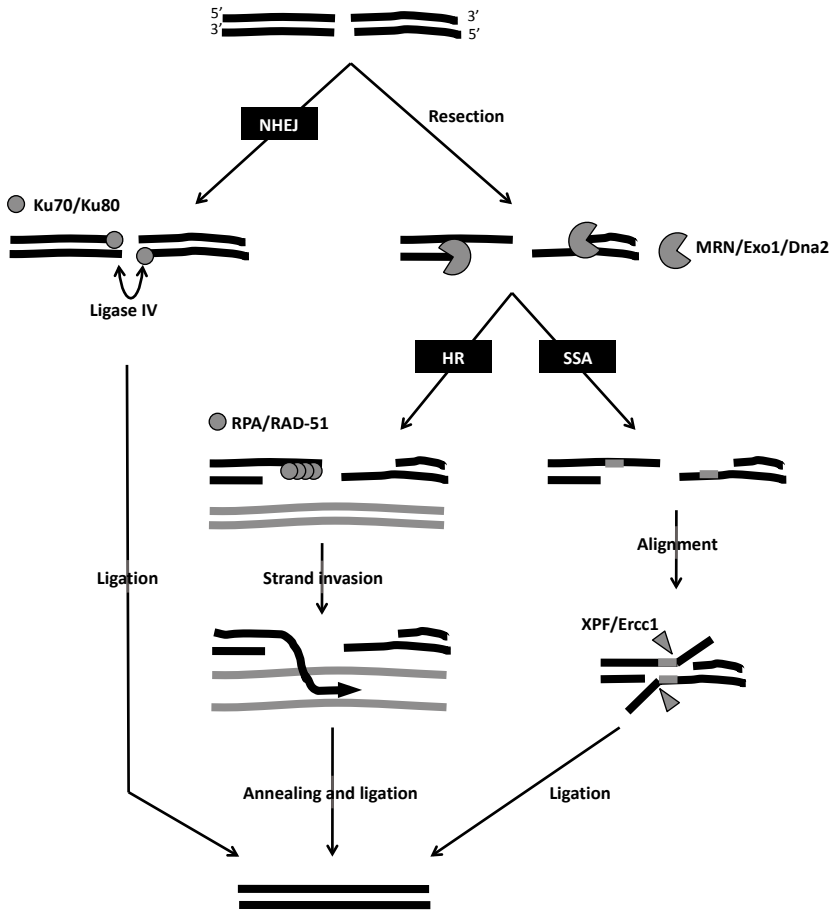

Figure 1 | Schematic overview of DSB repair pathways. Three DSB repair pathways are depicted: HR, Homologous Recombination; SSA, Single-strand Annealing; NHEJ, Non-homologous End-Joining. HR and SSA both require resection before further processing can occur, whereas NHEJ does not. Several key genes in the three pathways are indicated. See text for details.

Many genes have been described that operate in DSB repair. In mammalian HR and SSA, the MRN (for Mre11, Rad50 and Nbs1) complex binds the broken ends and initiates ssDNA resection from the 5'-sides, in cooperation with the nucleases Exo1 and Dna2, and the helicase Sgs1 (Mimitou and Symington, 2008; Tsubouchi and Ogawa, 1998; Zhu et al., 2008). The exposed ssDNA is coated and protected by the RPA protein complex (Fanning et al., 2006; Umezu et al., 1998). In HR, RPA is then displaced by Rad51, an enzyme that catalyzes strand exchange between DNA molecules, aided by cofactors that include BRCA1, BRCA2 and Rad52 (Sugiyama et al., 1998). Rad51 is dispensable for SSA since no strand invasion occurs here, but Rad52, which catalyzes annealing of homologous sequences, is required for both HR and SSA (Ivanov et al., 1996). After Rad51-mediated strand invasion, the invading 3'-end is extended

by specialized polymerases that use the invaded strand as a template (Kawamoto et al., 2005; McIlwraith et al., 2005). The extended DNA strand can then either release from the template and be captured by the other strand, or can remain annealed to the template, after which the structure needs processing by endonucleases to release the chromosomes. The former route does not lead to exchange of DNA between the chromosomes (called non-crossover, NCO), whereas the latter route results in crossing over (CO) of sequence information between chromosomes.

The relevance of the SSA pathway may be limited *in vivo* since repeats are required around the break site. However, repetitive DNA represents more than 40% of the human genome (Lander et al., 2001; Venter et al., 2001). In SSA, end-resection, coating of ssDNA and annealing of the repeats are performed by the same factors as in HR. One step specifically required for SSA is the removal of the 3'-overhanging flaps that arise after annealing of the repeats, but the complexes that are required for their removal, XPF/ERCC1 and Msh2/Msh3, are also required for nucleotide excision repair and mismatch repair, respectively (Alani et al., 1995; Mu et al., 1995; Sijbers et al., 1996; Strand et al., 1995). Altogether, no SSA-specific genes have been identified, since many of the proteins that contribute to the initial steps are shared with other repair pathways (Li et al., 2008).

In somatic cells, error-prone NHEJ is the main repair route. Here, an homologous template is usually not available and sequence errors that arise during inaccurate NHEJ repair are not propagated to the progeny of the organism (Rothkamm et al., 2003; Saleh-Gohari and Helleday, 2004). These inaccuracies may however contribute to tumorigenesis. In the NHEJ pathway, the broken ends are first recognized and protected from substantial resection by the Ku70/Ku80 heterodimer, which then recruits the catalytic subunit DNA-PK_{cs} (Wyman and Kanaar, 2006). DNA-PK_{cs} binds all other NHEJ proteins, including the recently identified protein Artemis (Moshous et al., 2001), and positions the ends in preparation for ligation. The enzyme Ligase IV is responsible for ligation (Rathmell and Chu, 1994; Wilson and Lieber, 1997) together with a dimer of XRCC4 and the novel XRCC4-Like Factor (XLF/Cernussos) in mammalian cells (Ahnesorg et al., 2006). Joining of the ends is generally sequence-independent, although microhomology-based joins also occur (Gaymes et al., 2002).

Besides this classic way of NHEJ, several variants on NHEJ with distinct genetic requirements have been uncovered. Recently, one variant pathway was described that operates predominantly in the absence of the canonical NHEJ factors Ligase IV and Xrcc4 (Corneo et al., 2007; Yan et al., 2007). This "alternative NHEJ" included increased insertions and chromosomal translocations and was strongly biased towards microhomology. Even though some of these characteristics may be reminiscent of SSA, it seems likely that these pathways are different on the genetic level, since another study detected similar events in the absence of the SSA genes Rad52 and Ercc1 (Bennardo et al., 2008). Before this pathway was described, Ku- and Ligase IV independent microhomology-mediated joining had also been observed to occur

in yeast cells (Boulton and Jackson, 1996; Moore and Haber, 1996). However, unlike alternative NHEJ, these events depended strongly on the Rad1/Rad10 endonuclease, the yeast homolog of XPF/ERCC1 (Colaiácovo et al., 2003), suggesting that these are distinct variants on classic NHEJ or SSA. No genetic requirements have been identified for alternative NHEJ and, its contribution to physiological repair remains unknown. Inconveniently, an accurate definition of alternative NHEJ is lacking; on one hand, it was found to be active only in cells that are deficient for classic NHEJ, whereas the term was also used to refer to microhomology joins in wild type background.

The main players of the above DSB repair pathways all have functional orthologues in *C. elegans*, and both HR and NHEJ operate similarly and depend on the same factors (Clejan et al., 2006), but a role for SSA has not yet been investigated. In **Chapter 2** of this thesis, we perform an extensive genetic analysis of DSB repair in *C. elegans*, revealing highly dynamic interactions between the canonical DSB repair pathways, and the presence of very robust alternative repair activity, which we term alternative end-joining (alt-EJ). We further characterize and define the molecular mechanism of this repair mode in **Chapter 3** and find that this pathway only operates in replicating cells.

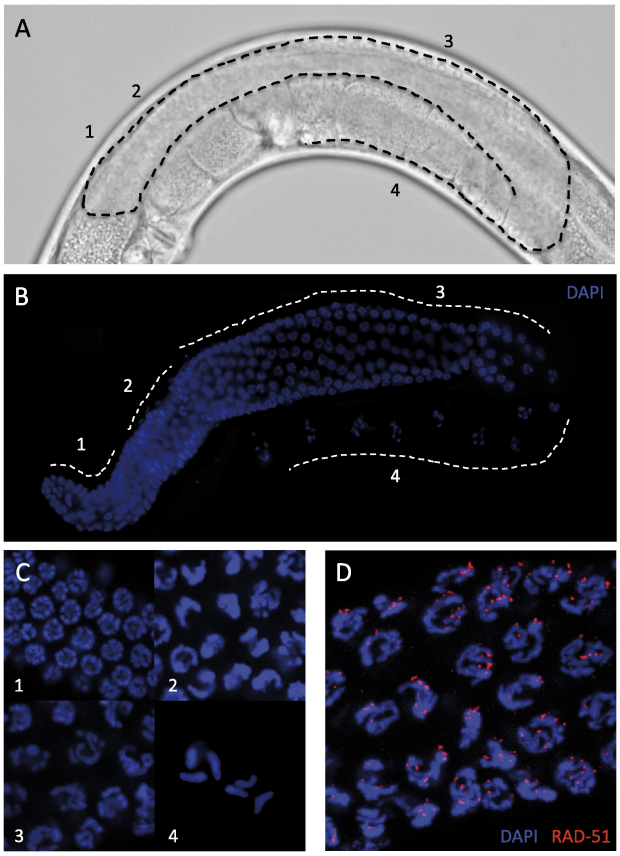

Figure 2 | Overview of *C. elegans* germline (A) *In vivo* DIC image of germline, outline indicated by the dotted line. The germline is divided into sub-zones: 1 premeiotic (mitotic) zone; 2 Transition zone (initiation of meiosis); 3 pachytene (synapsis and DSB resolvation); 4 diakinesis **(B)** DAPI staining on dissected germline. **(C)** Magnification of nuclei in zones 1-4 (see (A)). **(D)** Endogenous RAD-51 foci induced by SPO-11 in pachytene (zone 3) cells.

Despite their toxicity, DSBs are also required for several essential biological processes. One of these processes is meiosis, the reductional cell division that precedes gametogenesis. Meiotic cell division is a two-step process that generates haploid gametes from diploid precursors. To achieve this, two consecutive rounds of cell division without intervening DNA replication occur, where first the homologous chromosomes, and then the sister chromatids segregate. Meiotic cells are subject to relatively high levels of DSBs due to the activity of SPO-11, a topo-isomerase-like enzyme that generates programmed DSBs (Keeney et al., 1997), which are typically repaired by HR associated with crossovers. The transient physical connections between chromosomes (called chiasmata) that accompany crossovers are required for correct homologous chromosome segregation at this stage, and increase genetic diversity among the offspring.

C. elegans offers a convenient model to study meiosis: germline cells progress through the various stages of meiosis while they also progress physically through the gonad arm (Figure 2A-C) (Garcia-Muse and Boulton, 2007). In the first zone, the premeiotic tip, the pool of germ cells is replenished by mitotic cell divisions. Cells initiate meiosis in the transition zone, where they can be distinguished by their crescent shaped nuclei when chromatin condenses and homolog search is initiated (Figure 2C). SPO-11 activity can be visualized at this stage and at the subsequent pachytene stage by the formation of RAD-51 foci at sites of DSBs (Figure 2D) (Alpi et al., 2003). During pachytene, homologs are stably synapsed by a protein complex called the synaptonemal complex (SC) and crossovers are established here (MacQueen et al., 2002). In contrast to many other organisms, DSBs are not required to induce synapsis in *C. elegans*. In many organisms, the number and position of COs is regulated, a phenomenon called crossover interference. In *C. elegans*, this regulation is most extreme: in late pachytene, a single DSB per chromosome pair is resolved into a CO, whereas the other DSBs are resolved as NCOs (Hillers and Villeneuve, 2003). The chiasma formed by the CO holds the chromosomes together in diakinesis, where the SC is resolved and chromosomes strongly condense. Homolog segregation is initiated upon fertilization by sperm and quickly followed by the second round of division, upon which embryonic development can start.

Because the response to programmed DSBs may be different from the response to other DSBs, we investigated this further in meiotic cells using a targeted DSB in a known location in the genome. In **Chapter 4** of this thesis, we describe how we used this approach to show that the repair of DSBs is regulated differentially in different germline zones.

The Fanconi anemia pathway for crosslink repair

Fanconi anemia (FA) is a rare congenital disorder that is characterized by bone-marrow failure, various developmental abnormalities and cancer predisposition (Joenje and Patel, 2001; Moldovan and D'Andrea, 2009). Mutations in thirteen genes, representing thirteen complementation groups, have been identified as underlying

causes for this disease; Fanconi A, B, C, D1 (also known as BRCA2), D2, E, F, G (also known as XRCC9), I, J (also known as BRIP1 or BACH1), L, M and N (also known as PALB2) (Wang, 2007). Cells isolated from FA patients show increased sensitivity to DNA crosslinking agents such as cisplatin and nitrogen mustard, suggesting that these genes are involved in the repair of interstrand crosslinks (ICLs). The FA pathway operates in close collaboration with three other repair pathways to repair these ICLs: These are nucleotide excision repair (NER), translesion synthesis (TLS) and HR.

ICLs pose physical barriers to the DNA replication machinery, leading to replication fork stalling at the lesion, a situation that causes lethality if not resolved (Figure 3). Stalled forks first activate the FA pathway through phosphorylation of the replication-associated signaling kinase ATR. This leads to assembly of the FA core complex, consisting of FANC-A, B, C, E, F, G, L and M, at the damaged site (Wang, 2007). Through the ligase activity of FANCL, the complex is responsible for the subsequent monoubiquitination of the ID-complex, which consists of FANCD2 and its recently identified paralog FANCI (Garcia-Higuera et al., 2001; Smogorzewska et al., 2007). Each of the eight components of the core complex is required for monoubiquitination, and FANCD2 and FANCI monoubiquitination are mutually interdependent (Smogorzewska et al., 2007). This modification leads to relocalization of FANCD2 and FANCI into nuclear foci that colocalize with the breast cancer protein BRCA1 (Garcia-Higuera et al., 2001). The mechanism through which the ID-complex subsequently initiates repair remains largely unknown, but it was recently shown that in *Xenopus* egg extracts, both incision and translesion synthesis at the crosslink depend on ID-complex ubiquitination and localization (Knipscheer et al., 2009).

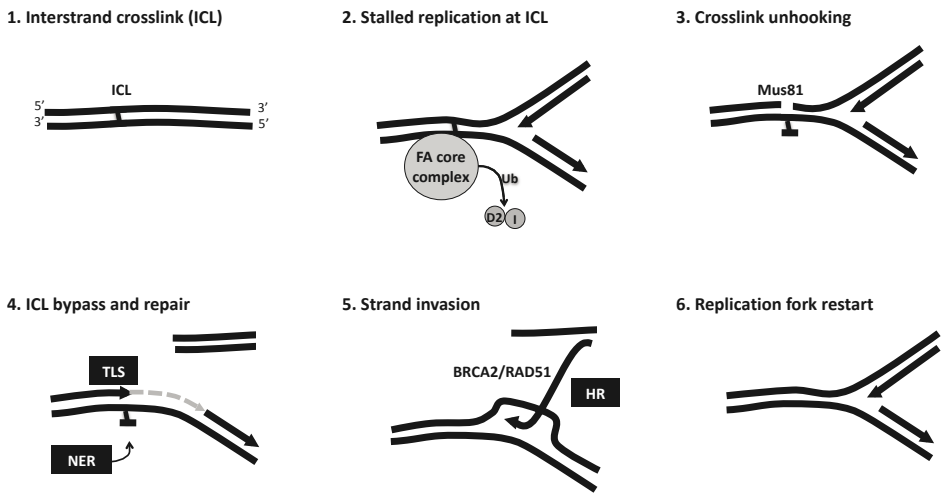

Figure 3 | Mechanism of crosslink resolvation by the Fanconi Anemia pathway. ICL, interstrand crosslink; TLS, translesion synthesis; NER, nucleotide excision repair; HR, homologous recombination. Several key genes in the processing steps are indicated. See text for details.

The endonuclease Mus81-Eme1 generates an initial incision at the crosslink that creates DSB (Hanada et al., 2006) (Figure 3). Studies in Xenopus egg extracts have shown that a nucleotide is then first inserted opposite the crosslink, followed by polymerase ζ-mediated extension (Räschle et al., 2008; Knipscheer et al., 2009). Next, NER is recruited for excision - likely by XPF-Ercc1 -and repair of the crosslink. However, the exact role of XPF and the possible involvement of other nucleases such as SLX1 are not entirely clear (Andersen et al., 2009; Fekairi et al., 2009; Muñoz et al., 2009; Svendsen et a;., 2009). Unhooking of the crosslinking by Mus81 results in a one-ended DSB, which requires HR for repair and replication fork restart (Hanada et al., 2006; Niedernhofer et al., 2005) (Figure 3). The last three FA genes (FANCD1, N and J, also called the BRCA-type FA proteins) are thought to have a role in this process. In contrast to the FA core complex, they are not required for monoubiquitination of the ID-complex (Garcia-Higuera et al., 2001; Litman et al., 2005; Reid et al., 2007), and their localization to a crosslink depends solely on DNA replication, not on core complex components or on monoubiquitination (Shen et al., 2009). This suggest that they function downstream of FANCD2 and that they may have additional or parallel functions that are unrelated to the FA-pathway. Indeed, FANCD1, N and J are all strongly related to the HR pathway that resolves DSBs: FANCJ is a binding partner for BRCA1 (Levitus et al., 2005; Levran et al., 2005; Litman et al., 2005), FANCD1 is allelic to BRCA2 (Howlett et al., 2002) and FANCN is an essential binding partner of BRCA2 (Rahman et al., 2007; Reid et al., 2007). Because of this strong relation with HR, this suggests that they are required for recombinatorial repair of the DSB that follows crosslink unhooking through Rad51-mediated invasion of the lagging strand (Figure 3).

G4 DNA instability

The recent identification of FANCJ as BACH1/BRIP1, a BRCA1 interacting protein underscored the strong intertwining of the FA and HR pathways (Levitus et al., 2005; Levran et al., 2005; Litman et al., 2005). BRCA1 had long been recognized to be intimately related to the FA-pathway, but until the finding that it binds directly to an FA-member, the functional relationship had remained unclear (Garcia-Higuera et al., 2001). Interestingly, studies in *C. elegans* have revealed that the highly conserved FANCJ orthologue, *dog-1*, is also required for resolving a very different type of lesion that occurs endogenously in the genome; G4 DNA, also called quadruplex or tetraplex DNA. G4 DNA is a highly stable secondary structure that can be formed by certain G-rich DNA sequences through Hoogsteen bonding of guanines in planar arrays (Gellert et al., 1962; Sen and Gilbert, 1988) (Figure 4A). A role for DOG-1 in the stability of G4 DNA first became apparent when a mutant strain of *C. elegans* was isolated in which frequent spontaneous deletions were observed, which all started at similar positions near 3'-ends of homoguanine stretches and ended at various positions upstream (Cheung et al., 2002). This unusual mutant activity was mapped to the DNA helicase *dog-1*, for deletions of guanines, the orthologue of BACH1, which was later shown to be allelic with FANCJ. It was suggested that homoguanine stretches can form

G4 DNA *in vivo*, posing blocks to the replication fork that require DOG-1 helicase activity to proceed (Figure 4B and C). The inability to solve this blocked fork in the absence of DOG-1 may result in joining of the stalled fragment to the next Okazaki fragment, which would explain both the orientation and length of the deletions in *dog-1* mutants (Figure 4D); deletions were always initiated near the 3' side of the G-tract, the location where lagging strand synthesis stalls, while the location of the upstream endpoint was highly variable, possibly reflecting the starting point of the next Okazaki fragment (Cheung et al., 2002; Kruisselbrink et al., 2008).

Further studies in our laboratory using unbiased array comparative genome hybridization (aCGH) analysis, subsequently revealed that not only homoguanine stretches, but all and only sequences that comply to the G4 signature $G_{3-5}N_{1-3}G_{3-5}N_{1-3}G_{3-5}N_{1-3}G_{3-5}$ are instable in *dog-1* mutants, albeit with varying frequencies (Kruisselbrink et al., 2008). These sequences all have the potential to form a quadruplex structure, strengthening the hypothesis that these structures can form *in vivo*. Interestingly, no other genes have been identified to date that show this G4 instability phenotype, including the conserved FA-genes in *C. elegans*, FANCD2 and BRCA2, and many known repair mutants in HR, mismatch repair and checkpoint signaling. An effort to identify *dog-1*-like genes through a forward genetic screen resulted in the isolation of five novel alleles, which all turned out to be mutated in *dog-1* (Kruisselbrink et al., 2008). Whereas the possibility remains that other, not yet identified proteins cooperate with DOG-1 to maintain G4 DNA stability, this suggests that this role for DOG-1 is unique amongst FA-proteins.

How DOG-1 exactly maintains G4 DNA is not entirely clear, although direct unwinding of G4 DNA by the helicase activity of DOG-1 seems the most plausible model. Indeed, FANCJ can unwind G4 DNA *in vitro* (London et al., 2008; Wu et al., 2008). However, FANCJ action is likely not limited to G4 DNA, because it interacts with a wide range

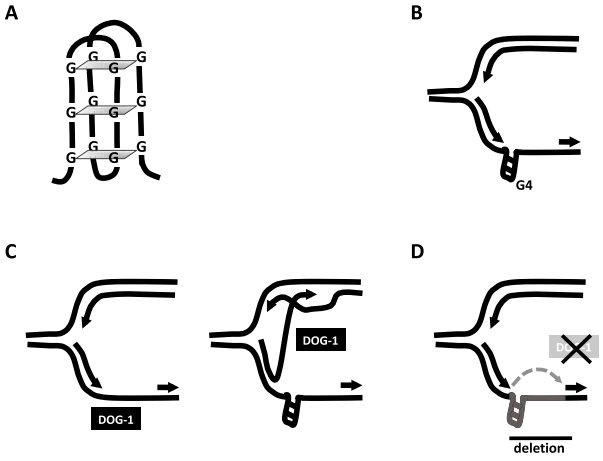

Figure 4 | G4 DNA formation and resolution. (A) Model of G4 DNA structure: ssDNA folding back on itself in planar arrays that each contain four guanines. **(B)** Model for stalling of lagging strand synthesis at G4 DNA. **(C)** Models of action for DOG-1: DOG-1 may either unwind G4 DNA (left), or mediate lesion bypass via the sister chromatid (right). **(D)** Model for deletion formation in *dog-1* mutants: deletions are thought to occur by joining of the stalled fragment to the next Okazaki fragment.

of DNA repair proteins *in vitro*. Besides BRCA1, these include MLH1, a protein that repairs mismatches (Peng et al., 2007), the ssDNA-binding protein RPA (Gupta et al., 2007; Suhasini et al., 2009) and TOPBP1, a topoisomerase involved in untangling supercoiled DNA (Greenberg et al., 2006). It has therefore been proposed that FANCJ may have a more general role, independent from the FA-pathway, in clearing the DNA of any secondary structures and proteins that may impede replication progression (Wang, 2007). However, some inconsistencies remain that may reflect an alternative mechanism for *dog-1*. First, similar to other FA-pathway mutants, *dog-1* mutants are sensitive to ICLs, and these can not be unwound (Youds et al., 2008). This would require a different role of DOG-1 in the repair of G4 DNA vs. ICLs. Moreover, other helicases that can unwind G4 DNA, such as BLM (Sun et al., 1998), do not cause a *dog-1* deletion phenotype (Kruisselbrink et al., 2008). Finally, DOG-1 possesses D-loop resolvation activity *in vitro* (Ali et al., 2009). An alternative mechanism that incorporates these properties and supports a similar mechanism of action for DOG-1 in the response to both types of lesions, would be DOG-1-mediated lesion bypass through strand invasion of the leading strand (Figure 4C, right).

The deletions that are formed in the absence of DOG-1 are thought to represent ligation of the stalled fork to the next Okazaki fragment (Figure 4D). Despite the testing of many candidates, no genes have yet been identified that are responsible for deletion formation (Kruisselbrink et al., 2008). These genes include NHEJ genes Ligase IV and Ku80, and MLH1, which interacts with FANCJ on the protein level (Peng et al., 2007). The highest deletion frequency observed for any G4 sequence was 4% per generation. If DOG-1 was required for G4 DNA unwinding, and if G4 DNA formation was very efficient, all G4 DNA would be deleted in these mutants. The fact that many G4 sites remain suggests that their formation is rather infrequent, or other mechanisms can bypass the lesion in a *dog-1*-independent manner. Although no genes have thus been identified that diminish the number of deletions in *dog-1*-mutants, mutations in HR genes slightly enhance the number of deletions. This suggests that in the absence of *dog-1*, HR is required for the error-free bypass of some G4 sequences (Youds et al., 2006).

In **Chapter 5** of this thesis, we address these issues by presenting a detailed analysis of the genetic interactants of *dog-1*, and by investigating the mechanism behind deletion formation in *dog-1* mutants. In **Chapter 6**, we show how G4 instability can be used as a tool to identify novel deletion alleles of many *C. elegans* genes.

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CHAPTER 2

A ROBUST NETWORK OF DOUBLE-STRAND BREAK REPAIR PATHWAYS GOVERNS GENOME INTEGRITY DURING *C. ELEGANS* DEVELOPMENT

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SUMMARY

To preserve genomic integrity, various mechanisms have evolved to repair DNA double-strand breaks (DSBs) (Wyman and Kanaar, 2006). Depending on cell type or cell cycle phase, DSBs can be repaired error-free, by homologous recombination (HR), or with concomitant loss of sequence information, via non-homologous end-joining (NHEJ) or single-strand annealing (SSA) (Pâques and Haber, 1999). Here, we created a transgenic reporter system in *C. elegans* to investigate the relative contribution of these pathways in somatic cells during animal development. Although all three canonical pathways contribute to repair in the soma, in their combined absence, animals develop without growth delay and breaks are still efficiently repaired, with a strong preference for homology. This residual repair, which we call alternative end-joining, dominates DSB repair only in the absence of NHEJ and resembles SSA but acts independently of the SSA nuclease XPF and repair proteins from other pathways. The dynamic interplay between repair pathways may be developmentally regulated, as it was lost from terminally differentiated cells in adult animals. Our results demonstrate profound versatility in DSB repair pathways for somatic cells of *C. elegans*, which are thus extremely fit to deal with chromosomal breaks.

RESULTS AND DISCUSSION

The NHEJ pathway is mechanistically conserved in *C. elegans*

To study DSB repair at the molecular level in *C. elegans*, we designed a transgenic assay in which DSBs can be introduced at a known location in the genome by a heat shock-inducible I-SceI transgene. The I-SceI restriction enzyme recognizes an 18-nt target sequence, which we included in a second transgene, the reporter, since this sequence does not occur in the *C. elegans* genome (Figure 1A). NHEJ is thought to be the main repair route in non-cycling somatic cells (Clejan et al., 2006; Rothkamm et al., 2003; Saleh-Gohari and Helleday, 2004) and often repairs DSBs inaccurately, leading to deletions. To detect whether this also occurs in *C. elegans* somatic cells, we induced I-SceI expression at the first larval (L1) stage and allowed two days for repair and growth, so that by the time of analysis, animals had grown into young adults. We then performed PCRs on genomic DNA from single animals using primers in the region flanking the I-SceI target site. Indeed, deletions of various sizes were observed within individual animals, indicative of frequent error-prone repair. Importantly, no deletions were observed in the absence of I-SceI induction (Figure 1B, top left panel). Sequence analysis of gel-purified deletion products showed that 62% of the deletions were the result of blunt repair events, 24% were generated through micro-homologies smaller than 5 bp, and 14% were insertions of 2-43 nt (Figure 2). It must be noted that small deletions or error-free repair (such as direct religation of the compatible ends that arise after I-SceI cutting) are not detected in this assay as they are not resolved by PCR.

To investigate the genetic requirements for deletion formation, we crossed in a null allele of the Ligase IV worm orthologue, *lig-4*, an important NHEJ factor in other organisms (Rathmell and Chu, 1994; Wilson and Lieber, 1997) as well as in *C. elegans* (Clejan et al., 2006; Morton et al., 2006). A profound reduction in the number of deletions was observed (Figure 1B, bottom left panel; Table S1). In agreement with other studies, some deletions were still formed in a LIG-4 independent manner (Schulte-Uentrop et al., 2008; Wang et al., 2003; Yan et al., 2007). Interestingly, the deletion products in NHEJ mutants were different from wild type on a molecular level; in *lig-4* mutants, deletion products were frequently characterized by long stretches of homology of up to 14 bp (Figure 2; Table S2). Homologies larger than 14 bp were not present in this amplicon, and deletions involving homology of more than 4 bp were never observed in wild type background. Similar results were obtained in *C. elegans* Ku80 (*cku-80*) mutants, which are also defective for classic NHEJ (data not shown) (Clejan et al., 2006). These results show that classic NHEJ is the main cause of large deletions in the reporter following a DSB in *C. elegans* somatic cells, and in its absence, another error-prone pathway that preferentially uses homology larger than 4 bp becomes apparent.

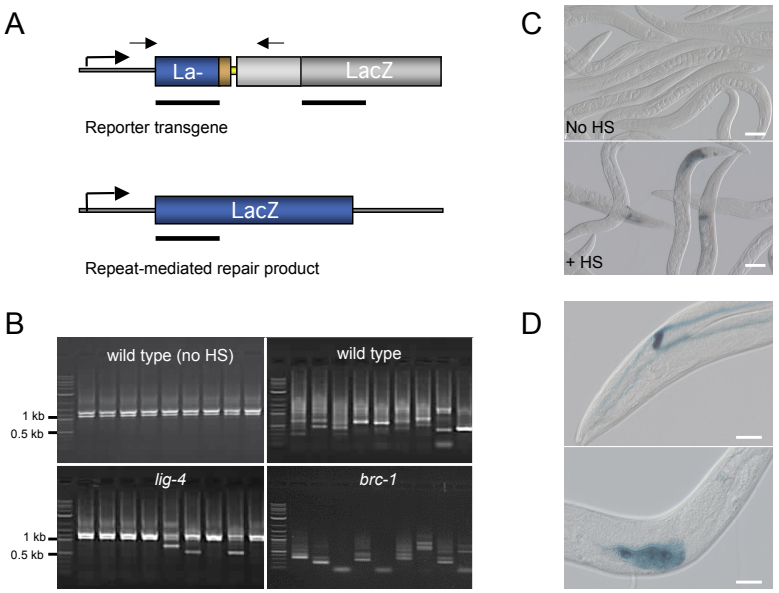

Figure 1 | Transgenic reporter system for I-SceI-induced DSBs. (A) Schematic representation of the DSB reporter transgene. Blue; in frame sequence. Grey; out-of-frame sequence. Orange; stop codons. Yellow; 18-nt I-SceI recognition site. Black lines represent homologous sequences. Arrows indicate primers used to amplify deletions (Figure 1B). **(B)** PCRs on DNA from single worms (consisting of 959 somatic cells) with the primers indicated in Figure 1A to detect deletions at the I-SceI site in the reporter transgene. "Wild type" in this figure refers to animals that carry both transgenes in otherwise wild type background. *Lig-4* and *brc-1* mutants have both transgenes in addition to a null mutation in the indicated gene. Top left panel; control on non-heatshocked animals. Top right panel; PCRs on heatshocked animals with both transgenes in otherwise wild type background. Bottom panels: deletions in *lig-4* and *brc-1* mutants (see also Table S1). A full-length band will always be detected in the absence of deletions and can be derived from uncut DNA, from germline tissue (which is mostly insensitive to heat shock treatment) or from error-free repair. **(C)** Representative images showing that ORF correction of the LacZ transgene depends on I-SceI expression. HS; heat shock. Bars 200 mm. **(D)** Examples of ORF correction in the reporter transgene in different cell types; H-shaped canal (excretory) cell (upper panel) and intestinal cells (lower panel). Bars 50 mm.

Error-prone homology-based DSB repair is frequent in *C. elegans* somatic cells

Two error-prone homology-based pathways have been described; alternative NHEJ (Corneo et al., 2007; Yan et al., 2007) and single-strand annealing (SSA) (Fishman-Lobell et al., 1992; Maryon and Carroll, 1991). Although both make use of homology in the flanks of the DSB and therefore seem very similar, there is evidence that they are different on a mechanistic level (Bennardo et al., 2008). However, no genes specific for the alternative NHEJ pathway have been identified and the pathway has not been well-defined. To further investigate these two forms of homology-driven repair we studied repair mediated by 251-nt repetitive LacZ-sequences that we placed in the flanks of the I-SceI site (Figure 1A). Only cells that repair the DSB using these repeats will delete the intervening sequence and restore a functional LacZ-ORF, resulting in beta-galactosidase (β-gal) expression. β-gal expression is thus a measure for homology-based

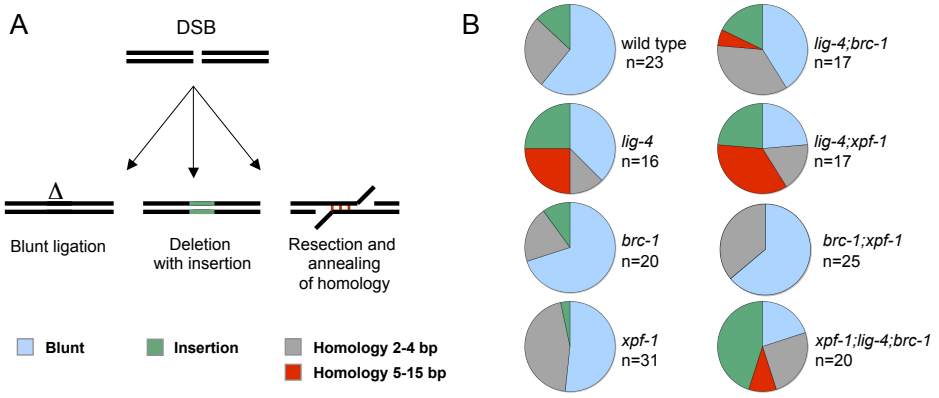

Figure 2 | Repair footprints of DSB-induced deletions. (A) Model explaining how the different types of deletions can be formed. Deletions that occur through blunt ligation do not make use of homology in region flanking the DSB. Deletions were sometimes accopanied by insertions that ranged in size from 1-41 nt. Insertions included duplications, insertion of transgenic, genomic, or seemingly random sequences. Repair using homology was divided in two classes; one class of 2-4 nt was frequently observed in wild type background, whereas the class of 5-15 nt was only observed in mutants that were *lig-4* (or *cku-80*, not shown) defective. **(B)** Deletion products as shown in Figure 1B were gel purified and sequenced. Deletion footprints for different mutants were attributed to one of the classes shown in the left panel.

repair by SSA, and potentially also for alternative NHEJ, depending on the window of homology that can be used by the latter pathway. Interestingly, we observed ample ORF correction after I-SceI induction in wild type background (Figure 1C). Staining was observed in many different cell types, for example intestinal and excretory cells (Figure 1D). This shows that in *C. elegans* somatic cells, besides NHEJ, error-prone, homology-based repair is common if homologous sequences are present at both sides of the break.

The PCR analysis of *lig-4* mutants had revealed a shift towards repair using homology (Figure 2). Simultaneously, we also observed greatly increased frequency of LacZ ORF correction in *lig-4* mutants (Figure 3A). β-gal expression was quantified by a biochemical approach where we measured the amount of enzymatic β-gal activity in a sample (see Supplemental Experimental Procedures for details), revealing a 3.1-fold increase in reporter expression in *lig-4* mutants versus wild type background (Figure 3B). This supports the previous conclusion that in the absence of canonical NHEJ, DSBs are re-routed to another repair mode that is characterized by the use of larger stretches of sequence homology than in wild type background.

HR is active in *C. elegans* somatic cells

We next investigated the contribution of HR to DSB repair. Even though the sister chromatid has been described to be the predominant template for repair in mitotic cells (Pâques and Haber, 1999), the homologous chromosome may also be used (Rong and Golic, 2003). To investigate this in *C. elegans*, we first prevented HR between homologous chromosomes by using males, which have only a single copy of the

X-chromosome on which the reporter transgene is located, but found no effect on ORF restoration (data not shown). However, in animals with a null mutation in *brc-1* - the *C. elegans* BRCA1 orthologue, which is required for HR between sister chromatids in meiotic cells (Adamo et al., 2008) - we found an almost 2-fold increase in β-gal expression (Figure 3B), suggesting that *brc-1* is also required for recombination between sister chromatids in mitotic cells of *C. elegans*. Also, RNAi against RAD-51, a recombinase essential for all types of HR (Wyman and Kanaar, 2006), resulted in greatly increased ORF restoration (Figure S1A). These results are in agreement with previous studies showing increased SSA in HR-deficient cells (Stark et al., 2004) and show that ORF restoration is not caused by HR, and that HR between sister chromatids is a common repair mechanism in somatic cells. When HR is unavailable, other types of homology-mediated repair are utilized.

The ssDNA-stabilizing protein complex RPA binds resected DNA and has been described to be necessary for both HR and SSA (Fanning et al., 2006; Umezu et al., 1998). Upon partial depletion of one RPA subunit (*rpa-2*, M04F3.1) by RNAi, we observed a clear and reproducible decrease in ORF restoration compared to control RNAi (Figure S1A). Since we showed that HR is not responsible for ORF correction, these data support a role for RPA in ORF restoration through SSA.

XPF-independent homology-mediated repair in NHEJ-deficient animals

We next investigated the genetic requirements for homology-driven repair. Homology of varying lengths in the flanks of a DSB can be a substrate for SSA (Sugawara et al., 2000). Annealing of these homologies causes 3'-overhanging flaps that need to be processed. The XPF/ERCC1 endonuclease has been described to remove 3'-flaps *in vitro*, and MUS81/EME1 has similar substrate specificity (Ciccia et al., 2008). SSA has been shown to be reduced in Ercc-1 deficient mammalian cells (Al-Minawi et al., 2008; Stark et al., 2004) and 3'-flaps persist in mutants of Rad1, the yeast XPF homolog (Li et al., 2008). We crossed in an *xpf-1* null allele, and observed a 7-fold reduction in the amount of LacZ-expression (Figure 3A and B), but β-gal expression was not completely abolished. We found similar results for a mutation in the binding partner of XPF, ERCC-1 (Figure 3A and B), whereas a *mus-81* null allele had no effect on reporter expression (data not shown), further supporting a role for SSA in ORF restoration.

If increased ORF restoration in HR and NHEJ mutants is the result of enhanced SSA, this should then depend on XPF/ERCC1. To address this, we made double mutants of *xpf-1* with *brc-1* and *lig-4*. The increase seen in *brc-1* single mutants was completely abolished in *brc-1;xpf-1* double mutants (Figure 3), indicating that DSB repair is indeed re-routed to *xpf-1*-dependent SSA in HR deficient animals. Surprisingly, this was not the case for *lig-4* mutants: LacZ ORF correction was only slightly reduced in *lig-4;xpf-1* double mutants as compared to *lig-4* single mutants (Figure 3A and B). This suggests that the majority of β-gal restoring events in *lig-4* mutants does not occur by SSA; instead, the breaks that would otherwise be a substrate for NHEJ are

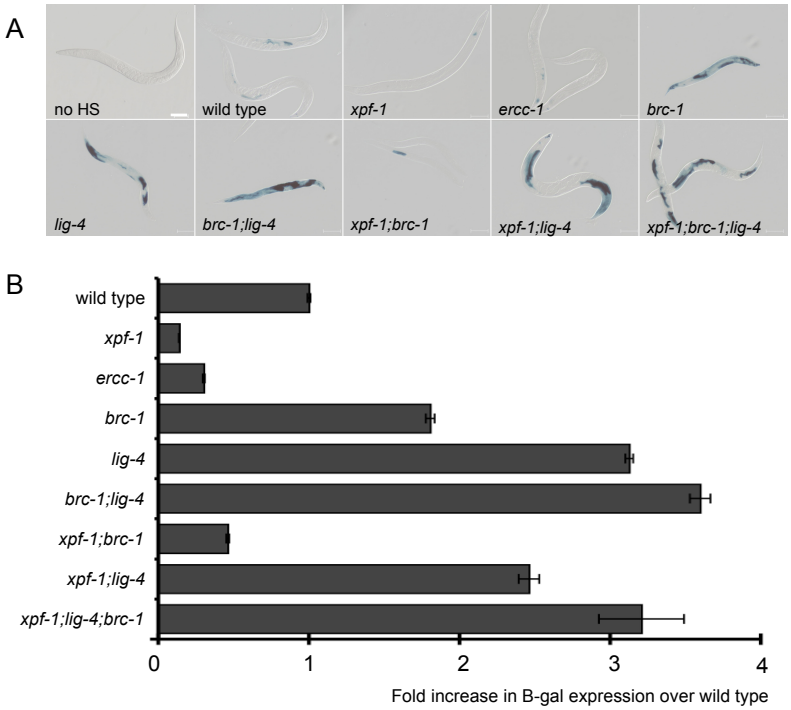

Figure 3 | Analyses of DSB repair pathway mutations. (**A**) Representative images of LacZ ORF restoration in transgenic animals visualized by blue staining with X-gal for indicated genetic mutants. Bars 200 mm. (**B**) Biochemical quantification of B-gal expression in I-SceI induced populations with indicated genetic backgrounds. Fold increase over wild type is shown. Error bars represent variation between 3 measurements. See Supplemental Experimental Procedures and Figure S3 for details.

repaired by another pathway capable of homology-mediated repair. In support of this, sequencing of deletion products revealed that the homology-based events of ≥ 5 bp were still found in the *lig-4;xpf-1* double mutant, so the formation of these also does not depend on *xpf-1*/SSA (Figure 2; Table S2). Moreover, while deletion products with ≥ 5 bp homology were observed in all backgrounds with defective NHEJ, we failed to observe these events in any of the NHEJ-proficient mutants (Figure 2). We conclude that in the absence of NHEJ, an SSA-like, but *xpf-1*-independent, pathway dominates the repair of DSBs. This pathway can lead to identical end products as SSA, but through a mechanistically different pathway. We refer to this pathway as alternative end-joining (alt-EJ).

Proficient repair and development in mutants defective for HR, NHEJ and SSA

To further validate the presence of an alternative DSB repair pathway in somatic cells, we created animals with combined mutations in components of SSA, NHEJ and HR. These *xpf-1;lig-4;brc-1* triple mutant animals were viable, and despite the severely

compromised ability to repair DSBs, induction of DSBs by I-SceI expression at the L1 stage did not affect the growth rate (Figure S2). We observed an increased level of LacZ ORF correction as compared to repair proficient wild type animals (Figure 3A and B). Only a very modest decrease in ORF restoration was observed in this triple mutant background compared to *brc-1;lig-4* mutant animals. Sequencing of deletion products revealed persistence of alt-EJ events of 5-14 bp in the triple mutants (Figure 2, Table S2). These data are in agreement with activation of *xpf-1*-dependent as well as *in*dependent pathways in *brc-1;lig-4* animals (Figure 3B). More importantly, this outcome also indicates that the pathway that is able to repair DSBs in the absence of HR, NHEJ and SSA, is sufficiently potent to repair a genomic break that occurs in many cells (many cells of the adult worm are positive for LacZ) to avoid developmental arrest.

Limited repair in non-dividing cells

Cell cycle stage plays an important role in the fate of a DSB (Barlow et al., 2008; Zierhut and Diffley, 2008). Whereas cells in G2 and S-phase have the possibility to use an homologous template for repair, cells in G1 do not generally have an homologous template within reach. In the experiments described above, DSBs were introduced in young L1 larvae and several days were allowed for repair, during which time cell divisions and endoreduplications occur. To study repair in non-replicating cells, we expressed I-SceI in young adults, where all somatic cells are post-mitotic. We quantified ORF correction by counting the number of positive cells per animal, assuming that each LacZ-expressing cell must be derived from an independent event. On average we observed only 0.4 blue patches per animal in a wild type background (Figure S1B) - a dramatic reduction compared to what was observed in developing animals, where multiple patches were observed in most animals. Interestingly, the number of blue patches was hardly different in *brc-1* or *lig-4* mutants (Figure S1B), thus a shift towards SSA or alt-EJ as observed in developing animals does not occur in non-cycling somatic cells, suggesting that these pathways require DNA replication and/or cell cycle progression.

In conclusion, our combination of transgenetics and conventional genetics reveals a highly dynamic and flexible response to DSBs, that changes at different developmental stages. Similarly, activity of HR and NHEJ has previously been shown to be differentially regulated in mouse development (Essers et al., 2000). It indicates that four pathways can interact on genomic breaks in somatic cells and that these pathways can functionally substitute for each other. These dynamics are especially underscored by the *xpf-1;lig-4;brc-1* triple mutants: because of their severely compromised repair abilities, we expected lethality or at least growth retardation when these mutants were subjected to DSBs. In contrast, we observed normal viability and abundant repair through alt-EJ. Perhaps, a dosage of one DSB per cell is insufficient to trigger cell cycle arrest or apoptotic response in *C. elegans*: in mammalian cells, a minimum of four DSBs per cell is required to trigger a detectable checkpoint response (Zierhut and Diffley, 2008), whereas in yeast, a single DSB is sufficient to cause lethality (Bennett et al., 1993).

Apparently, the alternative pathway is highly efficient to process a single DSB in *C. elegans*, since no growth retardation was observed and ORF correction was observed in many cells of the triple mutants. Altogether, all three canonical pathways contribute substantially to DSB repair during development, and a fourth pathway –alt-EJ- dominates repair if classic NHEJ is unavailable. Our data indicate that specifically during animal development, somatic cells are equipped with a tremendously robust network of repair pathways that can counteract the detrimental effects of DSBs.

MATERIALS AND METHODS

Constructs and transgenesis. Plasmid sequences are available upon request. The LacZ reporter transgene pkIs2170 [pRP1879: hsp-16.41::ATG-LacZ(first 251nt)-I-SceI site-stops-LacZ (complete); unc-119(+)] was integrated by micro particle bombardment. By Southern blotting, the copy number was determined to be three, present as a tandem array in one genomic locus on chromosome X. The I-SceI transgene pkIs2379 (pRP3001: hsp-16.41::I-SceI ORF) was injected into this strain and was integrated by irradiation at 40 Gy by a Cesium-137 source. The strain with both transgenes was subsequently outcrossed to wild type (N2) worms. To induce transgene expression, worms were incubated at 34°C for 1-3 hours. Because both transgenes are under the control of a heat shock promoter, we first tested whether the strain containing only the LacZ construct could express after heat shock independently of the I-SceI transgene, but this was not the case. In the presence of both transgenes, a single heat shock should not lead to LacZ expression, because the LacZ construct is not yet in frame. Indeed, no expression was observed when a single heat shock was applied to strains containing both transgenes. In most experiments, this first heat shock (to express I-SceI) was applied to young animals, and after allowing time for repair, the second heat shock (to express LacZ) was applied. Animals were washed and subsequently fixed or snap-frozen immediately after this second heat shock.

Strains and maintenance. General methods for culturing *C. elegans* strains were performed as described in (Brenner, 1974). The following alleles were crossed into the strain with pkIs2170 and pkIs2379: *lig-4* (ok716); *cku-80* (ok861); *xpf-1* (e1487); *brc-1* (tm1145); *mre-11* (ok179); F10G8.7 *ercc-1* (tm2073). Worms were grown at 20°C on OP50 or HT115 expressing the indicated RNAi clones from the Ahringer RNAi library as a food source, on 6 or 9 cm tissue culture dishes (Greiner Bio-One) with NGM.

X-gal staining procedure. To obtain a synchronized population of worms, eggs were obtained by bleach treatment. Eggs were allowed to hatch for 12-16 hours on OP50 and the population was then heat shocked to induce I-SceI expression. When fed on RNAi, bleached worms were allowed to hatch 12-16 hours in M9 buffer, put on RNAi food for 24 hours and then heat shocked for I-SceI induction. At the indicated repair time, B-gal expression was induced by heat shock treatment and expression was determined by fixation and staining in 5% X-gal or by an enzymatic B-gal activity assay (see below). We observed that the HT115 bacteria (used to express dsRNA for RNAi experiments), but not OP50, expressed B-gal in our X-gal staining procedure (see below), resulting in frequent background staining inside the intestine of HT115-fed worms. To ensure accurate quantification, we only used animals that were grown on OP50 for the biochemical quantification (see below). For animals fed on RNAi foods, B-gal expression was performed in a qualitative manner, since specific staining could easily be distinguished from bacterial background staining upon visual inspection.

Enzymatic assay for B-gal activity. We developed a quantitative biochemical method to determine and compare LacZ expression in *C. elegans*. This biochemical method allowed us to accurately measure the average ORF correction in a large population consisting of more than 200,000 animals. For this assay, worms were synchronized and heat shocked as above, but now worms were rinsed off the plates in M9 buffer directly after heat shock. Pellets were washed extensively in M9 and resuspended in IP-buffer (50 mM Hepes pH7.4; 1 mM EGTA; 1 mM $MgCl_2$; 100 mM KCl; 10% glycerol; 0.05% NP-40 and ½ tablet EDTA-free protein inhibitor (Roche)). Worms were then ground in liquid nitrogen and sonicated with a Gilson sonicator during 3 minutes where 10 sec of sonication was alternated by 30 sec cooling time. Cell debris was removed by two rounds of centrifugation and protein concentrations in the supernatant were determined using the Bradford method. We used B-Glo reagent (Promega, Madison USA), to convert B-gal enzymatic activity into luciferase activity, of which the activity can then be measured with a luminometer. Negligible levels of expression were observed in wild type (N2) worms or in non-induced controls (Figure S3A). We determined the optimal range of protein concentrations wherein the measurements followed a linear correlation (Figure S3B). 25 ml of B-Glo reagent was added to 10 or 100 ng of protein in a total volume of 50 ml, and this was done in triplicate. After 45 minutes incubation at room temperature, luciferase activity was measured.

Deletion analysis. Single worms were picked into lysis buffer (50 mM KCl; 2,5% mM $MgCl_2$; 10mM Tris-HCl pH8; 0.45% Nonidet P40; 0.45% Tween-20; 0.01% Gelatin; 200 mg/ml proteinase-K) and lysed for 60 minutes at 60°C. Proteinase K was inactivated for 15 minutes at 95°C and 1/10 of the lysis mixture was used for a nested PCR with primers in the flanks of the I-SceI site. Deletion products were excised from 1% agarose gels, purified over gel extraction columns (Qiagen) and sequenced.

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SUPPLEMENTAL FIGURES

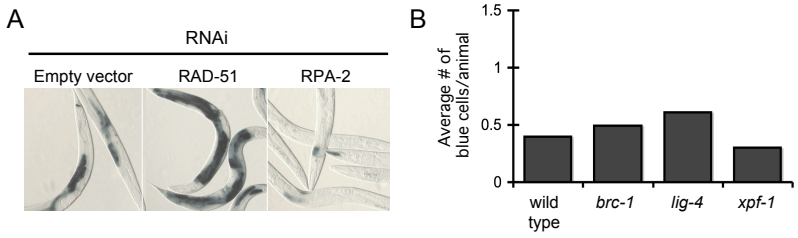

Figure S1 | (A) Representative images of animals fed on RNAi against the indicated gene. **(B)** Low numbers of blue patches per animal when I-SceI is expressed in adult animals devoid of dividing cells.

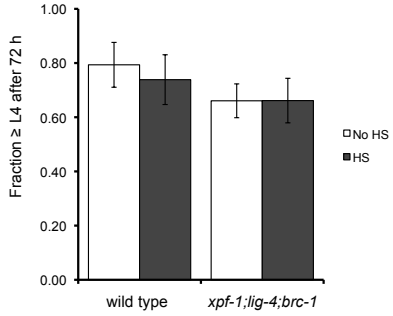

Figure S2 | Growth progression in triple mutants. Animal growth (measured as the number of animals that have reached the L4 larval stage after 72 hours) in wild type and triple mutant background is not affected by heat shock inducible I-SceI expression.

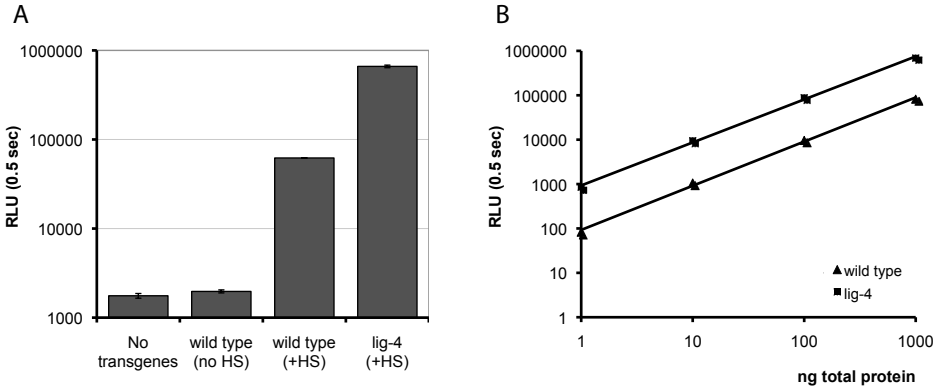

Figure S3 | Validation of quantitative luciferase assay to measure B-gal activity. (A) Graph representing relative B-gal expression of 1000 ng of the indicated strains on a logarithmic scale after subtraction of background levels. See Supplemental Experimental Procedures for details. HS; heat shock. "No transgenes" refers to the wild type N2 strain without transgenes. "Wild type no HS" is a non-heatshocked control that carries both the reporter and I-SceI transgenes. RLU in wild type is increased 35 times compared to the no transgene control, and 376 times in *lig-4*. RLU: Relative Light Units. **(B)** Graph showing the range of protein concentrations wherein measurements follow a linear pattern. Correlation coefficients are r^2(wild type) = 0.9983, r^2(*lig-4*) = 0.9988.

Table S1 | Number of DSB induced deletions

Genotype	% of animals with large deletions	n
wild type	100	86
xpf-1	93	96
brc-1	96	48
lig-4	39	77
brc-1;lig-4	37	126
xpf-1;brc-1	87	114
xpf-1;lig-4	39	41
xpf-1;lig-4;brc-1	47	102

Table S2 | Summary of alt-EJ specific deletion events

No	Homology used (bp)	Deletion size (bp) *	Sequence (homology indicated in bold, deleted sequence in grey)		Observed in
1	14	812	5' flank	cccaacagttgccaag**gtaagtttaaacag**atccatactaactaact	*lig-4* (1x)
			Deletion product	cccaacagttgccaag**gtaagtttaaacag**ttcggtactaactaacc	
			3' flank	cccaactacaagacac**gtaagtttaaacag**ttcggtactaactaacc	
2	13	1027	5' flank	cccaacagttgccaag**gtaagtttaaaca**gatccatactaactaact	*lig-4* (3x); *lig-4;xpf-1* (2x)
			Deletion product	cccaacagttgccaag**gtaagtttaaaca**tgattttactaactaact	*lig-4;brc-1* (1x); *cku-80* (3x)
			3' flank	gaatggaatcaaagtt**gtaagtttaaaca**tgattttactaactaact	*lig-4;brc-1;xpf-1* (1x)
3	12	1027	5' flank	agtttaaacagatcca**tactaactaact**tgttctgacataattttca	*lig-4;xpf-1* (3x)
			Deletion product	agtttaaacagatcca**tactaactaact**aatctgatttaaattttca	
			3' flank	agtttaaacatgattt**tactaactaact**aatctgatttaaattttca	
4	9	1027	5' flank	taacttgttctgacat**aattttcag**cttgaatggcgaatggcgcttt	*lig-4;xpf-1* (1x)
			Deletion product	taacttgttctgacat**aattttcag**aacttcaaaattagacacaaca	
			3' flank	taactaatctgattta**aattttcag**aacttcaaaattagacacaaca	
5	6	1173	5' flank	atgactgtcgttttac**ttttac**aacgtcgtgactgggaaaaccctgg	*lig-1;xpf-1;brc-1* (1x)
			Deletion product	atgactgtcgttttac**ttttac**taactaactaatctgatttaaattt	
			3' flank	gtaagtttaaacatga**ttttac**taactaactaatctgatttaaattt	
6	5	1026	5' flank	atactaactaacttgt**tctga**cataattttcagcttgaatggcgaat	*cku-80* (2x)
			Deletion product	atactaactaacttgt**tctga**tttaaattttcagaacttcaaaatta	
			3' flank	tttactaactaactaa**tctga**tttaaattttcagaacttcaaaatta	

* Several alt-EJ events (numbers 1-4) are derived from homologies in one of the 51-nt artificial introns that are present in the reporter transgene. These introns are similar but not 100% homologous, explaining why deletion products of the same size (1027 bp) can arise even when different stretches of homology are used.

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CHAPTER 3

ALTERNATIVE END-JOINING OF DOUBLE-STRAND BREAKS IS DEPENDENT ON DNA REPLICATION

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SUMMARY

Double strand breaks are highly toxic DNA lesions and besides three canonical pathways, an alternative end-joining (alt-EJ) pathway was recently described. In *C. elegans,* alt-EJ activity is limited in wild type cells, but becomes more prominent in NHEJ deficient cells. Whereas NHEJ can operate throughout the cell cycle, we show that alt-EJ activity is cell cycle dependent and only occurs in replicating cells. This suggests that resection is required for alt-EJ, but alt-EJ efficiency was not affected in mutants for the resection endonuclease *mre-11*. Despite efficient alt-EJ in replicating cells, almost all DSBs persist up to 24 hours in *lig-4* deficient larvae. *Pme-1*, the ortholog of PARP1, a protein previously implicated in alt-EJ, had no effect of alt-EJ in *C. elegans,* suggesting high redundancy among alt-EJ pathways.

INTRODUCTION

Double-strand breaks (DSBs) are highly toxic DNA lesions that can be repaired by a wide variety of DNA repair pathways. The lesion can be repaired in an error-free fashion by homologous recombination (HR) (West, 2003), or in an error-prone way, by non-homologous end-joining (NHEJ) or single-strand annealing (SSA) (Fishman-Lobell et al., 1992; Hefferin and Tomkinson, 2005). In the case of HR, the original sequence is restored at the break site by making use of the sister chromatid or the homologous chromosome as a template. NHEJ directly ligates the broken ends with little or no sequence homology, which frequently leads to small deletions or insertions at the site of the DSB. Finally, SSA combines features of both other pathways: SSA is an error-prone pathway that uses sequence homology within the same chromosome. For this, repetitive sequences are required at both sides of the DSB and SSA invariably leads to deletions of one of the repeats and the intervening sequence.

In canonical NHEJ, a heterodimer of the Ku70 and Ku80 proteins recognizes and binds broken DSB ends, protecting them from nucleolytic degradation (Rathmell and Chu, 1994; Roth et al., 1995). The Ku-complex then recruits the kinase DNA-PKcs (catalytic subunit) to form the DNA-PK complex, which is required for end bridging and subsequent ligation by Ligase IV and XRCC4 (Hefferin and Tomkinson, 2005). Even though NHEJ is generally sequence-independent, small sequence homologies in the flanks of the DSB can be used, which requires the activities of nucleases and polymerases for flap processing and gap filling, respectively (Ma et al., 2003). It has long been thought that NHEJ occurs predominantly in G1 cells, whereas HR would be the most prominent route in G2 and S phase because of the availability of an homologous template at these stages. However, it recently became clear that both the mechanism and the regulation of NHEJ are not as straightforward as previously recognized. First, several studies showed that the type of DSB – "dirty" DSBs introduced by ionizing radiation (IR) or "clean" endonuclease-induced DSBs – strongly influences which proteins are recruited to the DSB and which repair pathway will be used (Barlow et al., 2008). Secondly, another study found that NHEJ can be used throughout the cell cycle, being the predominant repair route even in G2 phase cells (Beucher et al., 2009). Finally, the recent identification of novel NHEJ factors such as Artemis (Moshous et al., 2001) and the observation of abundant end joining activity in the absence of canonical NHEJ components (Pontier and Tijsterman, 2009; Wang et al., 2003), show that the separation of function in DSB repair pathways is complicated and versatile.

Ku-independent end-joining has been observed in several studies and is frequently characterized by the use of microhomology at the break site (McVey and Lee, 2008). Nevertheless, the nature of this activity, which is often termed alternative-EJ (alt-EJ), remains elusive and ill-defined. For example, some studies report the observation of micro-homology mediated end-joining in wild type cells, and show that this occurs independently of Ku (Bennardo et al., 2008; Ma et al., 2003). In contrast, we and others have found a form of alt-EJ that only becomes apparent in the absence of classic NHEJ,

and that is almost undetectable in the wild type situation (Corneo et al., 2007; Pontier and Tijsterman, 2009; Yan et al., 2007). Also, the genetic requirements for this pathway are not yet clear. The homology usage suggests that resection occurs, but whereas some studies reported a role for the MRN complex (Mre11/Rad50/Nbs1) in micro-homology-mediated repair in various organisms (Delmas et al., 2009; Deriano et al., 2009; Ma et al., 2003), others reported that this complex may promote – but is not essential for – alt-EJ (Zhang and Paull, 2005). These differences may either depend on the experimental approach, such as the organism that is used for the study, the stage of the cell cycle at the time of detection, or on the mechanism by which DSBs are introduced, or they may reflect distinct alternatives to classic NHEJ.

The use of homology within the same DNA strand is also a characteristic of the SSA pathway. However, SSA generally uses longer homology of up to 1200 bp (Sugawara et al., 2000) and requires nuclease activity from the XPF/ERCC1 nuclease to remove overhanging flaps that arise after annealing of the homology (Al-Minawi et al., 2008). Some studies report alternative end-joining activity in yeast that was dependent on Rad1 (the ortholog of XPF) (Boulton and Jackson, 1996; Moore and Haber, 1996) and therefore reminiscent of SSA. We and others have shown that alt-EJ and SSA are genetically distinct in mammalian cells and in *C. elegans*, because alternative end-joining does not require the activity of XPF (Bennardo et al., 2008; Pontier and Tijsterman, 2009). We have previously proposed three characteristics to define the alt-EJ pathway in *C. elegans*: i) Independency from the classic NHEJ genes Ku and Ligase IV; ii) Frequent use of larger homologies than in NHEJ; iii) Distinct from XPF-dependent SSA.

Whereas NHEJ can repair DSBs throughout the cell cycle (Beucher et al., 2009), HR usually uses a sister chromatid for repair, which is only available in replicating cells. Moreover, non-replicating cells show limited SSA activity (Al-Minawi et al., 2008; Pontier and Tijsterman, 2009). NHEJ thus dominates DSB repair in non-replicating cells. Nevertheless, the homology-requirements of the alt-EJ pathway are not necessarily replication dependent: Homology-mediated repair by intramolecular recombination as in alt-EJ does not require an homologous chromosome nor a sister chromatid, and end resection has been shown to occur in non-replicating cells in yeast (Barlow et al., 2008).

In this study, we investigate the genetic and cell cycle requirements for alt-EJ in *C. elegans*. We find evidence of highly redundant end-joining activity: both SSA and alt-EJ do not require nuclease activity by MRE11 (*mre-11*) or PARP-1 (*pme-1*) activity. We employ several approaches to show that efficient repair by SSA as well as by alt-EJ is promoted by DNA replication. Remarkably, repair in replicating and non-replicating cells is largely dependent on *lig-4*, and DSBs persist in *lig-4* deficient animals during animal development. Together, these data show that alt-EJ can be separated from canonical NHEJ and requires replication.

RESULTS

All types of homology-mediated repair are replication-dependent

To compare repair in replicating versus non-replicating cells of *C. elegans*, we used a construct that expresses the rare-cutting yeast endonuclease I-SceI behind an inducible heat shock promoter, in combination with a reporter transgene that contains the 18-nt recognition site for this enzyme in front of an out-of-frame LacZ gene. The first 251 nt of the LacZ gene were also cloned upstream of the I-SceI site, so that homology-mediated repair driven by this repeat will result in a functional LacZ ORF (Figure 1A). We constructed the reporters behind the *elt-2* and the *myo-2* promoters, which are expressed in distinct tissues: *myo-2* expression is restricted to the pharynx, whereas *elt-2* is expressed only in the intestine (Figure 1B) (Fukushige et al., 1999; Okkema and Fire, 1994). These tissues are formed at different times in development: Cells in the pharynx have finished their divisions at the time of embryo hatching and the pharynx then contains 25 *myo-2* expressing cells (Albertson and Thomson, 1976). At the time of hatching, 20 intestinal cells are present, and this increases to 34 cells in adults through divisions at the transition from the first (L1) to the second (L2) larval stage. In addition to these cell divisions, the DNA content of intestinal cells doubles at each larval stage transition by endoreplication, so several DNA replication rounds occur in all intestinal cells between hatching and adulthood (Hedgecock and White, 1985; Sulston and Horvitz, 1977). Although potential differences in I-SceI expression do not allow for direct comparison between these two tissues, this system can be used to study repair independently in replicating and in non-replicating tissues, by modulating the timing of I-SceI induction and quantification of LacZ expression in the clearly distinguishable pharyngeal and intestinal tissues.

Populations of worms were synchronized and I-SceI expression was induced at various developmental time points: (i) young eggs: wherein both *elt-2* and *myo-2* expressing cells are replicating. (ii) L1 larvae: at this stage all *myo-2* expressing cells have finished their divisions, whereas *elt-2* expressing cells are still replicating and becoming polyploid: (iii) young adults (1 day post L4-stage): in these animals, both *elt-2* and *myo-2* expressing cells have stopped replicating. LacZ stainings were performed when the animals had grown into young adults, with a minimum of 24 hours between I-SceI expression and staining procedure to allow time for repair. We consistently found high levels of ORF restoration in cells that were replicating at the time of I-SceI induction, whereas non-replicating cells showed very low levels (Figure 1C): When I-SceI was expressed in eggs, when *myo-2* cells are replicating, nearly all pharynxes had blue cells, indicating that repair in the pharynx had taken place through homology-mediated repair. Similarly, when I-SceI was expressed in L1's, the replicating *elt-2* expressing cells showed abundant ORF restoration. In contrast, induction of I-SceI at this stage led to low levels of ORF restoration in *myo-2* expressing cells, which at this stage are terminally differentiated and do not proliferate. Finally, adults, where all cell types are

in a quiescent state, showed no ORF restoration in any tissues. Together, these data suggest that ORF restoration can only occur in cells that are able to replicate.

Alt-EJ is active when NHEJ is compromised

We have previously shown that ORF restoration can occur either by XPF-1 dependent SSA, or by XPF-1 independent alt-EJ (Pontier and Tijsterman, 2009). To determine the genetic requirements for ORF restoration in replicating and non-replicating cells, we expressed I-SceI in wild type and repair mutant L1 larvae, when *elt-2*-expressing cells are replicating and *myo-2* cells are not. We allowed several days for growth and repair and quantified ORF restoration in *elt-2* or in *myo-2* tissues when animals had grown into young adults.

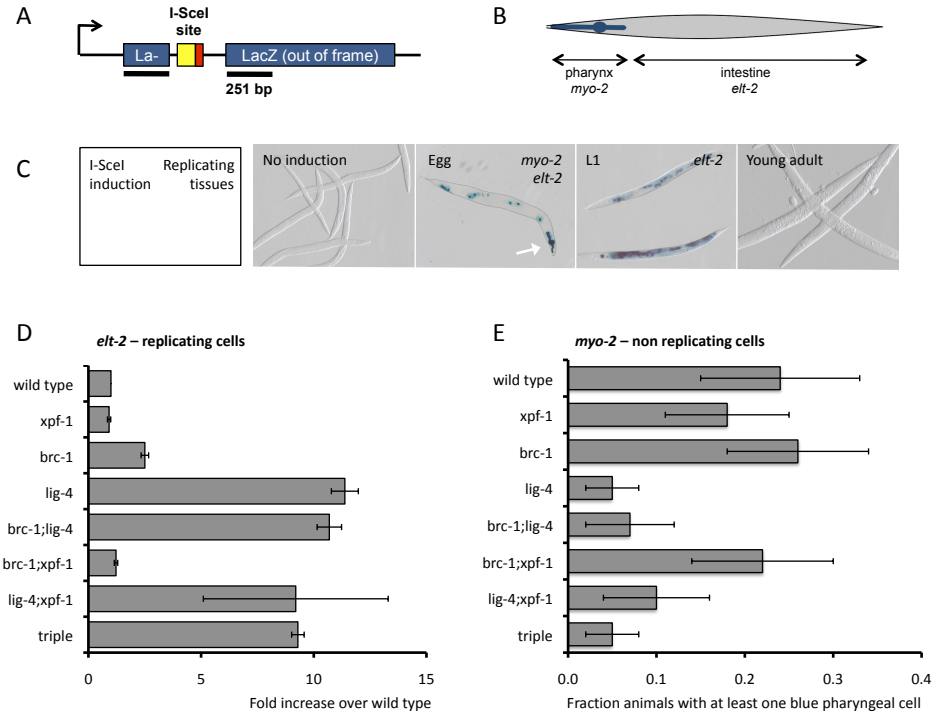

Figure 1 | Quantification of LacZ expression in candidate alt-EJ genes. (A) Schematic representation of the LacZ reporter transgene. Black bars indicate homologous sequences. Yellow; I-SceI recognition site. Red; stop codons. **(B)** Scheme showing regions of expression of the *myo-2* (pharynx) and *elt*-2 (intestine) promoters. **(C)** Representative images of LacZ ORF restoration in pharyngeal (arrows) and intestinal tissues after I-SceI induction at different developmental time points. Only tissues that were replicating at the time of I-SceI induction show ORF restoration. **(D)** Quantification of LacZ expression in cells that were replicating at the time of I-SceI induction (*elt-2* cells). Animals were heat shocked to induce I-SceI as L1 larvae and grown to adulthood before subjected to analysis. **(E)** Quantification of LacZ expression in cells that were not replicating at the time of I-SceI induction (*myo-2* cells). Experiment was performed in a similar way as for replicating cells in **(D)**.

Interestingly, both in replicating and non-replicating cells, ORF restoration was unaltered in *xpf-1* mutants (Figure 1D and E). This suggests that ORF restoration is the result of NHEJ or alt-EJ, not SSA. In *lig-4* mutants, ORF restoration in *elt-2* expressing replicating cells was increased. This was the result of elevated alt-EJ, because the increase was independent from *xpf-1* (Figure 1D). ORF restoration was also increased in replicating cells of mutants for the HR gene *brc-1*. Here, the increase was dependent on *xpf-1*, suggesting that HR deficiency leads to a switch to SSA, not alt-EJ. In conclusion, these data show that in replicating cells, alt-EJ is active when NHEJ activity is compromised. In replicating cells, NHEJ deficiency lead to increased alt-EJ, whereas HR deficiency causes a shift towards SSA.

Homology-mediated repair can not be induced in non-replicating cells

To see whether these findings were specific for replicating cells, we also quantified ORF restoration in non-replicating *myo-2* expressing cells. In wild type background, low numbers of ORF restoration events were observed (Figure 1E); positive animals often contained only a single blue cell. These events were not the result of SSA, because there was no change in *xpf-1* mutants. Strikingly, ORF restoration was reduced in *lig-4* mutants (Figure 1E). Thus, the infrequent ORF restoration in non-dividing cells depends largely on classic NHEJ, and mutation of classic NHEJ can not activate alt-EJ here. NHEJ causes frequent deletions in the reporter transgene that can be detected by PCR (Pontier and Tijsterman, 2009). To investigate how NHEJ can cause ORF restoration, we sequenced NHEJ-induced deletions. These sequences showed that some of the deletions are predicted to give rise to an in-frame and presumably functional ORF, even though substantial homology is not used for repair (data not shown). Classic NHEJ thus results in occasional ORF restoration by large in frame deletions that remove the stop codons that were placed up- and downstream of the I-SceI site.

Interestingly, the data for ORF restoration that we obtained in these non-replicating cells is an exact mirror-image of the data for replicating cells (Figure 1D and E), which emphasizes the clearly distinct repair characteristics of both cell types. In *myo-2* expressing cells, mutations in none of the genes tested, either in HR (*brc-1*), SSA (*xpf-1*) or NHEJ (*lig-4*) resulted in increased ORF restoration relative to the wild type, suggesting that a shift towards SSA or alt-EJ is absent in non-replicating cells. Remaining ORF restoration was mostly caused by classic NHEJ-induced deletions, as all mutants in *lig-4*, either single or in combination with other repair mutants, invariably showed the lowest levels of ORF restoration.

Homology-based repair in somatic cells is *mre-11* independent

For homologous repair by HR and SSA, the repeats present in the reporter first need to be resected to generate ssDNA, before annealing of homologous sequences can occur (Yang et al., 2006). Alt-EJ frequently uses homology, and combined with the replication-dependency, this suggests that resection may be required for alt-EJ. Mre11 is a resection endonuclease that is required for HR (Tsubouchi and Ogawa, 1998)

and for SSA (Yang et al., 2006), and a role in alternative end-joining has also been suggested (Delmas et al., 2009; Deriano et al., 2009; Ma et al., 2003). In *C. elegans*, the observation of increased defects in vulval development after ionizing radiation also suggest a somatic role in DSB repair for this nuclease (Weidhaas et al., 2006). *Mre-11* null mutants are sterile due to an essential role of this nuclease in repair of meiotic DSBs in the germline (Chin and Villeneuve, 2001). We obtained an *mre-11* allele that is viable in the first generation due to maternally contributed protein. In contrast to the increase observed for other HR genes like *rad-51* and *brc-1* (Pontier and Tijsterman, 2009), no change was observed in LacZ ORF restoration in *mre-11* mutants (Figure 3B). Similar results were obtained from the rare progeny of homozygous *mre-11* mothers, eliminating maternally contributed MRE-11 protein in the soma as a reason for the absence of an effect (results not shown). This shows that *mre-11* is not involved in HR or in SSA of an I-SceI-induced DSB in *C. elegans* somatic cells. We previously showed that increased ORF correction in *lig-4* mutants can be attributed to alt-EJ because it is largely independent from *xpf-1* (Pontier and Tijsterman, 2009). Using *mre-11;lig-4* double mutants, we found that the increase in *lig-4* mutants occurred independently of *mre-11* (Figure 3B). We thus conclude that in *C. elegans*, *mre-11* does not function in

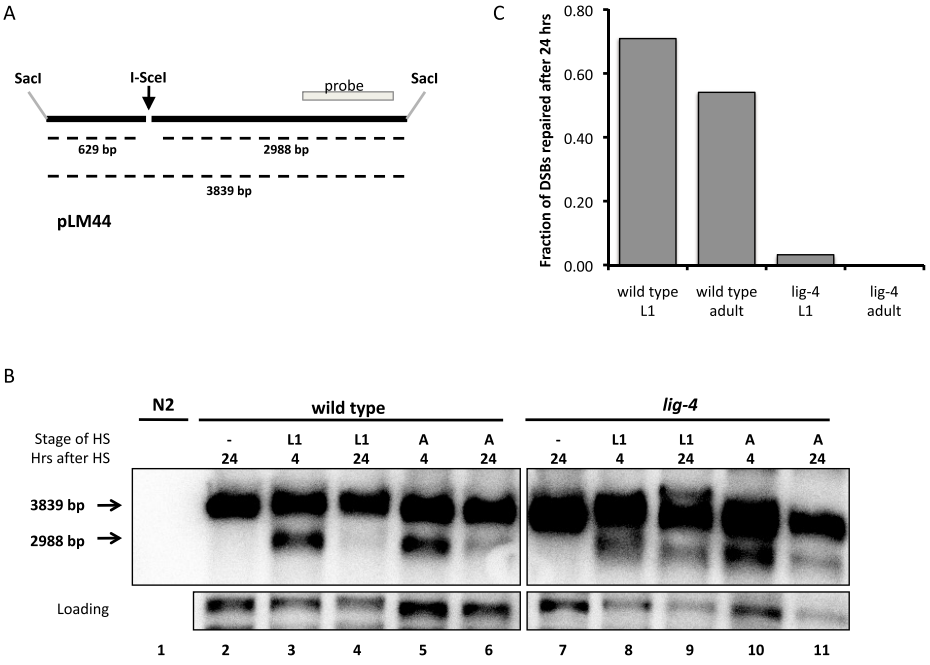

Figure 2 | DSBs persist in non-replicating cells. (A) Schematic representation of the reporter transgene after digestion with SacI. The complete fragment is 3839 bp, but after I-SceI digestion a fragment of 2988 bp will be detected with the probe indicated in the figure. **(B)** Southern blot of SacI-digested DNA. Animals were heat shocked to induce I-SceI expression at the indicated stages (L1, L1 larvae; A, adult), and DNA was isolated 4 or 24 hours later. **(C)** Quantification of repair of DSB product; relative amount of DSB product at 24 hours compared with relative amount at 4 hours.

repair of the DSB-reporter in the soma, and that it is dispensable for homology-based repair of the LacZ-reporter by SSA and alt-EJ.

DSBs persist in somatic cells in adults

Next, we investigated the efficiency of alt-EJ. Our previous data had shown that growth was not impaired even in mutants that were defective for HR, SSA and NHEJ (Pontier and Tijsterman, 2009). This suggests either highly efficient alt-EJ or tolerance to DSBs. We induced I-SceI in synchronized populations of larvae at the first larval stage (L1) or in terminally differentiated young adults, and let these grow on food for 4 or 24 hours prior to DNA isolation. During this 24 hour time interval, several cells of the L1 but not the adult population go through replication rounds. DNA from these worms was then digested, separated on agarose gels and subjected to Southern blotting. We used a probe that hybridizes to the I-SceI-digested fragment of 2988 bp as well as to the intact fragment of 3839 bp of the reporter transgene (Figure 2A). Importantly, no signal was observed in wild type (N2) animals without a transgene, and no DSB product was observed in the absence of I-SceI induction (Figure 2B, lanes 1 and 2, respectively).

Four hours after DSB induction, we observed very efficient *in vivo* digestion of the reporter transgene by I-SceI (Figure 2B, lane 3). Intact reporter transgene is expected to remain at any time point because of incomplete efficiency of I-SceI, repair, and from tissues that are relatively resistant to heatshock treatment, such as the germline. After 24 hours of growth on food, approximately 70% of DSBs had been repaired (Figure 2B, lane 4 and Figure 2C). L1 larvae consist of 558 cells at the time of I-SceI induction, and only several of these cells, most notably the intestinal cells, will undergo DNA replication during the 24-hour time interval. Therefore, these data indicate that repair occurs in developing animals within 24 hours.

In adults, ~50% of the DSBs were repaired after 24 hours (Figure 2B, lanes 5 and 6 and Figure 2C). We could not determine whether the remaining DSBs were repaired at later time points, because DNA from embryos starting to develop within the adults would dilute I-SceI exposed genomes. The 50% residual repair was completely dependent on

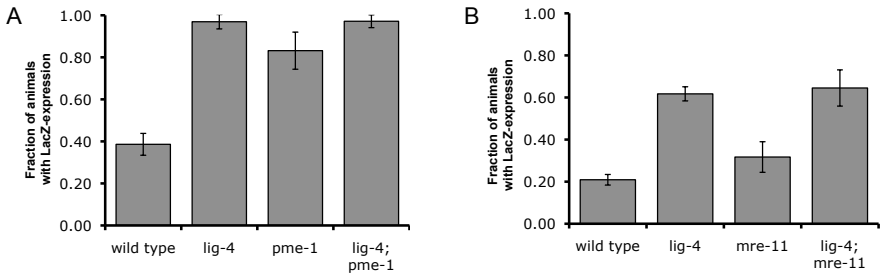

Figure 3 | LacZ ORF restoration in mutants in candidate alt-EJ genes. (A) Quantification of LacZ-expression in *pme-1*, *lig-4* and *pme-1;lig-4* double mutants. **(B)** Quantification of LacZ-expression in *mre-11*, *lig-4* and *mre-11;lig-4* double mutants.

classic NHEJ: Repair in *lig-4* mutant adults dropped to undetectable levels (Figure 2B, lanes 10 and 11, Figure 2C). These data show a dominant role for NHEJ at the adult stage, and point towards NHEJ as the only available route for repair in non-replicating adult cells. Strikingly, after 24 hours, repair was also very limited in L1 *lig-4* mutants (Figure 2C), despite proficient homology-mediated repair that we observed in LacZ stained animals (Figure 2D). These unrepaired DSBs did not cause a delay in growth: While *lig-4* mutants have a slightly slower overall growth rate, we verified that the progression from the L1 to the L2 stage within 24 hours was not impaired (data not shown). Altogether, these data show that NHEJ is the predominant repair pathway in L1 and in adult animals. Also, they suggest extreme tolerance to high levels of DSBs and place growth progression above the necessity to repair DSBs.

Alt-EJ is independent of *C. elegans* PARP1 (*pme-1*)

PARP1, a protein involved in base excision repair (BER) and single-strand break (SSB) repair, has previously been implicated in Ku-independent end-joining pathways in other organisms: Rodent cells pretreated with PARP1 inhibitors showed reduced Ku-independent DSB rejoining (Audebert et al., 2004), and others showed that PARP-1 and Ku compete for binding to DSB ends in mammalian cells (Wang et al., 2006). This led us to ask whether the *C. elegans* PARP1 ortholog, *pme-1* (Gagnon et al., 2002), is involved in alt-EJ. Similar to HR and NHEJ mutants, we found that *pme-1* mutants displayed elevated ORF correction when assayed in replicating cells as compared with *pme-1* proficient cells (Figure 3A). As discussed above, increased expression can be caused by a shift towards repair by SSA or alt-EJ. If *pme-1* functions in alt-EJ, *lig-4;pme-1* double mutants should have reduced ORF restoration compared with *lig-4* or *pme-1* single mutants. However, similar to *lig-4* single mutants, *lig-4;pme-1* double mutants showed ORF restoration in all animals (Figure 3A), suggesting that *pme-1* does not function in alt-EJ. We previously showed that besides increased LacZ ORF restoration, a 13-bp repeat that is present at both sides of the I-SceI site is also used more frequently for repair (25 and 47% of the deletions in *lig-4* and *cku-80* mutants, respectively) (Pontier and Tijsterman, 2009). We sequenced deletion products from *pme-1* mutant animals to see whether these alt-EJ-specific repair products also occurred here, but found no such events (n=13). In addition, sequencing of deletion products showed persistence of alt-EJ events in *lig-4;pme-1* double mutants in 3 out of 6 sequenced deletions. This shows that although *pme-1* and *lig-4* deficiency both cause increased ORF restoration, they do so by different mechanisms: NHEJ deficiency triggers alt-EJ, whereas *pme-1* deficiency does not.

DISCUSSION

DSBs can lead to deletions, translocations or other DNA rearrangements. Upon DNA breakage, proteins are recruited to the break site. Which proteins bind and how the break is being processed depends on the stage of the cell cycle at which the

DSB occurred. One way of processing is end resection, which is counteracted by the protective binding of Ku during the G1 phase of the cell cycle. Resected ends can be processed by HR, SSA and presumably also by alt-EJ in replicating cells, but the latter two pathways are intrinsically error-prone. NHEJ joins ends without resection or extensive sequence homology, but in its absence, DSBs persist until they can be processed by one of the resection-dependent pathways.

Several studies have reported alt-EJ activity in the absence of classic NHEJ. Despite its role as a "backup" for NHEJ – which can operate throughout the cell cycle – we show here that alt-EJ activity is limited to replicating cells. Moreover, our data suggest that alt-EJ is not restricted to NHEJ-deficient cells, but may also be activated whenever NHEJ factors become limiting. Many studies make use of low numbers of site-specific DSBs, because the repair products can then easily be characterized at the molecular level. In this study we make use of higher copy number arrays in highly polyploid intestinal cells. The relatively high number of DSBs caused per cell by this approach may explain why we observe alt-EJ here in wild type background; DSBs may be too numerous for classic NHEJ, resulting in a role for alt-EJ. Such a role for alt-EJ is thus easily overlooked when low numbers of DSBs are introduced.

How alt-EJ activity is restricted to replicating cells is unclear, but similar cell cycle limitations have been observed for repair by SSA in mammalian cells (Al-Minawi et al., 2008). One possibility is that proteins involved in alignment, annealing and/or ligation may be present or active only at these stages. Alternatively, the strict regulation could be enforced by an inhibitory signal that binds to DSBs that are not bound by Ku to prevent premature repair. Upon entry into S-phase, this signal may be removed by phosphorylation or other modification to make the ends available for repair.

PARP1 has high affinity for ssDNA nicks and blunt-end DSBs, but does not bind efficiently to DSBs that involve overhangs of a few nucleotides as created by I-SceI (D'Silva et al., 1999). The nucleotides thus first have to anneal, either spontaneously or aided by annealing enzymes, to create ssDNA nicks that can be bound by PARP-1. PARP1 has previously been implicated in alt-EJ: First, Ku-deficient cells that were treated with PARP-inhibitors were less efficient in repairing a DSB than wild type cells. PARP collaborates with the XRCC1/Ligase III complex to ligate breaks, and XRCC1 deficient cell extracts also showed impaired end-joining activity (Audebert et al., 2004). Others then showed that PARP possibly competes with the classic NHEJ protein Ku80 for end binding (Wang et al., 2006). We found no evidence for a role for *pme-1* in alt-EJ in *C. elegans*. This suggests that alt-EJ in our assay is different from the end-joining activity observed in other studies. Also, the type of DSB can determine whether PARP-1 has a role in alt-EJ.

The increased ORF restoration of *pme-1* mutants suggest that *pme-1* functions in another alternative DSB repair pathway. I-SceI cleaves with 4-nt compatible overhangs that can re-anneal in the absence of processing or Ku-binding. This results in a situation

where two single-strand breaks are interspaced by four nucleotides; a likely substrate for PME-1. In the absence of *pme-1*, this intermediate is instable, and re-opened DSBs will eventually be processed by classic NHEJ, whereas some may enter the SSA pathway in dividing cells. In *lig-4* mutants, the ends are bound by Ku or processed, preventing PME-1 dependent repair. This model predicts that remaining repair by *pme-1* should be possible in *lig-4* mutants. However, the Southern data showed that in the absence of *lig-4*, no significant repair was observed. We envisage three possible explanations: First, the contribution of *pme-1* dependent repair is relatively small compared with NHEJ (Figure 3A). Second, *pme-1* dependent repair restores an intact I-SceI site, that may be cleaved repeatedly as long as I-SceI is present: This also results in DSB persistence. Finally, slower kinetics of *pme-1* dependent repair may also play a role.

To circumvent SSB repair by re-annealing of complementary overhangs, tandem I-SceI or HO sites in opposite orientations can be used. I-SceI then creates incompatible ends that are not susceptible to direct re-annealing. DSBs that are repaired by PME-1 in our study should then be repaired by classic NHEJ or by alt-EJ in the case of many DSBs. Others have previously shown that non-complementary ends indeed more frequently give rise to deletions, and these were predominantly caused by Ku-independent alt-EJ and by XPF dependent SSA (Ma et al., 2003).

The MRN complex has been implicated in microhomology-mediated and Ku-independent joining in various organisms (Delmas et al., 2009; Deriano et al., 2009; Ma et al., 2003), as well as in SSA. However, our data indicate that *mre-11* is not required for SSA or alt-EJ in the soma. Redundancy for mre11 at different types of DSBs has been reported previously: Despite rapid recruitment of Mre11 to I-SceI induced DSBs in budding yeast (Barlow et al., 2008), Mre11 is not required for end resection and repair of an HO-induced DSB (Llorente and Symington, 2004). These divergent findings could reflect redundancy of *mre-11* with other nucleases, such as Dna2 and Exo1, or with helicases, such as Sgs1 (Mimitou and Symington, 2008; Zhu et al., 2008).

Together, our data show that alt-EJ is restricted to replicating cells and that repair by this pathway is delayed compared with NHEJ. Nevertheless, the genetic requirements remain unclear, and different genes have been found to be involved in different studies. This suggests that highly redundant factors may be involved, and no single gene may completely disrupt alt-EJ activity. Possibly, genes that are involved in HR or SSA may have a dual role in alt-EJ. However, the lethality that is associated with most HR genes complicates the analysis of these genes.

MATERIALS AND METHODS

Strains and constructs. All strains were cultured under standard conditions at 15°C (Brenner, 1974). Mutant alleles used were *mus-81* (tm1937); *pme-1* (ok988); *lig-4* (ok716); *xpf-1* (e1487); *brc-1* (tm1145); *mre-11* (ok179). Strains were grown on 6 or 9 cm culture dishes (Greiner Bio-One) with NGM-agar and OP50 as a food source.

Plasmid sequences are available upon request. Plasmids pLM44 (P*elt-2*::LacZ::ISceI-site::LacZ-ORF), pLM45 (P*myo-2*::LacZ::I-SceI site::LacZ-ORF), pLM17 (HSP::I-SceI::mCherry) and pRF4 (*rol-6*(su1006)) were co injected at 2 ng/µL together with genomic DNA to form high-copy complex extrachromosomal arrays. These arrays were integrated by treatment with ionizing radiation (50 Gy from a Cs-137 source) followed by selection for 100% inheritance of the array in the F2 generation of the irradiated animals. Two integrated strains that were obtained independently with this method were used in this study for LacZ analysis and genetic crosses.

X-gal staining and quantification. Populations were synchronized by bleach treatment, left to hatch overnight on OP50 and heatshock was performed to induce I-SceI expression at 34°C for two hours. Animals were rinsed off plates as young adults, dried well in a speedvac and fixed in acetone. LacZ staining was performed using a 5% X-gal solution. The quantitative analysis of LacZ expression for *elt-2* expressing cells was performed as described previously (Pontier and Tijsterman, 2009). To quantify expression in *myo-2* expressing cells, the number of animals with LacZ-expressing cells in the pharynx was scored using Nomarski microscopy.

DNA isolation and Southern blotting. Animals were synchronized by bleach treatment, heat shocked when indicated and rinsed off plates in M9 buffer, washed 4 times and snap-frozen in liquid nitrogen at the indicated time points. Pellets were lysed in 750 µL SDS lysis buffer (0.2 M NaCl; 0.1 M Tris.HCl pH8.5; 50 mM EDTA (Mallinckrodt-Baker B.V); 0.5% SDS; 100 µL/ml proteinase K) for 4 hours at 60°C. To isolate DNA, one part PCI (25:24:1; phenol pH 7.9:chloroform:isoamylalcohol) was added to the lysates and incubated at RT in a spinning wheel for 10 minutes. After centrifugation (12k x *g* for 10 minutes), sups were incubated with RNaseA for 30 minutes at 37°C and the PCI step was repeated once with PCI and once with chloroform (Mallinckrodt Baker B.V.). DNA was precipitated from the sup by adding 2.5 parts 100% ethanol and incubation at -80°C overnight. DNA was collected by centrifugation and the pellets were washed once in 500 µL 70% ethanol. Pellets were left to dry and redissolved in 100 µL milliQ. 10 µg DNA per sample was digested with SacI and XBaI in a total volume of 150-200 µL overnight at 37°C. Samples were loaded onto 1% agarose gels and run overnight at 40V. DNA was fragmented on a UV-tray and gels were incubated in denaturing buffer (0.4 M NaOH; 1.5 M NaCl) and neutralizing buffer (0.5 M Tris.HCl;1.5 M NaCl) for 30 minutes each. DNA was then blotted onto positively charged nylon membranes (Roche) overnight. DNA was crosslinked to the blot with a UV crosslinker at 1200 J/m^2. and incubated in Church buffer (0.36 mM Na_2HPO_4; 0.14 mM NaH_2PO_4; 1 mM EDTA (Mallinckrodt-Baker B.V.) and 7% SDS; pH7.2) at 65°C for 1 hour. Probes were generated by PCR, purified on a PCR purification column (Qiagen) and labeled with 3 µL ^{32}P-dCTP using a DNA labeling kit (Roche). Labeled probes were purified over G25 columns (GE Bioscience), denatured at 95°C and incubated with the blot in Church buffer for 24-72 hours at 65°C. Blots were washed at 65°C in 2xSSC;0.2% SDS, 1xSSC;0.1% SDS and 0.5xSSC;0.5% SDS, respectively, for 30 minutes each, exposed to PhosphoImage screens for 24-72 hours and scanned using a Cyclone scanner. Quantification of repair was performed as follows: Total signal of the bands on the Southern blot (intact reporter and DSB product) was quantified using OptiQuant software. Background signal was subtracted from all bands. The fraction of DSB per lane was calculated by the formula [signal DSB/(signal reporter + signal DSB)]. The fraction repair was then calculated by the formula [1- (fraction DSB 24 hrs/fraction DSB 4 hrs)].

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CHAPTER 4

DIFFERENTIAL USE OF DOUBLE-STRAND BREAK REPAIR PATHWAYS DURING *C. ELEGANS* GERM CELL DEVELOPMENT

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SUMMARY

Meiosis is a specialized form of cell division that generates haploid gametes, and is accompanied by the programmed introduction of DNA double-strand breaks (DSBs). DSBs are normally highly toxic for cells, but they are required in meiosis for proper chromosome segregation. By studying the formation and repair of an endonuclease-generated DSB in a known location in the genome, we were able to study the response to DSBs at different stages of mitotic and meiotic cell division. We found that the response is predominantly determined by the stage at which DSBs are introduced, and likely not by their nature or number. DSBs introduced in mitosis trigger increased cell cycle arrest and apoptosis, two hallmarks of a DNA damage response, whereas programmed meiotic DSBs do not result in such a response. We found evidence for a second repair mode switch at the diakinesis stadium: The association of the repair protein RAD-51 with broken chromatin is completely lost during oocyte maturation and early embryonic development, even in the presence of numerous DSBs. This loss is dependent on the translesion synthesis polymerase eta, polη. Together, our data support a model where germ cells switch between several different repair modes, depending on the stage of the cell cycle.

INTRODUCTION

Meiosis is a specialized form of cell division in which the chromosomal content of the cell is reduced by half to produce haploid (1N) gametes for subsequent fertilization and reproduction (reviewed in Longhese et al., 2009). This is achieved by a single round of DNA replication followed by two rounds of cell division, where first the homologous chromosomes and then the sister chromatids segregate (Petronczki et al., 2003). Preceding homolog segregation, the paternal and maternal chromosomes exchange DNA material in a crossover reaction, which is initiated by the tightly regulated introduction of double-strand breaks (DSBs) (Keeney et al., 1997). Besides increasing genetic diversity among the offspring, crossovers also establish a transient physical bond, called chiasma, between the chromosomes that is required for correct homolog segregation (Bishop and Zickler, 2004; Page and Hawley, 2003).

The *C. elegans* germline is a particularly suitable system to study meiosis, because of the well-organized spatio-temporal orientation of the cells while they progress through the various stages of meiosis (Garcia-Muse and Boulton, 2007). The germ cell population is maintained through mitotic cell divisions in the premeiotic zone at the distal tip. Further from the distal tip, in the transition zone, cells convert to the meiotic program. From this stage onwards, programmed meiotic DSBs are generated by the conserved SPO-11 enzyme (Dernburg et al., 1998; Keeney et al., 1997), and they are a substrate for interhomolog homologous recombination (HR) throughout the subsequent pachytene stage. In HR, the DSB is first resected by the MRN (Mre-11, Rad-50 and Nbs1) complex, after which strand exchange with the homologous chromosome is catalyzed by the activity of RAD-51, a homolog of bacterial recA that accumulates at resected DSB ends (Alpi et al., 2003; Ogawa et al., 1993). HR can result either in a crossover (CO) or in a non-crossover (NCO), depending on how the intermediate intertwined DNA molecules are resolved. The number of COs is subject to stringent regulation in all organisms, but to an extreme level in *C. elegans*, where only a single DSB per chromosome pair results in a crossover; the remaining DSBs are resolved as non-crossovers (Hillers and Villeneuve, 2003; Wood, 1988). Other DSB repair pathways than HR are intrinsically error-prone and do not establish chiasmata. These pathways have a very limited role in the germline which only becomes apparent in complex genetic backgrounds (Martin et al., 2005), but they are important routes for DSB repair in somatic tissues (Clejan et al., 2006; Pontier and Tijsterman, 2009). Non-homologous end-joining (NHEJ) is the major pathway that operates throughout the cell cycle in somatic cells, and simply joins the broken ends using no or only small patches of sequences homology (Hefferin and Tomkinson, 2005). The single-strand annealing (SSA) pathway requires homology in the sequences flanking the DSB, which, upon alignment and ligation, results in deletion of the intervening sequence and one of the repeats (Pâques and Haber, 1999).

Besides endogenous processes, DSBs can also result from DNA damaging agents such as certain chemicals and ionizing radiation. DNA damage leads to activation of the

ATM and ATR kinases, which phosphorylate downstream proteins that constitute a checkpoint response. ATM is mainly involved in the response to DSBs, whereas ATR more specifically targets lesions that are encountered during DNA replication, but redundancy between the two kinases exists (Abraham, 2001; Kastan and Bartek, 2004; Shiloh, 2003). Checkpoint activation is normally required for the DNA damage response (DDR), which involves DNA damage-induced cell cycle arrest, apoptosis and DNA repair. As a consequence, DNA repair, in particular HR, is severely impaired in mammalian cells that lack ATM (Lobrich and Jeggo, 2005; Morrison et al., 2000). ATM and ATR are conserved in *C. elegans*, and as expected, mutants in these genes (*atm-1* and *atl-1*, respectively) are exquisitely sensitive to ionizing radiation (IR) (Garcia-Muse and Boulton, 2005). Interestingly however, these genes are not required for *spo-11* induced HR and crossover formation in the *C. elegans* germline: *atm-1* mutants are normally viable and fertile, and despite complex mitotic phenotypes, *atl-1* mutants show normal crossover formation (Garcia-Muse and Boulton, 2005).

While programmed DSBs thus do not induce checkpoint activation, low doses of ionizing radiation, an external trigger of DSBs, initiate a DNA damage response characterized by apoptosis, cell cycle arrest and lethality among the progeny (Gartner et al., 2000). Alternative sources of DSBs can also result in CO formation: *spo-11* mutants, which lack meiotic DSBs, fail to form RAD-51 foci and chiasmata, but these phenotypes can be largely rescued when DSBs are introduced by ionizing radiation or deaminases (Dernburg et al., 1998; Pauklin et al., 2009). Also, in yeast, HO-induced DSBs are as efficient in inducing crossovers as Spo-11 (Malkova et al., 2000). This suggests that the different responses may instead be regulated by the time of the cycle at which the DSBs are introduced.

We investigated the fate of DSBs in the germline by introducing them at a known location in the genome by expression of the rare-cutting endonuclease I-SceI in animals that also contain the 18-nt recognition site of this enzyme. Here, we show that SSA and possibly NHEJ are both occasionally used for germline DSB repair. Moreover, we found that I-SceI induced DSBs, in contrast to SPO-11-induced DSBs, trigger cell cycle arrest, apoptosis, chromosomal missegregation and lethality amongst the progeny. We argue that some of these responses are the result of I-SceI-induced DSBs that occur in the mitotic, not in the meiotic zone. Moreover, we find that late pachytene cells again switch to a different repair mode, which is characterized by the failure to induce RAD-51 foci formation by ionizing radiation. Altogether, our results support a dynamic model of DSB repair in the germline, where the repair mode is highly dependent on the stage of the cell cycle at which the DSB is introduced.

RESULTS AND DISCUSSION

I-SceI efficiently introduces DSBs in the *C. elegans* germline

To study the fate of a DSB in germ cells, we constructed two transgenes to introduce a targeted DSB in a known location in the germline: One transgene (the reporter) contains the 18-nt recognition site for I-SceI in an out-of-frame LacZ gene (Figure 1A), and I-SceI was expressed from a second transgene under control of the *pie-1* promoter, which expresses constitutively throughout the germline (Merritt et al., 2008). We performed RAD-51 antibody stainings on dissected germlines to determine I-SceI cutting efficiency (Figure 1B). The strand exchange protein RAD-51 accumulates at resected DSBs and foci are normally present as a result of endogenous SPO-11 activity in the pachytene stage: they first appear in the transition zone and disappear well before the diakinesis stadium, where chromosomes condense and oocytes are formed (Alpi et al., 2003). DSB induction by I-SceI can thus most easily be scored in the mitotic zone, where the *pie-1* promoter is active but where no endogenous DSBs are present. Control animals that contain only the *pie-1*::I-SceI construct but not the reporter transgene showed a similar staining pattern as wild type worms, indicating that there are no endogenous sequences in the *C. elegans* genome that can be recognized and cleaved by I-SceI (Figure 1B). However, efficient DSB induction was observed in animals that had both transgenes: RAD-51 foci were now also observed in mitotic cells, and they were more frequent in meiotic cells compared to the controls (Figure 1B).

Depending on the stage of the cell cycle, ploidy of cells in the mitotic zone can be 2N (in G1 phase) or 4N (in S/G2 phase). The maximum number of DSBs introduced by I-SceI in one nucleus is thus four. However, ploidy of 4N is unlikely to result in detection of four foci because the sister chromatids localize closely together at this stage and can probably not be resolved individually. We counted an average of 0.5 foci per nucleus (n=150) in the mitotic zone (Figure 1C). A single focus was observed in 35% of the nuclei, but we observed two foci in only 6% of the nuclei, and foci were absent in 59% of nuclei. No mitotic nuclei with more than two foci were observed. In conclusion, I-SceI cuts specific and frequent in *C. elegans* germlines.

A single DSB in *C. elegans* mitotic cells induces a DNA damage response

The reporter system introduces a maximum of four DSBs per meiotic nucleus, a 25% increase compared to the estimated number of endogenous SPO-11-induced DSBs per nucleus, which is twelve (Mets and Meyer, 2009). The real increase must be even lower, since RAD-51 staining showed RAD-51 foci only in a subset of cells. Relatively, a more severe change is introduced by the reporter in mitosis, where DSBs are normally completely absent. We investigated the effect of this low level of DSBs on the germline DNA damage response (DDR) by using two hallmarks of a DDR in the germline: cell cycle arrest and apoptosis.

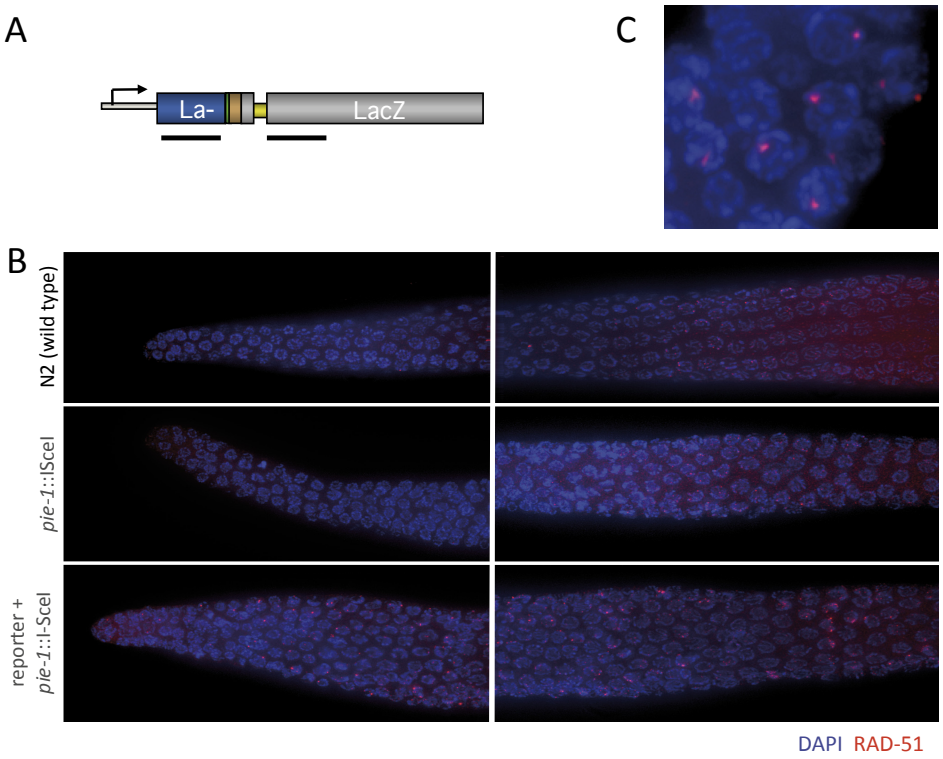

Figure 1 | Germline DSB reporter (A) Schematic representation of the DSB-repair reporter. Blue, in-frame sequence; orange, stop codons; yellow, I-SceI recognition site; grey, out-of-frame sequence. Black lines represent homologous sequences. **(B)** Germlines were immunostained for RAD-51 to determine introduction of DSBs in double transgenic animals and controls. Representative images are shown for the premeiotic (left) and meiotic (pachytene) stage (right) **(C)** Close-up of premeiotic RAD-51 foci induced in the reporter transgene by I-SceI.

Cell cycle arrest results in enlarged mitotic nuclei, because the cells continue to grow but do not go through divisions (Gartner et al., 2000). We scored for the presence of these enlarged nuclei in transgenic animals by DIC optics (Figure 2A) and we found that the number of germlines that contain arrested cells was significantly increased. This was specific for animals containing both transgenes (Figure 2B), and never occurred in wild type or control animals. Still, cell cycle arrest occurred in only one or two cells per germline, in less than 50% of the germlines scored. RAD-51 foci were much more frequent, thus RAD-51 foci induction is not necessarily associated with cell cycle arrest, possibly because the number of DSBs is lower than four in most cells. This result shows that low doses of DSBs in the mitotic stage of the germline is sufficient to trigger cell cycle arrest to prevent entry into meiosis with a damaged genome.

In *C. elegans*, a certain fraction of germ cells dies by physiological apoptosis, which occurs only in the late pachytene region in the germline bend. Whereas apoptosis is

usually associated with a DNA damage response, *spo-11* mutants - which lack germline DSBs - display wild type levels of apoptosis (Gartner et al., 2000). Elimination of physiological apoptosis has only been observed in mutants defective for core apoptotic machinery, such as the caspase *ced-3*, but not for any of the checkpoint genes *atm-1*, *atr-1*, *chk-2*, or the 9-1-1 complex components *hus-1* and *mrt-2* (Gartner et al., 2000; Stergiou et al., 2007). Strikingly, apoptosis is not increased in strains with persistent RAD-51 foci such as *rec-8*, *him-3* [RNAi] or in strains that lack a repair template due to heterozygous translocations (Alpi et al., 2003). Altogether, this indicates that physiological apoptosis is not caused by SPO-11 induced DSBs, even when these can not be repaired. Only two sources of apoptosis in germ cells have currently been described: First, ionizing radiation and other DNA damaging agents increase the number of apoptotic cells, and this is further enhanced in mutants defective for DNA repair, such as *rad-51* and checkpoint mutants. Second, inhibition of synapsis by RNAi against synaptonemal complex components has also been shown to result in increased apoptosis (Jaramillo-Lambert and Engebrecht, 2009). Both DNA damage and failure to synapse can occur endogenously at a certain level, and this raises the question whether

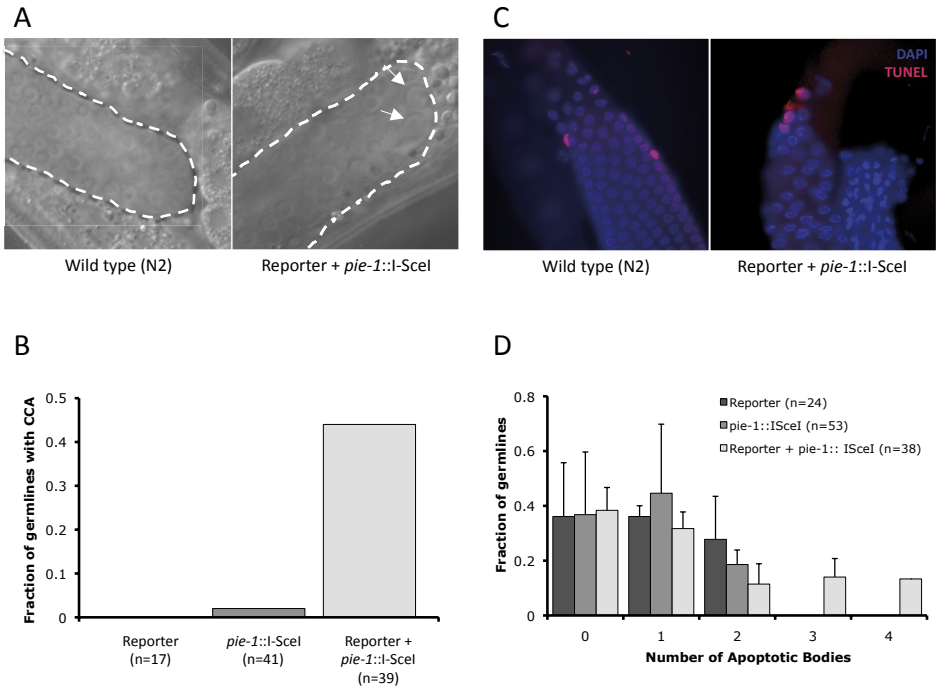

Figure 2 | Cell cycle arrest and apoptosis are induced by mitotic DSBs. (A) DIC images of premeiotic cells in wild type (left) and double transgenic animals (right). Arrow indicate enlarged, arrested cells. **(B)** Quantification of the number of germlines in which arrested cells were observed. **(C)** TUNEL labeling of apoptotic cells in wild type (left) and double transgenic animals (right). **(D)** Quantification of the number of apoptotic cells in control and double transgenic animals.

physiological apoptosis may then be the result of endogenous levels of either of these two processes.

We developed a TUNEL assay (terminal dUTP transferase nick end labeling) to stain *C. elegans* germlines for apoptotic cells. In this assay, the terminal dUTP transferase (TdT) enzyme transfers labeled nucleotides to DNA ends, which occur abundantly in apoptotic cells because this is accompanied by massive DNA fragmentation. Apoptotic nuclei are therefore generally completely stained with this method. We quantified the number of apoptotic cells in animals with both the I-SceI expression construct and the reporter transgene, and in animals with only one of the transgenes as controls. Wild type (N2) animals show 0, 1 or 2 apoptotic bodies per germline (data not shown), and this was unchanged in control animals that contain one of the transgenes. In double transgenic animals however, we observed germlines with 3 or 4 apoptotic cells, which was never observed in the controls ($p < 0.05$ when double transgenic animals are compared with control animals that only express *pie-1*::ISceI without the reporter; Figure 2C and D). Thus, apoptosis is increased by I-SceI-induced DSBs. While synapsis defects as a result of these DSBs are one possible explanation, the number of meiotic DSBs is only marginally increased by the reporter and meiotic DSBs do not prevent (nor are they required for) synapsis. Mitotic DSBs are a more likely cause: We therefore postulate that the increased apoptosis that we observe upon I-SceI expression may be the result of the subset of DSBs that are induced by I-SceI in the mitotic zone. This conclusion is also supported by the notion that *dog-1* mutants, which show a DNA replication-associated -and thus, mitosis-specific- DNA damage phenotype, also display increased apoptosis (Cheung et al., 2002; Youds et al., 2008), despite efficient repair of these lesions (Kruisselbrink et al., 2008; Youds et al., 2006).

Interestingly, if the apoptotic fate is determined early in the mitotic zone, the execution must be actively postponed until the cell reaches a certain zone: Apoptosis is never observed outside of the late pachytene region. It remains unknown how this delay is regulated and why late pachytene is the preferred zone for apoptosis, but a plausible explanation is that contents from the dying cells may be required for filling and maturation of the rapidly growing oocytes directly downstream. Oocytes - which grow much larger than their pachytene precursors - have been shown to be filled by cytoplasmic streaming of products from transcriptionally active pachytene cells (Wolke et al., 2007), and apoptotic cells may also contribute to this process. This is supported by the fact that physiological germ cell death does not occur during spermatogenesis - sperm contains very little cytoplasm. Moreover, mutants of *ced-3*, a caspase essential for apoptosis, exhibit increased embryonic lethality and smaller oocytes in ageing animals (Gartner et al., 2000; Gumienny et al., 1999), supporting the hypotheses that apoptosis eliminates damaged cells and that their contents can be used to nurture oocytes.

RAD-51 is removed from chromatin during oogenesis and early embryogenesis

The above results show that low levels of DSBs are sufficient to trigger a DDR that is characterized by cell cycle arrest and apoptosis. In yeast, a single DSB in the genome is sufficient to induce a DNA response and lethality, and four DSBs can induce lethality in mammalian cells (Bennett et al., 1993; Zierhut and Diffley, 2008). We investigated whether lethality can also be induced by low numbers of DSBs in *C. elegans* germ cells. Indeed, whereas 99% of embryos were viable in controls that express I-SceI but lack the reporter transgene, only 68 ±6% survivors were observed in double transgenic animals ($p=2.5 \times 10^{-6}$).

Increased embryonic lethality suggests that DSBs are still present at later stages of germ cell development or lead to permanent genomic changes such as rearrangements. Indeed, *pie-1* is expressed throughout the germline (Merritt et al., 2008). Remarkably however, in double transgenic animals, all RAD-51 foci had completely disappeared at the diakinesis stadium (data not shown). To address whether this is specific for I-SceI induced DSBs, we subjected animals to 50 Gy IR as an alternative source of DSBs and looked until what stage RAD-51 foci were observed. IR caused ubiquitous foci throughout the germline, but they were never observed after the -3 oocyte, even though IR-induced DSBs must be present here (Figure 3A). RAD-51 was instead detected as a diffuse nuclear signal in these later oocytes, similar to non-irradiated animals. Interestingly, RAD-51 foci reappeared in irradiated late-stage embryos (~40 nuclei), whereas earlier stage embryos were devoid of foci, except from occasional foci in the germline precursor cells P1 and P2 (Figure 3B). RAD-51 is thus removed from chromatin or prevented from binding in the stages between the third oocyte and late embryos, regardless of the source of DSBs. This suggests that DSBs at these stages are repaired by a RAD-51 independent (thus error-prone) repair pathway in this interval, or repair may be postponed until HR is again available.

Three other studies have reported evidence for a change in repair mode around the stage where we observe loss of RAD-51 foci: First, it has been shown that RAD-51 focus formation following IR depends on RAD-50 in the meiotic, but not in the premeiotic and late pachytene zones (Hayashi et al., 2007). RAD-50 is part of the MRN complex, and the nuclease activity of this complex is required for removal of SPO-11 from DSBs, but is dispensable for the repair of HO-endonuclease DSBs in fission yeast (Llorente and Symington, 2004; Rothenberg et al., 2009). Therefore, RAD-50 dependency may be restricted to the region where SPO-11 is active because other DSBs do normally not occur here. The RAD-50 dependency of IR-induced RAD-51 loading is released at the late pachytene stage (Hayashi et al., 2007). This switch coincides with the loss of the ability to form interhomolog COs. Whether these are causative relationships remains to be determined, but the reason for the loss of interhomolog recombination at this stage can be understood considering the following: Upon fertilization, oocytes immediately initiate the first meiotic division, rapidly followed by the second division. Because the activity of RAD-51 establishes physical connections between chromosomes, this could

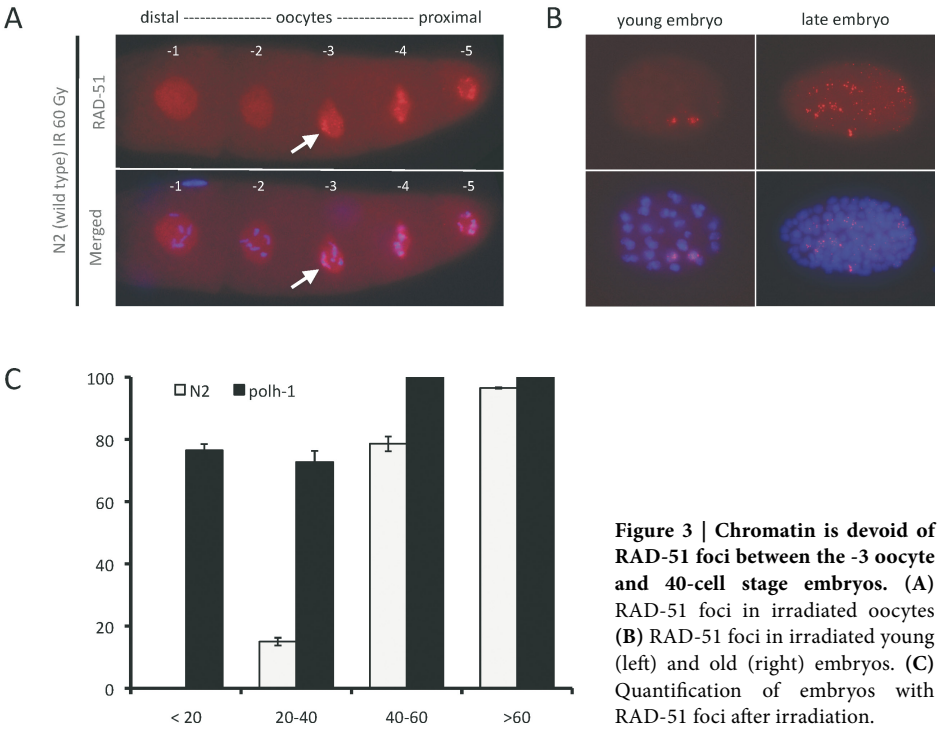

Figure 3 | Chromatin is devoid of RAD-51 foci between the -3 oocyte and 40-cell stage embryos. (A) RAD-51 foci in irradiated oocytes **(B)** RAD-51 foci in irradiated young (left) and old (right) embryos. **(C)** Quantification of embryos with RAD-51 foci after irradiation.

interfere with chromosome segregation. It is thought that the loss of interhomolog recombination is accompanied by an increase in intersister recombination (Martinez-Perez and Villeneuve, 2005; Zetka et al., 1999). Intersister recombination would not interfere with meiosis I, where homologous chromosomes segregate. If intersister recombination is faster than interhomolog recombination and involves shorter ranges of sequence homology, this could account for the lack of detectable RAD-51 foci at this stage. This would also explain why RAD-51 foci are not observed in somatic cells (our unpublished data), whereas HR is used here: somatic cells preferentially use the sister chromatid as a repair template (Pontier and Tijsterman, 2009).

A second finding that supports a repair mode change is that chromatin undergoes major remodeling at the diakinesis stage (Rogers et al., 2002; Severson et al., 2009). This could affect accessibility of repair factors. Indeed, the switch from RAD-50 dependency for RAD-51 loading seems to be regulated by the chromatin modifying genes *him-3* and *htp-1* (Hayashi et al., 2007). Moreover, these genes have been suggested to regulate the transition from interhomolog to intersister recombination (Martinez-Perez and Villeneuve, 2005; Zetka et al., 1999). Finally, oocytes are remarkably more sensitive to ionizing radiation than pachytene cells: The first brood laid after IR, which is derived from gametes that were oocytes at the time of irradiation, has a significant higher lethality rate than brood laid at later time points. While this could reflect the

fact that oocytes have less time for repair before they start their first divisions, our observation that RAD-51 foci are absent during these stages, together with the notion that RAD-51 [RNAi] has a smaller effect on survival of oocytes and early embryos than on survival of pachytene cells after IR (Takanami et al., 2000) suggest that oocytes respond differently than pachytene cells to DSBs. The difference in sensitivity between oocytes and pachytene cells could be due to differential regulation of proteins that have access to the DSB.

In conclusion, in addition to a repair mode switch between the premeiotic and meiotic stage, *C. elegans* germ cells undergo a second switch at the diakinesis stage, characterized by loss of RAD-51 from DSBs and either persistence of DSBs or error-prone repair. The loss of RAD-51 foci in oocytes may prevent chromosome segregation defects during meiosis I and II , whereas in embryos, the highly coordinated timing of cell divisions is a possible reason to limit RAD-51 activity. Checkpoint activation and repair may interfere with this coordination and lead to mis timing of divisions, resulting in embryonic lethality.

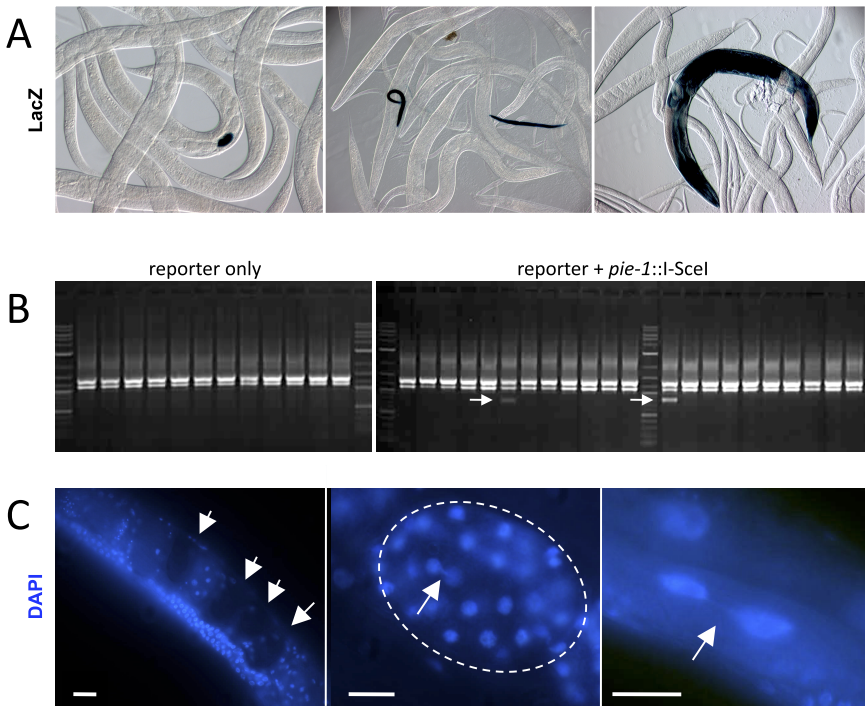

Figure 4 | Error-prone repair and chromosomal missegregation due to a germline DSB. (A) Germline SSA events in embryo (left) and young worms (middle), and late SSA event in cell lineage (right). **(B)** PCRs with primers in the flanks of the DSB in DNA from single double transgenic (right) and control animals (left). Arrows indicate deletions. **(C)** DAPI stainings showing embryos with no DNA content (left) and chromatin bridges in embryo (middle) and adult somatic cells (right).

A role for *polh-1* in preventing IR-induced RAD-51 foci

It has previously been shown that mutants for the translesion synthesis polymerase eta (*polh-1*) or *gei-17* [RNAi] show RAD-51 foci formation in MMS-treated embryos, whereas foci were absent from embryos treated with control RNAi. In addition, MMS treatment also led to cell cycle arrest in mutants for *gei-17* and *polh-1*, but not in wild type animals (Holway et al., 2006). This likely occurs because impaired repair in these mutants leads to persistence of damage that is sufficient to trigger arrest. This suggests that *polh-1* bypasses lesions at MMS-stalled replication forks in early embryos and thereby prevents checkpoint activation and subsequent mistiming of cell divisions. If this is the case, we do not expect that DSB induction leads to RAD-51 in *polh-1* mutants, because DSBs cannot be bypassed. We obtained a null allele of *polh-1* (S.F.R., unpublished results), treated these with IR and performed RAD-51 immunostainings. Similar to wild type, IR-induced foci disappeared in late oocytes of *polh-1* mutants. Remarkably however, we observed frequent foci in young *polh-1* embryos at all stages after IR treatment, whereas the same dose of IR did not induce foci in a wild type control (Figure 3C). This suggests that the diminished checkpoint in *polh-1* mutant embryos may not occur through lesion bypass, but through an unknown mechanism that may involve correct localization or activation of other repair proteins. However, we cannot exclude the possibility that these foci occur as a result of IR-induced damage other than DSBs: IR induces a wide range of DNA damages (Henner et al., 1982), and only a subset of those can be bypassed by POLH-1. RAD-51 foci can represent these lesions, whereas the DSBs induced by IR may remain devoid of RAD-51 protein. To investigate whether this is the case, DSBs should be introduced more specifically in embryos of *polh-1* mutants, e.g. by I-SceI expression controlled by an embryo-specific promoter, or by treatment with camptothecin. If RAD-51 foci also persist here, this would point towards a role for *polh-1* in preventing RAD-51 foci at sites of DSBs.

Our data support previous findings that suggested a role for polη in DSB repair. First, polη is predominantly known as a polymerase capable of bypassing UV-induced lesions (Masutani et al., 1999), but polh-1 mutants of C. elegans are also sensitive to DSBs: lethality is observed after treatment with IR and in strains where transposons are excised leaving DSBs (S.F.R., unpublished data). Moreover, mammalian polymerase eta (polη) colocalizes with RAD-51 after damage in mammalian cells (Kannouche et al., 2001) and these proteins physically interact in cell extracts (McIlwraith et al., 2005). Furthermore, polη can uniquely extend D-loops using the invading strand as a primer in human cell extracts, and this activity is stimulated by Rad51 (McIlwraith et al., 2005). These data, together with our findings, support two alternative mechanisms: First, POLH-1 and RAD-51 may promote DSB repair in wild type embryos in an extremely rapid manner, possibly by intersister HR, which does not allow for the detection of RAD-51 foci. In polh-1 mutants, RAD-51 induced D-loops would then not be extended and persist, explaining why foci can be observed here. Alternatively, polh-1 could be important for correct localization or modification of other proteins in checkpoint silencing.

Error-prone repair pathways are active at stages of *pie-1* expression

If absence of RAD-51 foci in the oocyte and early embryonic stages is caused by lack of HR, this should result either in persistence of the DSB throughout subsequent cell divisions, which is obviously instable, or other, RAD-51-independent, repair pathways must be employed, which are all intrinsically mutagenic. Also, the absence of foci in almost 60% of all nuclei can reflect loss of the I-SceI site in a subset of cells due to error-prone repair or incomplete I-SceI cutting efficiency, or a combination of both. Finally, the fact that more than 40% of the cells show RAD-51 foci whereas only very few cells exhibit cell cycle arrest, suggests that many DSBs may be repaired effectively.

We investigated which pathways are used to repair the I-SceI-induced DSBs. Repair by HR would restore an intact I-SceI site that can be cleaved again, resulting in the constitutive presence of a DSB in these cells. In contrast, error-prone repair by SSA or NHEJ would lead to disruption of the I-SceI site, possibly posing a selective advantage for these cells by release from the recurrent DSBs. Activity of the error-prone repair pathways SSA and NHEJ have not yet been observed in the germline, but with this reporter system we can detect these events, even when they are rare. To detect SSA, we made use of two 251-nt LacZ-repeats that are present in the reporter transgene (marked with black bars in Figure 1A). SSA joins DSBs by annealing and ligation of repeats, and in this process, one of the repeats and the intervening sequence are lost. In our reporter, SSA through these repeats will result in expression of LacZ, and we can thus study repair by SSA by scoring LacZ expression.

When we stained populations of double transgenic worms for LacZ expression, we observed several animals that stained 100% blue (Figure 4A), indicative of germline ORF restoration events by SSA. Quantification of the frequency by which this occurs was complicated by the fact that different populations show greatly varying levels of LacZ expression: When populations were clonally grown from single animals, only 9% (n=32) of these populations showed LacZ expression amongst the progeny. If ORF restoration occurs as a stochastic event, we would expect similar frequencies of LacZ expression in the different populations. The fact that we see a few populations with high expression whereas many populations have none suggests that LacZ expression likely results from events that occurred in mitotic germ cells. All the daughter cells that arose after the event during subsequent mitotic divisions will give rise to LacZ-positive progeny, explaining why some populations showed multiple LacZ positive worms whereas other populations were negative. However, the overall frequency of SSA in germline cells appears very low.

If SSA is not a major route leading to I-SceI site loss, an alternative explanation for the lack of LacZ expression in most of the strains and the absence of foci in 60% of germ cells is that the I-SceI site is frequently lost by other error-prone repair, such as NHEJ. We obtained preliminary evidence that NHEJ indeed occurs, because occasional deletions were detected by PCR that could be the result of NHEJ (Figure 4B). However, it should be noted that these events are not necessarily derived from germline events,

because PCRs were performed on DNA from whole worms, including somatic cells. Long-term stability of I-SceI into somatic tissues could thus also cause these deletions. Smaller deletions than detected here likely occur with higher frequency but are missed in this approach because they lack the amplification advantage in the PCR. These results show that SSA and perhaps NHEJ can operate in the germline in the case of mitotic or recurrent DSBs, and at least partially explain the lack of RAD-51 foci in a subset of germline cells as well as the loss of the ability to express LacZ in a subset of populations.

DSBs lead to chromosomal missegregation in meiosis I and during embryonic development

In addition to the above repair events that had occurred in the germline, we also detected repair events that were derived from later stages. First, besides the germline events discussed earlier, LacZ expression was sometimes observed to be restricted to particular cell lineages (Figure 4A), suggesting that these error-prone repair events do not only occur in the germline, but can also arise early in embryonic development. Secondly, we observed frequent chromatin bridges that connect two nuclei in embryos and adults (Figure 4C), an indicator of DNA damage and chromosomal missegregation (Collis et al., 2007; Youds et al., 2008). Finally, we observed a high incidence of males in the double transgenic strain, which indicates chromosomal missegregation: In *C. elegans*, males are characterized by having a single X-chromosome, whereas hermaphrodites have two. Males are rare in the general population because they arise only from occasional X-chromosome nondisjunction. A high incidence of males (*him*) thus indicates missegregation of the X-chromosome. Our reporter transgene is located on the X-chromosome and shows a *him*-phenotype, rising the hypothesis that a single DSB induces chromosomal missegregation. We found that in the population of double transgenic animals 4.8 ± 2.8% were males, vs. 0.1 ±0.02% in control animals that express I-SceI but lack the reporter (p=0.009). These results indicate that the effects of the I-SceI-induced DSB can persist until later stages, resulting in error-prone repair, aneuploidy and missegregation by chromatin bridging. The absence of RAD-51 foci in later stages supports this hypothesis.

MATERIALS AND METHODS

Strains, maintenance and constructs. All strains were maintained under standard culturing conditions on agar plates at 15°C or 20°C with OP50 as a food source on 6 or 9 cm tissue culture dishes with NGM (Greiner-Bio One B.V.). Ionizing radiation treatments were performed using a Cs^{137} source with a dose rate of 4.37 Gy/min. The *pie-1*::I-SceI (pRP1886) and reporter (pRP1879) constructs were cloned with the Gateway system and integrated independently by bombardment (plasmid sequences available upon request). Double transgenic strains were obtained by crossing of the two bombarded lines. Crossing leads to transgene instability because of the heterozygous situation combined with transgene cutting that occurs in the germlines of the F1 progeny. We therefore selected F2 populations that expressed LacZ in a subset of their offspring, and obtained two independent strains that were both used in this study.

We must note that we were unable to use the reporter strain for genetic crosses, because the DSBs will lead to loss of the reporter sequence in the heterozygous situation that occurs in the germlines of F1 animals: the default repair template, the homologous chromosome, does not contain the reporter, which will therefore be lost. The repair in various repair deficient backgrounds could therefore not be studied.

Cell cycle arrest. Animals were anaesthetized in 0.1M sodium azide and immobilized on 2% agarose pads under a cover glass. Germlines were scored *in vivo* for the presence of enlarged mitotic cells using DIC optics (Leica).

DAPI stainings. Animals were rinsed off plates in demineralized H_2O, washed 3 times and incubated in 70% ethanol with 1:1000 DAPI for 30 minutes. Worms were washed in PBS-0.1% Tween-20 three times 10 minutes and mounted in 20% glycerol.

Immunostainings. Gonads from young adult animals (1 day post-L4) were dissected in egg salts (0.12 M NaCl; 48 mM KCl; 2 mM CaCl2; 2 mM MgCl2 (Mallinckrodt-Baker B.V.); 25 mM Hepes (pH7.4); 0.1% Tween-20 and 0.2 mM levamisole), fixed in 1.8% paraformaldehyde in egg salts for 5 minutes and snap-frozen onto poly-lysine coated glass slides in liquid nitrogen for 10 minutes. Slides were incubated in 100% methanol at -20oC for 20 minutes and then washed in PBS with 1% Triton three times, 10 minutes. Blocking was performed in PBSTB (PBS supplemented with 0.1% Tween and 1% BSA) for 30 minutes at RT, followed by three 10-minute washes in PBS-0.1% Tween-20 (PBST). First antibody incubation (1:200 αRAD-51 in PBSTB) was performed o/n at 4oC. Slides were washed 3 times in PBST and incubated in secondary antibody (1:1000 goat Alexa-44 conjugated anti-rabbit) with DAPI in PBST for 3 hours at RT in the dark. Slides were washed 3 times in PBST, mounted in Vectashield and observed using a Leica SP5 confocal microscope.

Deletion analysis. Single worms were picked into lysis buffer (50mM KCl; 2,5% mM MgCl2; 10mM Tris-HCl pH8; 0.45% Nonidet P40; 0.45% Tween-20; 0.01% Gelatin; 200 µg/ml proteinase-K) and lysed for 60 minutes at 60oC. Proteinase K was inactivated for 15 minutes at 95oC and 1/10 of the lysis mixture was used for a nested PCR with primers in the flanks of the I-SceI site.

X-gal staining procedure. Populations were heat shocked for 2 hrs at 34ºC to induce β-galactosidase expression and immediately subjected to X-gal staining as described in Pontier and Tijsterman (2009).

TUNEL assay. Gonads were prepared as described for the immunostainings. After incubation in liquid nitrogen, slides were washed in PTX (PBS with 0.4% Triton) three times and permeabilized in 100 mM sodium citrate for 20 minutes at 65oC. Slides were rinsed in PTX 2 times and blocked for 30 minutes at RT in blocking buffer (0.1 M Tris-HCl pH7.5 containing 3% BSA and 20% sheep serum), followed by two 10-minute washes in PBS. Excess buffer was wiped off with a Kim wipe and gonads were incubated in 30 µL TUNEL reaction mixture (27 µL labeling buffer with 3 µL enzyme mix) (In situ Cell Death Detection Kit, TMR Red from Roche) for 1.5 hrs at 37oC. The reaction was stopped by washing in PTX once, followed by two washes in PBST, incubation in PBST with DAPI for 1 minute and three additional washes in PBST. Germlines were mounted in Vectashield and apoptotic cells were scored in the germline bend (late pachytene region) using a DM6000 deconvolution microscope from Leica.

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CHAPTER 5

DOG-1 PREVENTS DOUBLE-STRAND BREAKS AT G4 DNA-INDUCED STALLED REPLICATION FORKS

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SUMMARY

The helicase DOG-1, the *C. elegans* orthologue of human Fanconi J protein, is involved in the repair of DNA crosslinks and in the maintenance of G4 DNA, stable secondary structures that can be adopted by particular G-rich ssDNA sequences. Mutants for *dog-1* show unidirectional deletions that initiate at G4 sequences and end at various locations upstream. Here, we show that these deletions are generated through a DSB intermediate. First, deletions in *dog-1* mutants have distinctive repair footprints that are reminiscent of footprints at sites of transposon excision. Second, spontaneous RAD-51 foci are increased almost twofold in *dog-1* mutants. Third, we observed that homology-driven repair at G4 DNA resembles repair of an endonuclease-induced double-strand break (DSB). Finally, homology-driven repair did not occur when the homology was placed close to the 5' end of the G4 sequence, suggesting that the DSB is generated through 5'-incision and removal of the sequence between the G4 sequence and the next Okazaki fragment.

INTRODUCTION

DNA intra- and interstrand crosslinks covalently link two bases within or between DNA strands, respectively, and thereby pose blocks to approaching replication forks. Interstrand crosslinks activate a complicated response that removes the crosslink and allows stalled replication to proceed. This response consists of the activation of an intricate network of repair pathways that includes the Fanconi Anemia (FA) pathway, translesion synthesis (TLS) and nucleotide excision repair (NER).

Fanconi Anemia is a recessive and phenotypically heterogeneous disease caused by mutations in any of the thirteen genes FANCA, B, C, D1, D2, E, F, G, I, J, L, M or N (D'Andrea and Grompe, 2003). Cells from Fanconi Anemia patients display spontaneous chromosomal aberrations and increased sensitivity to DNA crosslinking agents. When the DNA replication fork encounters the crosslink, the FA-core complex is recruited. This complex, composed of FANC-A, B, C, E, F, G, L and M, monoubiquitinates the ID-complex, which consists of the closely related paralogs FANCD2 and FANCI (Smogorzewska et al., 2007). Studies in *Xenopus* cell extracts showed that the ID-complex is subsequently required for recruitment of nucleases, which incise the DNA to release the crosslink (Knipscheer et al., 2009). The crosslink is then bypassed by a TLS polymerase and the crosslink is further removed by NER, after which replication can be restarted by homologous recombination (HR). Whereas the use of site-specific interstrand crosslinks have firmly established that the FA-pathway responds to these lesions in this manner, it remains to be determined whether intrastrand crosslinks elicit a similar response: These lesions can also be repaired in a replication-independent manner by NER (De Silva, NAR 2002).

The last three FANC proteins, FANCJ, FANCD1 and FANCN, function downstream of or parallel to monoubiquitination of the ID-complex and are thought to be involved in re-establishing the replication fork by homologous recombination (HR). This hypothesis is strengthened by the fact that these proteins have all been found to be allelic with known HR factors, being BRIP1/BACH1 (BRCA1 interacting protein), BRCA2 and PALB2 (PArtner and Localizer of BRCA2), respectively (Howlett et al., 2002; Levitus et al., 2005; Levran et al., 2005; Litman et al., 2005; Rahman et al., 2007; Reid et al., 2007). Despite this dual role that is specific for these three genes, mutations result in similar phenotypes as the other Fanconi genes. Many genes in the FA-pathway are functionally conserved in the nematode *C. elegans*, including FANCJ (*dog-1*), BRCA2 (*brc-2*), FANCD2 (*fcd-2*), FANCM (*fncm-1*) and FANCI (*fnci-1*) (Collis et al., 2006; Lee et al., 2010; Martin et al., 2005; Youds et al., 2008).

FANCJ, a helicase belonging to the recQ family, recently became of particular interest because of an unusual phenotype that was described for this gene in *C. elegans*. Worms defective for the FANCJ ortholog, *dog-1*, display genome instability of quadruplex or G4 DNA, which is a stable secondary structure of ssDNA that can be adopted by particular G-rich sequences (Cheung et al., 2002; Kruisselbrink et al., 2008). *Dog-1*

mutants showed frequent deletions of these sequences, which always initiated near the 3'-end of G4 sequence and ended at various locations upstream, with an average deletion size of ~120 bp (Kruisselbrink et al., 2008). The G4 secondary structure can only be adopted by ssDNA, and together with the unidirectional orientation of the deletions relative to the G4 sequence, this led to the hypothesis that G4 DNA can form in the lagging strand during DNA replication, and requires DOG-1 to be bypassed. Interestingly, FANCD2, which is absolutely required for the canonical FA pathway also in *C. elegans* (Lee et al., 2010), is not involved in G4 DNA stability (Youds et al., 2008). This points towards an FA-independent function for *dog-1* in the maintenance of G4 DNA.

How *dog-1* functions exactly to resolve G4 DNA is unclear. The first, and perhaps most likely, explanation is unwinding of G4 DNA by the helicase activity of DOG-1. Indeed, FANCJ has been shown to display G4-unwinding capacity *in vitro* (London et al., 2008; Wu et al., 2008). Mutants in the ortholog of Bloom syndrome helicase (*him-6*), which also display this activity, have no G4 DNA phenotype, but a high percentage of embryonic lethality is observed in double mutants of *dog-1* with *him-6*. FANCJ is the binding partner of BRCA1, which could also suggest HR-mediated bypass of the lesion through the sister chromatid. However, DOG-1 lacks the domain with which human FANCJ interacts with its binding partner BRCA1, and human FANCJ constructs that lack the BRCA1 interacting domain can still efficiently rescue the crosslink sensitivity of FANCJ deficient cells (Bridge et al., 2005). In agreement with this, *dog-1* and *brc-1* have non-epistatic roles in the response to crosslinking agents (Youds et al., 2008), and *brc-1* mutants do not display G4 instability (Kruisselbrink et al., 2008). This suggests that HR does not play a role together with *dog-1* in the repair of G4 DNA.

Which mechanism is responsible for deletion formation in *dog-1* mutants remains unknown. Deletions still occur in double mutants of *dog-1* with the homologous recombination genes *brc-1* and *brc-2*, with the non-homologous end joining gene

Table 1 | Insertions in G4-induced deletions in *dog-1* mutants

Name	5'-flank*	Deletion 5'	Deletion 3'
19B_ggg333	ATACTAACCACTCCCCCCCC	CCCCCCCCCCCCC	GTTTTTTAATGC
2_qua262	AAAACTCTTTGAAATCCCCT	CCCCCCCCCCCCC	TGCAATCGA
Human_ggg268	CCGTCTGCCGGGTTTTTGTG	CCCCCCCCCCCCC	CTGGTTCTAT
Human_ggg6	GTGAAAATTCAAAACATTCC	CCCCCCTCCCTCC	TTTATTTGCCAC
17_ggg106	ACATTCATAAACTTACCCCC	CCCCCCCCCCCCC	CAGAGCTCTG
14_qua1215	GATTTTTATTTGAATTTTTC	TCCCCCCCCCCCC	TTCTCAAATATT
15_ggg385	GTCTTTTTTCTCCAGTACTC	CCCCCCCCCCCCC	GTGTACGTGTGC
4_ggg389	CCTTCATTTTCACGTCGCGC	CCCCCCCCCCCCC	TTGATTTGGAGA
16_qua0066	GCTCTTTTCTTTAAAAGTGC	CCCACCCCCCCCC	GCTATTGATATC

* sequences from the flank that are duplicated in insertions are underlined

lig-4 and with the nucleotide excision repair and single-strand annealing gene *xpf-1* (Kruisselbrink et al., 2008). This indicates either that these pathways do not contribute to deletion formation, or that the underlying pathways are highly redundant.

We investigated the mechanism of deletion formation at G4 DNA. Using DNA sequencing and a wide range of genetic mutants combined with transgenic reporter assays, we found that these deletions strongly resemble deletions generated at known DSBs; similar repair footprints were observed at G4 DNA as at sites of transposon excision, and homology-mediated repair at G4 DNA and at endonuclease-induced DSBs showed similar genetic requirements. These findings, together with an increase in the number of spontaneous RAD-51 foci, a hallmark of DSBs, in *dog-1* mutants, lead us to propose a model for the generation of deletions at G4 DNA through DSB intermediates.

RESULTS

G4 DNA deletions resemble sites of transposon excision

When we studied the footprints of 63 germline deletions at G4 DNA sequences in *dog-1* mutants that were identified by comparative genome hybridization (Kruisselbrink et al., 2008), two noteworthy features were observed: First, none of the footprints revealed repair via flanking microhomology. Secondly, we observed insertions accompanying the deletions in 15 out of 63 deletions, and these insertions ranged from 1-8 nucleotides. Of these, eight insertions consisted of sequences that were derived from upstream and downstream flanking DNA (Table 1), and the remaining seven were composed of AT-rich sequences that could not be directly mapped to one of the flanks. Interestingly, a very similar deletion/insertion pattern has previously been observed at excision sites of the Mos1 transposon (Robert and Bessereau, 2007) and in *C. elegans* at excision sites of the Tc1 transposon (Brouwer, 2009). Transposons are mobile DNA elements that can be excised by the enzymatic activity of a transposase protein, leaving a DSB at

Inserted sequence	**3'-flank***
ATTTTTATAGAGCTTTTATGCAATAT	TTTCATAGAGCTTTTATGCAATAAATAC
TTTTAACAGAAAAAT	TTTTTAACAGAAAAATTTGAAAATCACG
TTATTTTTGTG	TGATTAATTCTTATTTTATAAAACTTAG
AAAAAACA	TTTTTTATTGACTACTGTAAAGCCAATT
TGAAATT	ACTGAAATTGATCTTGGCGCCTTTGAAC
ATCA	TATACGAAAGAAAATCCAGAAAAAGCCA
AATATTTT	TCATATATTTGTTTTTACAGATCCGCCG
GAAG	AAGTGAAGAAGGGGCAGCTAGGAGAAAA
AAG(A)AAAAG	TGTCGGCATCTAAATGTTCCCAAGAGAG

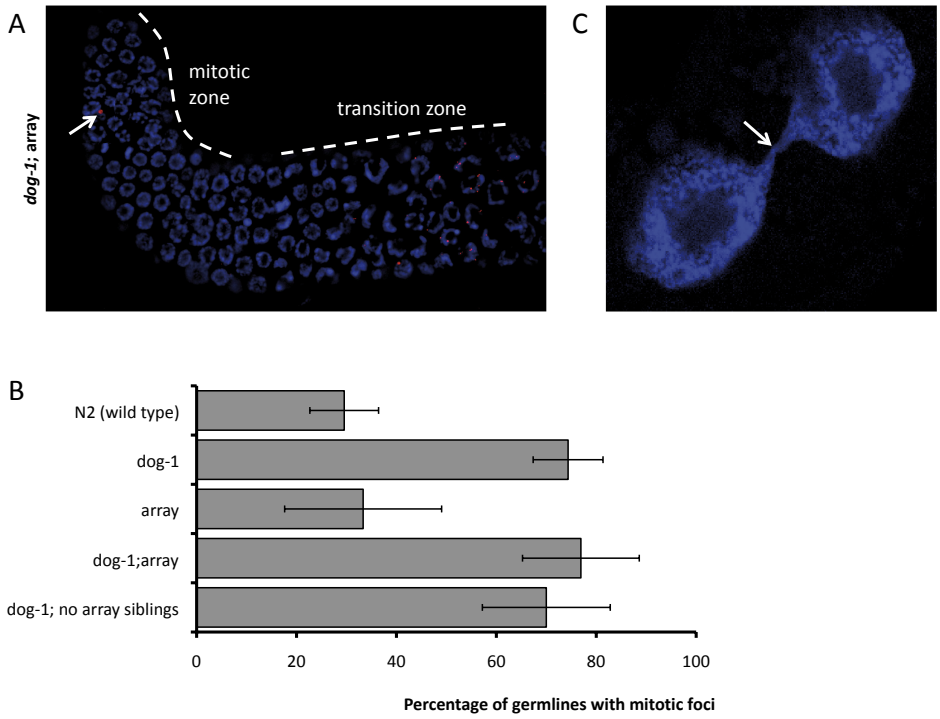


Figure 1 | Hallmarks of spontaneous DNA damage in *dog-1* mutants. **(A)** Representative image of RAD-51 staining of untreated *C. elegans dog-1* germline with mitotic and meiotic compartment indicated. Foci from the transition zone onwards are caused endogenously by SPO-11, whereas mitotic foci (arrow) represent spontaneous damage during DNA replication. **(B)** Quantification of spontaneous foci in wild type and *dog-1* mutant germlines, as well as germlines carrying an extrachromosomal array with a C_{23}-tract. The array is not inherited 100%, and as a control, siblings not carrying the array were also stained. **(C)** Spontaneous DNA bridging (arrow) in *dog-1* mutant intestinal cells.

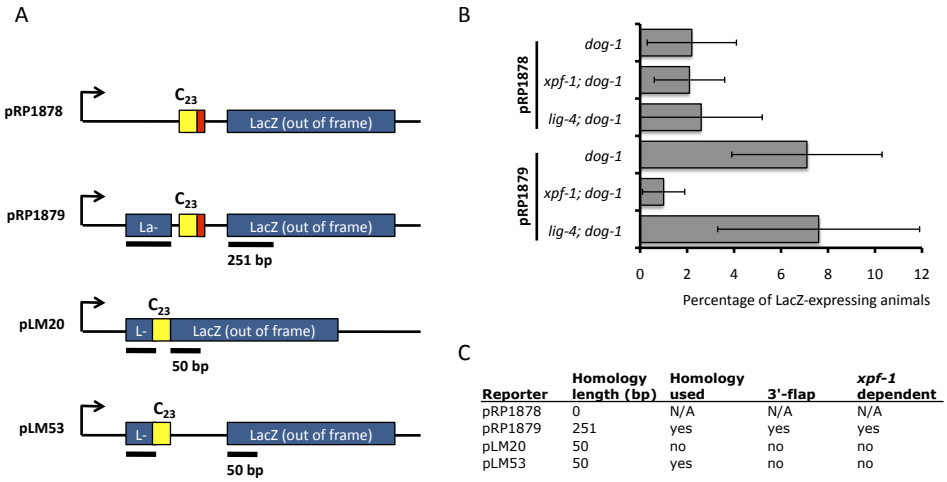
the excision site (Plasterk, 1993). Thus, the fact that footprints derived from germline transposon activity closely resemble the footprints that occur at G4 DNA deletions, suggests that G4 DNA deletions may be also derived from a DSB intermediate.

Spontaneous DNA damage foci occur in *dog-1* mutants

To repair DSBs by homologous recombination (HR), the broken ends are resected from the 5'-ends. The free ssDNA 3'-ends that arise by resection can subsequently serve as a primer for DNA synthesis from an homologous template (Wyman and Kanaar, 2006). The invasion into the homologous template requires the enzymatic activity of the strand exchange protein RAD-51, which is required for repair of DSBs by HR, and focus formation of this protein is a hallmark of the presence of DSBs. In the *C. elegans* germline, cells are oriented in a spatio-temporal organization, where cell cycle progression through mitosis and meiosis I is accompanied by physical movement of the cells through the germline (Garcia-Muse and Boulton, 2007). The physical position of

a cell in a particular germline compartment is thus linked to the cell cycle stage. In the meiotic compartment of the germline, spontaneous RAD-51 foci form abundantly as a result of endogenous SPO-11 activity, an enzyme that cleaves DNA to induce crossover formation in meiosis (Alpi et al., 2003). However, in the premeiotic compartment of the germline, RAD-51 foci do not normally occur, except as the result of DNA damaging agents, such as ionizing radiation or crosslinking agents. This region of the germline is therefore very well suited for the study of damage-induced RAD-51 foci.

We performed RAD-51 antibody stainings on dissected germlines from wild type and *dog-1* mutant animals, and scored the number of germlines that contained one or more RAD-51 foci in the mitotic compartment (Figure 1A). We found that the number of spontaneous RAD-51 foci was significantly increased in *dog-1* mutants: ~30% of wild type germlines contain mitotic RAD-51 foci, whereas foci were detected in ~74 % of *dog-1* mutant germlines (Figure 1B), suggestive of spontaneous DSBs in *dog-1* mutants. This percentage remained unaltered in strains carrying a high copy-number extrachromosomal array containing a homoguanine tract (G_{23}) (Figure 1B), probably because the relative increase in G4 DNA is small. These data support the notion that a DSB can be formed at G4 DNA, which can be either followed either by binding of RAD-51 and error-free repair by HR, or by deletion formation through an unknown mechanism.



C

Reporter	Homology length (bp)	Homology used	3'-flap	*xpf-1* dependent
pRP1878	0	N/A	N/A	N/A
pRP1879	251	yes	yes	yes
pLM20	50	no	no	no
pLM53	50	yes	no	no

Figure 2 | Reporter assay for deletion formation. (A) Schematic representation of the four reporters used to study deletion formation in *dog-1* mutants. Yellow, C_{23} tract; red, stop codons. Black bars indicate homologous sequences. **(B)** Quantification of LacZ expression in two low-copy reporter strains. Expression is more frequent in the reporter that contains homology, and this expression depends on the SSA nuclease XPF. **(C)** Overview of results for the four reporters. Homology is not used when placed directly downstream of the C_{23}-tract, and homology-mediated repair only depends on XPF when flaps are present after annealing of homologous sequences.

Homology-requirements

To further investigate whether G4 DNA deletions arise from DSB intermediates in germline and somatic tissues, we compared G4-induced deletion formation with deletions derived from a known DSB-inducing enzyme; I-SceI. We constructed several G4 DNA containing transgenes (Figure 2A), using a similar transgenic approach as described previously, where a C_{23}-tract was placed downstream of the first part of a LacZ ORF (Kruisselbrink et al., 2008). Deletions of certain sizes that occur at the G4 sequence, or homology-mediated repair that uses repetitive sequences that were placed upstream and downstream of the G4 sequence, will result in an intact LacZ ORF. ORF restoration can be observed by staining for LacZ and can be used to study the requirements for deletion formation at the G-tract in the transgene.

First, we investigated whether the flanking sequence homology around the G4 site could be used for repair in somatic cells. Although the exact copy number has not been determined, we observed more frequent LacZ expression in the reporter with homology (pRP1879) than in the one without (pRP1878) (Figure 2B), suggesting that homology is preferentially used when available. Using a similar transgenic approach, we have previously shown that repair through such homology largely depends on the nuclease XPF-1 in the case of I-SceI-induced DSBs in somatic cells (Pontier and Tijsterman, 2009). If G4 DNA is processed through a DSB intermediate, homology-mediated repair is predicted to depend on XPF-1. Indeed, when we assayed LacZ expression in *dog-1;xpf-1* double mutants in both transgenes, we found that homology-mediated repair of pRP1879 was greatly reduced, whereas non homology-mediated repair in pRP1878 remained unaffected (Figure 2B). Homology-mediated repair at G4 DNA and at I-SceI-induced DSBs thus have similar genetic requirements, strengthening the hypothesis of a DSB intermediate in G4 DNA deletions.

Formation of the DSB through incision or breakage

We have previously shown that in the absence of *lig-4*, repair of an I-SceI induced DSB leads to fewer deletions and to more frequent use of (micro)homology among the remaining deletions in somatic cells (Pontier and Tijsterman, 2009). However, we observed no change in the frequency of LacZ expression in both *dog-1* reporters in the absence of *lig-4*, suggesting that NHEJ is not acting on G4-induced DSBs (Figure 2B). This could be due to the cell cycle phase at the time of induction or because of different substrates posed by *dog-1* induced DSBs versus I-SceI induced DSBs: A DSB intermediate at a fork stalled at G4 DNA could be generated by incision or breakage at either the 5' of the 3' side of the G4 sequence. In both cases, a large flap of ssDNA results that stretches from the stalled fragment up to the next Okazaki fragment, which possibly prevents NHEJ from acting on this lesion. This flap is absent in the case of I-SceI-induced DSBs.

To further investigate how G4 DNA leads to deletions, we constructed a transgene where the upstream homologous sequence ends at the start of the G4 sequence,

whereas the downstream homology starts within 24 nt from the end of the G4 site (Figure 2A, pLM20). In this scenario, the 3'-overhang can directly anneal to the downstream homology without causing a 3'-flap, leaving no substrate for XPF. We started two thousand independent populations and assayed these for germline ORF restoration. Nine populations were identified where a germline ORF restoration event had occurred, which makes sequencing analysis possible. Interestingly, sequencing showed that none of these were caused by repair using the homologous flanking sequences; instead, these nine populations contained non-homologous deletions that also resulted in an in frame and functional LacZ product. This could indicate that

Table 2 | Positive clones from genome wide RNAi screen for synthetic lethality with *dog-1*

A	**Most reproducible hits**			
	Cosmid	**Gene**	***H. sapiens***	**Function**
	M04F3.1	*rpa-2*	RPA2	Replication protein A2; ssDNA binding protein for repair
	Y48G1C.7		-	Uncharacterized unconserved
	Y48G1C.8		-	Uncharacterized unconserved
B	**Intermediate hits**			
	Cosmid	**Gene**	***H. sapiens***	**Function**
	Cell cycle, apoptosis and repair			
	Y43C5A.6	*rad-51*	RAD51	Double-strand break repair recombination enzyme
	C54G10.2	*rfc-1*	RFC1	Replication factor C, subunit RFC1 (large subunit)
	F59E12.4/5	*npl-4.1/2*	NPLOC4	Nuclear Pore Complex
	C45G3.1	*aspm-1*	ASPM	Microtubule-binding spindle protein
	F25H2.11	*tct-1*	TCTP	Microtubule-binding protein, translationally controlled tumour protein
	W02a2.6	*rec-8*	REC8	Meiosis-specific cohesin complex component
	Y38f1a.5	*cyd-1*	CCND2	Cyclin D homolog
	ZK328.1a	*cyk-3*	UBP32	Ubiquitin C-terminal hydrolase
	F37A4.8	*isw-1*	ISWI	SWI/SNF regulator of chromatin
	F55g1.11	*his-60*	HIST1H4H	Histone H4
	K03A1.6	*his-38*	HIST1H4H	Histone H4
	Other			
	F46A9.4	*skr-2*	SKP1	Core component of SCF Ubiquitin ligase complex
	f33g12.4	*lrr-1*	PPIL5	Leucine rich repeat protein
	F29E12.12	*bli-2*	COL3A1	Collagen
	K07f5.14		NOL8	Uncharacterized nucleolar protein
	K11B4.1		RT27	Ribosomal protein S27
	B0361.6		C9orf114	Uncharacterized conserved protein w/ DUF171 domain
	K07A1.1		-	Uncharacterized unconserved protein

homology close to the G4 sequence is not used for repair, or the homology of 50 bp may be too small to be used for repair. To distinguish between these possibilities, we constructed a similar transgene where the same 50 bp homology was placed further downstream of the C_{23}-tract (Figure 2A, pLM53). Here, we observed five ORF restoring events, and sequencing showed that these had all used the homology (Figure 2C).

These data show that homology close to the G4 sequence can not be used for repair as opposed to homology placed more downstream. This suggests that the sequence

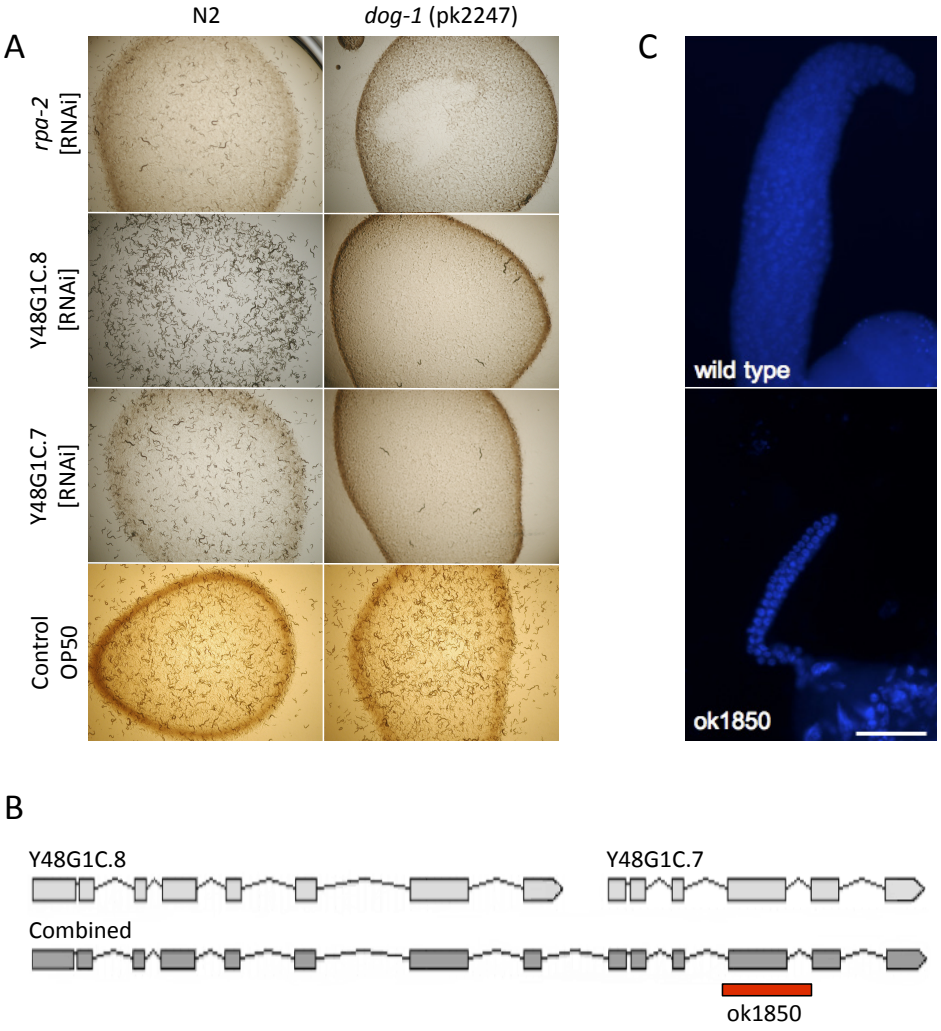


Figure 3 | Synthetic lethal interactions with *dog-1*. **(A)** Growth of wild type versus *dog-1* mutants on indicated RNAi or control foods on plates. **(B)** rtPCR was used to show that predicted genes Y48G1C.7 and Y48G1C.8 (top) actually belong to a single ORF (bottom). Allele ok1850 is a deletion of the 12th exon and is indicated in red. **(C)** Germline morphology is abnormal in homozygous ok1850 mutants.

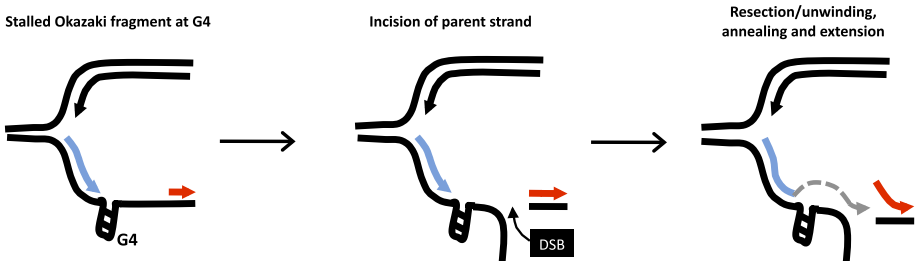


Figure 4 | Model for deletion formation at G4 DNA. We propose the following model for G4 DNA induced deletion formation in *dog-1* mutants. Because of the prolonged exposure of ssDNA in the lagging strand, G4 DNA will preferentially form here. The replication fork stalls at this structure and can not proceed in the absence of DOG-1. This situation in instable and can lead to a DSB 5' to the G4 sequence. Resection or unwinding of the 3'-ends at either side of the break can then lead to invasion and minimally templated extension of free 3'-ends, leading to deletions that may be associated with insertions if this occurs in multiple rounds of annealing, extension and release.

directly downstream of the G4 sequence is removed before homology-mediated repair takes place, or that the DSB is created upstream of the second repeat, so that both homologies are present at the same side of the DSB. This is compatible with a model where the DSB arises 5' to the G_{23} tract: in this model, the DNA is incised or broken at the fragile RNA/DNA hybrid of the next Okazaki fragment, causing a DSB. Further processing can be done by resection or unwinding of the downstream fragment, so that the stalled 3'-end can invade – with or without using homology – to resume replication. This way, homologies that are located more distantly can be used for repair, whereas nearby homology is lost in the first incision (Figure 4, bottom). Also, the observation that insertions can arise from sequences on either flank can be explained by this model, because free 3'-end arise at both sides of the DSB. In an alternative scenario, incision at the 3' end and subsequent 5' resection would result in a free 3'-overhang of the stalled fragment. Then, we would expect nearby homologous sequences to serve as repair templates, but we observed none such events (pLM20, Figure 2C). Therefore, we propose that 5' incision or breakage is the most likely route of action.

At present, we do not know whether these DSBs are generated via nuclease action (in a regulated repair pathway), or if they are the outcome of physical stress generated at the unresolved replication intermediate. It has previously been shown that *dog-1* mutants display DNA bridges that were formed between two cells in response to crosslinking treatment (Youds et al., 2008). These bridges generally arise from aberrant chromosome segregation during cell division, and they occur spontaneously in e.g. in cells deficient for Bloom (BLM), a helicase that is required for proper DNA replication (Chan et al., 2007). Here, it was proposed that these bridges represent sites of unresolved replication intermediates. Because G4 DNA is also a type of unresolved replication intermediate in *dog-1* mutants, we investigated whether these bridges can also form spontaneously in *dog-1* mutants. Indeed, we frequently observed intestinal cells that were connected

through a fine thread of DNA specifically in *dog-1* mutants (Figure 1C). We did not observe such bridges in wild type. Speculatively, this may indicate that the DSBs are formed by breakage instead of incision; the bridges may occur in loci where tension has not increased sufficiently for DNA breakage.

Synthetic lethality with *rpa-2*

Despite extensive screening, no other genes have been found with a phenotype similar to *dog-1*. Moreover, no genes that are responsible for deletion formation have been identified in *dog-1* mutants. We hypothesized that blocking the pathway that leads to deletions in *dog-1* mutants may lead to lethality, because stalled forks would persist, leading to cell death. We thus performed a genome-wide RNAi screen for synthetic lethality to identify these factors. Wild type and *dog-1* (pk2247) deficient worms were grown in 96-well format and RNAi was performed by feeding bacterial clones from the Ahringer library, which covers almost 90% of the worm genome (Fraser et al., 2000). Wells were scored positive if growth was normal in wild type animals, but lethal in *dog-1* deficient worms. All hits were confirmed with a second *dog-1* allele (pk2178) to exclude synthetic lethality with mutations that accumulate in the background of this strain.

From a total of ~17.000 clones, we obtained only three clear hits that showed a strong effect and that were reproducibly synthetic lethal with both *dog-1* alleles (Table 2A). Besides these, we also identified a set of "intermediate" clones of which the phenotypes were less penetrant or more variable (Table 2B). All these clones have been described to confer substantial lethality on their own. The penetrance of RNAi varies between experiments, and partial depletion can lead to intermediate phenotypes that are more difficult to reproduce consistently. Interestingly, many of the hits are conserved genes that are involved in cell cycle regulation and DNA replication and repair. For example, we identified microtubule-binding proteins, a cyclin and a cohesin protein. Also, we found several genes that are involved in chromatin remodeling during cell cycle progression or DNA repair, such as histone H4 and *isw-1*, and we found a component of the nuclear pore complex, which has also been identified in previous screens for DNA repair factors (Van Haaften et al., 2006). Also, we found *rad-51*. This gene is likely not involved in deletion formation, but in error-free bypass of G4 DNA (Youds et al., 2006). There is considerable overlap between genes that we identify here and genes that were previously identified to be required for ionizing radiation protection, suggesting similar underlying repair mechanisms for IR-induced lesions, of which the DSB is the most abundant and toxic, and for G4 DNA in the absence of DOG-1.

Next, we further examined the most prominent three hits. First, we found that *rpa-2* [RNAi] leads to synthetic lethality in *dog-1* mutants. While complete depletion of *rpa-2* causes 100% sterility, the RNAi knockdown consistently revealed synthetic lethality with *dog-1*. This interaction was confirmed when RNAi was performed on plates instead of liquid culture (Figure 3A). Functional interactions between the DOG-1 ortholog,

FANCJ, and the ssDNA-binding protein RPA2 have been described previously (Gupta et al., 2007; Suhasini et al., 2009). These results are compatible with the function of DOG-1 on ssDNA.

The second and third hits, Y48G1C.7 and Y48G1C.8, represent two neighboring genes. Their adjacent positions together with their similar phenotypes led us to hypothesize that these two genes may actually represent a single ORF. We used rtPCR to define the transcript borders and found that a splice site had been missed in the gene prediction of Y48G1C.8: Incorporation of this splice site indeed leads to a single ORF for Y48G1C.7 and Y48G1C.8 (Figure 3B). The newly identified single ORF contains a predicted nuclear localization signal and a putative DNA binding domain and is conserved in nematodes, but lacks a clear mammalian homolog. Although sequence analysis thus failed to identify conservation, functional homologs that lack sequence similarity have previously been identified in *C. elegans* (e.g. p53/*cep-1* and BRCA2/*brc-2*) and a metazoan functional homolog may be identified for this gene in the future.

We obtained a putative null allele of Y48G1C.7/8, ok1850, which deletes 985 bp of the ORF (Figure 3B). Homozygous null mutants of this allele were sterile, but viable, likely due to functional protein contributed from the heterozygous mother. DAPI stainings on adult worms revealed that germlines were small and lacked the characteristic meiotic stages (Figure 3C). Possibly, Y48G1C.7/8 is required for replication progression and subsequent entry into meiosis. Other phenotypes were also reminiscent of proliferation defects; animals were *pvl* (protruding vulva) and showed DNA bridges between intestinal cells (data not shown). We had anticipated that genes that are involved in deletion formation could cause synthetic lethality with *dog-1* mutants. To investigate this, we made double mutants of *dog-1* with ok1850 and performed deletion PCRs on two endogenous G-tracts that are frequently deleted in *dog-1* single mutants. However, deletions were still formed with similar frequency in the double mutant (data not shown), possibly due to maternal contribution of intact protein. Also, the phenotype of the double mutant was similar to the ok1850 double mutant with respect to the germline and vulval defects. Further investigation of this allele is necessary to establish the value of this synthetic interaction.

DISCUSSION

We investigated the mechanism by which deletions are formed at G4 DNA in *dog-1* mutants, and found four lines of evidence that support a model where G4 DNA is processed through a DSB intermediate (Figure 4): First, we identified numerous germline deletions that were accompanied by insertions in *dog-1* mutants, and these insertions closely resemble insertions at sites of transposon excision (Brouwer, 2009). Second, we observed more frequent spontaneous RAD-51 foci in *dog-1* mutants. RAD-51 foci are a general hallmark of DSBs, and are also observed in response to DSBs induced by ionizing radiation or by the endonuclease I-SceI (our unpublished

data, (Alpi et al., 2003). Third, homology can be used for repair in somatic cells in an XPF-dependent manner. Similar observations have previously been made in somatic cells where a DSB was introduced by I-SceI (Pontier and Tijsterman, 2009). Fourth, we found that homology close to the G4 sequence cannot be used for repair of G4 DNA. This can be explained by the generation of a DSB by 5'-incision or breakage at the side of the next Okazaki fragment, as this will cleave off any homology close to the G4 sequence (Figure 4), while incision at the other end would not have such an effect. Our data suggest that once the DSB is formed, repair can take place in an error-free fashion by RAD-51-mediated strand invasion and homologous recombination, or can lead to deletions by an unknown mechanism.

The genetic requirements for deletion formation remain elusive. For transposons, it does not depend on the NHEJ factor *lig-4* in germline cells. Germline G4 deletions have not been tested for *lig-4* dependency, but deletions form independently from *lig-4* in somatic cells, possibly because the substrate that arises contains a long ssDNA flap that can not be processed by NHEJ. Several pathways alternative to classic NHEJ have been described that could be involved here. Whereas some of these pathways depend on PARP1 and Ligase III, other pathways have unknown genetic requirements (Corneo et al., 2007; Pontier and Tijsterman, 2009; Schulte-Uentrop et al., 2008; Wang et al., 2003; Yan et al., 2007). For insertions at sites of transposon excision, a model has been proposed where insertions are generated by annealing and extension of a 3'-end with minimal priming (Brouwer, 2009). Insertions of multiple sequences can be explained by cycles of annealing, extension, release and re-annealing. In our model of how DSBs are formed at G4 DNA, a similar mechanism can be envisaged. Resection or unwinding is first required to release free 3'-ends. This can occur at both ends of the DSB (Figure 4). This way, this mechanism can explain how insertions of both flanks can arise and how the G4 sequence is lost through bypass.

If permanently blocked forks at G4 DNA lead to DSBs and loss of the sequence between the stalled fork and the next Okazaki fragment, this could well occur at any lesion that poses such a barrier. Besides G4 DNA, palindromes or intrastrand crosslinks may pose similar blocks to the approaching replication fork, and our model therefore possibly applies to a wider range of lesions. Palindromic sequences have previously been investigated for instability in *dog-1* mutants, but no evidence for deletions was found (Kruisselbrink et al., 2008), so these may be stabilized by other proteins than DOG-1. Intrastrand crosslinks – covalent linkage of two bases within the same DNA strand – are harder to study because they can not be generated in a site-specific way. Similar to G4 DNA in *dog-1* mutants, intrastrand crosslinks cannot be unwound and involve only one DNA strand. It is well-established that interstrand crosslinks – linkage between bases in opposing strands – lead to DSBs, but DSBs have not been observed after exposure to cisplatin, a chemical that induces predominantly intrastrand crosslinks (De Silva et al., 2002; Eastman, 1987; Fichtinger-Schepman et al., 1985). Nevertheless, deletions do occur in response to cisplatin treatment (our unpublished data). Possibly, DSBs at

intrastrand crosslinks are below the detection threshold because the majority is likely repaired in a replication-independent manner by Nucleotide Excision Repair (NER) (Zamble et al., 1996). Therefore, NER may prevent deletion formation at intrastrand crosslinks by removing them before DNA replication stalls at these sites. Indeed, null mutants for the NER gene *xpa-1* are more sensitive to cisplatin than *dog-1* or *fcd-2* mutants (Kruisselbrink et al., 2008), suggesting a more important role for NER than for the FA-pathway in intrastrand crosslink repair. Crosslinks that are not repaired by NER, for example in NER mutants, may be processed similar to G4 DNA. If this is the case, NER mutants should show DSBs and deletions in response to intrastrand crosslinks with the typical 5' unidirectional orientation. Site-specific intrastrand crosslinks are needed to further investigate this hypothesis, e.g. by determining the orientation, replication-dependency and average size of the deletions.

We observed synthetic lethality of the ssDNA binding protein RPA-2 with DOG-1. RPA-2 binds ssDNA that arises during replication, transcription and repair (Fanning et al., 2006). Interestingly, mammalian FANCJ has been shown to interact with RPA (Gupta et al., 2007; Suhasini et al., 2009). RPA may thus act to prevent the formation of G4 DNA, or to stabilize the ssDNA that arises after fork stalling. Interestingly, both RPA and FANCJ have been shown to resolve G4 DNA *in vitro* (London et al., 2008; Salas et al., 2006), suggesting partial redundancy as a potential cause of the synthetic lethality. Alternatively, RPA may be involved in the repair process once the G4 DNA has formed, e.g. as a stimulator for RAD-51 loading and HR repair of the stalled fragment. The significance of the synthetic lethality with the unconserved gene Y48G1C.7/8 therefore remains unknown.

In summary, we conclude that deletions in *dog-1* mutants are most likely generated through DSB intermediates, thereby explaining the resemblance of repair footprints to transposon excision, the spontaneous occurrence of RAD-51 foci and the failure to form deletions using a nearby repeat, while more distant repeats require similar genetic requirements as an endonuclease induced DSB.

MATERIALS AND METHODS

Constructs and strains. Constructs pRP1878 and pRP1879 were both integrated in low copy number by bombardment, whereas constructs pLM20 and pLM53 were integrated by injection and irradiation to generate high copy number extrachromosomal arrays. Plasmid sequences are available upon request. Alleles used were: Y48G1C.7/8 (ok1850, balanced by a *bli-4* and GFP-marked translocation, strain VC1360), *dog-1* (pk2247). Strains were maintained on 6 or 9 cm plates (Greiner Bio-One) on OP50 under standard conditions as described in Brenner (1974) unless otherwise indicated.

Immunostainings. Gonads from young adult animals (1 day post-L4) were dissected in egg salts (0.12 M NaCl; 48 mM KCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 25 mM Hepes (pH7.4); 0.1% Tween-20 and 0.2 mM levamisole), fixed in 1.8% paraformaldehyde in egg salts for 5 minutes and snap-frozen onto poly-lysine coated glass slides in liquid nitrogen for 10 minutes. Slides were incubated in

100% methanol at -20°C for 20 minutes and then washed in PBS with 1% Triton three times, 10 minutes. Blocking was performed in PBSTB (PBS supplemented with 0.1% Tween and 1% BSA) for 30 minutes at RT, followed by three 10-minute washes in PBS-0.1% Tween-20 (PBST). First antibody incubation (1:200 αRAD-51 in PBSTB) was performed o/n at 4°C. Slides were washed 3 times in PBST and incubated in secondary antibody (1:1000 goat Alexa-44 conjugated anti-rabbit) with DAPI in PBST for 3 hours at RT in the dark. Slides were washed 3 times in PBST, mounted in Vectashield and observed using a Leica SP5 confocal microscope.

X-gal staining procedure. B-gal expression was induced in transgenic strains by heat shock treatment for 2 hours at 34°C. Animals were fixed in acetone and stained in 5% X-gal overnight in the dark. The amount of LacZ expression was quantified by scoring of the percentage of animals that contained at least one blue patch.

RNAi screening in liquid. RNAi screening was performed in liquid cultures in 96-well tissue culture plates (Greiner Bio-One B.V.) as described previously (van Haaften et al., 2004). Positive hits were repeated at least twice in liquid and once on RNAi plates. All clones were sequenced to confirm their identity.

RNA isolation and rtPCR. To isolate RNA, worm pellets from N2 and from VC1360 GFP negative (ok1850/ok1850) animals were snap frozen in liquid nitrogen. Four volumes of trizol with β-mercaptoethanol (12.5 µL for every 1 mL trizol) were added and samples were subjected to three cycles of freeze/thawing. Samples were vortexed at RT for 30s and placed on ice for 30s, these steps were repeated 5 times. After incubation at RT for 5 minutes, cellular debris was spun down at 12k x *g* at 4°C for 10 minutes. One worm-pellet volume of chloroform was added to the supernatant, sample was vortexed, incubated at RT for 10 minutes and spun down at 12k x *g* at 4°C for 15 minutes. Chloroform extraction was repeated once with the supernatant and RNA was precipitated in an equal volume of isopropanol. Pellets were redissolved in 25 µL H_2O.

Primer (10 nmol, sequence GTGATGCCGTCAGGATTGG) and RNA (1 µg) were incubated for 5 min at 65°C and for 15 min at 4°C. cDNA was made by incubation with MLV1 reverse transcriptase and dNTPs at 37°C for 2 hours. cDNA samples were diluted 25 times and 10 µL of the dilution was used in a PCR reaction using primers around the predicted stop codon of Y48G1C.8 (sequences GTGATGCCGTCAGGATTGG and AATGTTGTCGAAGAACGTGG). We observed a band of the expected size for a combined Y48G1C.7/8 ORF in the N2 sample only. This band was purified from gel using a gel purification kit (Qiagen) and sequenced. Sequencing revealed that a splice site upstream of the putative Y48G1C.8 stop codon had been missed. The combined protein is 1426 amino acids and consists of 14 exons. The sequence around the novel splice site is: (Y48G1C.8 - GGA TCA CAG TTT CAG/ACC AAA GAC AAC ATG GAC – Y48G1C.7)* *Underlined; previously predicted ATG start site of Y48G1C.7; the position of the novel splice site is indicated by the /.

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CHAPTER 6

ISOLATION OF DELETION ALLELES BY G4 DNA-INDUCED MUTAGENESIS

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SUMMARY

Metazoan genomes contain thousands of sequence tracts that match the guanine-quadruplex (G4) DNA signature $G_3N_xG_3N_xG_3N_xG_3$, a motif that is intrinsically mutagenic, probably because it can form secondary structures during DNA replication. Here we show how and to what extent this feature can be used to generate deletion alleles of many *Caenorhabditis elegans* genes.

RESULTS AND DISCUSSION

The ability of DNA sequences to form structures other than canonical Watson-Crick duplexes is potentially dangerous for the faithful segregation of genetic information. Such structures may form during processes that involve transient denaturation of the DNA duplex, such as DNA replication, transcription or DNA repair (Maizels, 2006).

Thermodynamically stable secondary structures could impede the progression of DNA and RNA polymerases (Tornaletti et al., 2008; Voineagu et al., 2008), and it has been suggested that specialized proteins exist that resolve or repair such structural impediments. One structure that readily forms *in vitro* in guanine-rich DNA is guanine-quadruplex DNA (G4 DNA)(Gellert et al., 1962; Sen and Gilbert, 1988): guanine-rich ssDNA can adopt a four-stranded configuration, which consists of square arrangements of guanines, stabilized by Hoogsteen hydrogen bonding (Figure 1A). After observations that poly(G) tracts are deleted in *C. elegans* strains with mutations in the DNA helicase *dog-1*, the orthologue of the human Fanconi J (FANCJ) gene (Cheung et al., 2002; Youds et al., 2008), we recently showed that G4 DNA is mutagenic in this genetic background *in vivo*. We found that the G4 DNA signature $G_3N_xG_3N_xG_3N_xG_3$ is the best predictor of *dog-1*–dependent mutagenicity (Kruisselbrink et al., 2008). Array Comparative Genome Hybridization (CGH) analysis of so-called mutator accumulation lines (worms were clonally grown for ten generations, allowing DNA replication errors to accumulate) demonstrated that G4 DNA sequences can be deleted in germ nuclei at very high frequency (Kruisselbrink et al., 2008). One specific guanine tract (ggg317) was lost in 3 of 16 *dog-1* substrains after just ten generations of growth. Such great extent of site-specific mutagenesis suggests that deletion alleles of genes that flank a G4 DNA site can be isolated in populations of *dog-1* worms. Here we describe the methodology to do so, provide proof of principle by isolating deletion alleles in various genes and list all *C. elegans* G4 DNA sites for researchers to inspect whether their genes of interest are close to one of these sites. The G4 sites from this list are also included in WormBase.

We identified 2,907 sites in the *C. elegans* genome that matched the G4 DNA signature $G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}$ (four stretches of at least three consecutive guanines, alternated by 1-7 nucleotides of any kind). We named these sites *qua (or ggg)1–qua2907,* where prefix ggg refers to homoguanine tracts, and qua to non-homoguanine G4 DNA. Increasing tract length and G-richness may increase the probability of G4 DNA formation and we thus ranked these endogenous sites according to the number of possible different G4 DNA configurations. As an example, there is only one way to fold qua129, GGGTGGGAGGGAGGG, into a four-stranded configuration with three planar guanine quartets (with one nucleotide in the loop), whereas there are 83 different ways to fold qua1442, GGGAGGGGGTGGGGGAGGGGGGGG, into a structure with three guanine quartets. Along the same rationale, a homoguanine tract of similar size ranks very high.

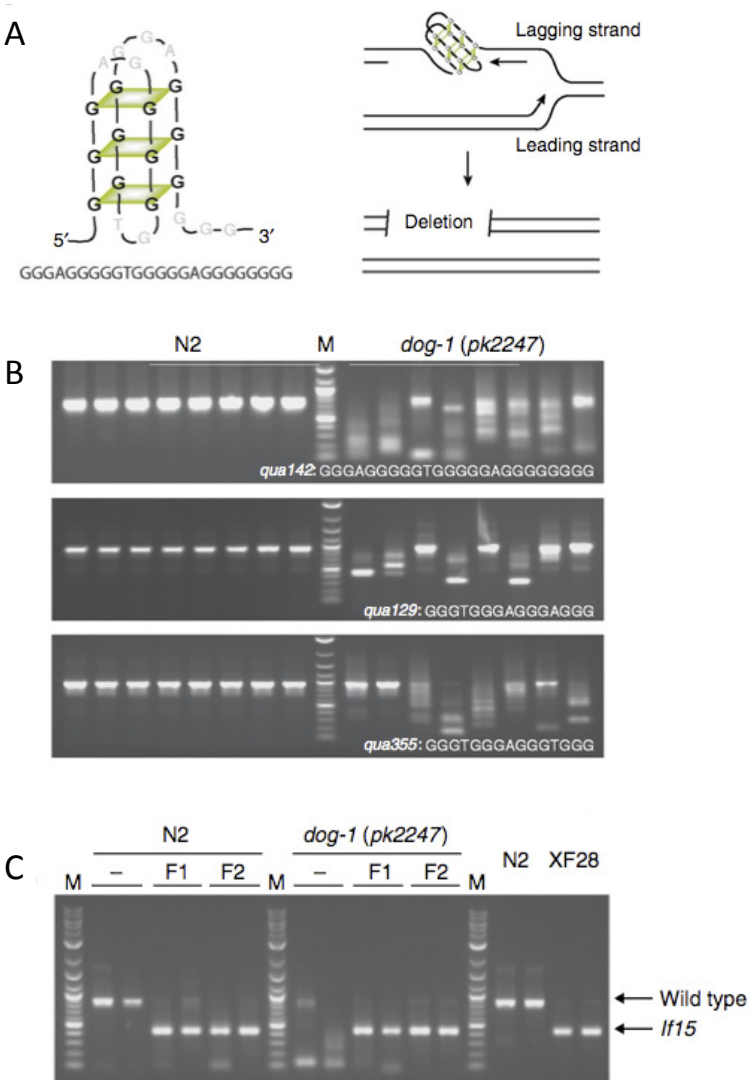

Figure 1 | Mutagenic potential of G4 DNA tracts. (A) One possible configuration of *qua1442*, GGGAGGGGGTGGGGGAGGGGGGGG, in which planar rings of four guanines (guanine quartets) can stack on top of each other, thereby forming a DNA structure that is thermodynamically stable under physiological conditions (left). Model to explain G4 DNA–induced deletion formation. A G4 DNA structure in the lagging strand blocks progression of DNA replication. In the absence of FANCJ/*dog-1* this problem is overcome (via an unknown error-prone repair mechanism) at the expense of sequence information. **(B)** Results of a PCR analysis on DNA samples derived from >10^4 wild type (N2) or from worms containing an early stop mutation (pk2247) in the *dog-1* gene (lanes reflect separate populations). Wild type products are ~1 kilobase. **(C)** PCR analysis results for *qua1442*, in a background of DNA derived from duplicate wild type (N2) and DOG-1–deficient (*dog-1*) cultures. For each genotype we added in a worm carrying a known 443-base-pair deletion allele of *qua1442* (*lf15*) when the population consisted of predominantly F1 or F2 progeny worms (–, DNA from populations without the worm with the mutant allele added). Arrows, reference PCR products, for which PCR was performed on DNA from pure N2 or qua1442 (*lf15*) mutant worms (strain XF28). M, DNA size marker.

We next inspected the mutagenic potential of several of these G4 DNA sites by performing nested PCR analyses with primers that we positioned ~1 kilobase 5' and 0.1 kilobase 3' of the G4 sequence, as we and others have found that deleted sequences are almost exclusively positioned 5' of the guanine tract (Cheung et al., 2002; Kruisselbrink et al., 2008). We identified 3 mutagenic G4 DNA sites in which we observed (somatic) deletions in either 5 adult *dog-1* worms or in DNA isolated from populations of approximately 10,000 worms (Figure 1B). We postulate that we can identify minute amounts of smaller-than-wild type products because these preferably amplify under suboptimal PCR conditions and also because they lack the potentially replication-blocking G-rich sequence. In our analysis of whole populations by nested PCR and subsequent gel electrophoresis we frequently observed smears instead of single bands in the gels, which we interpret as amplification of many bands of different size. Now little is known about possible mutagenic determinants in the G4 DNA structure *in vivo* (for example, loop size and number of possible planar rings), and ongoing work is aimed at addressing this in a systematic way. In this analysis we found that sites which minimally comply with the G4 DNA consensus $G_3N_xG_3N_xG_3N_xG_3$ are mutagenic *in vivo* (Figure 1B), which suggests that many of the predicted G4 DNA sites have deletion-forming potential.

We then searched for assay conditions that would allow us to distinguish germline deletion events (which will propagate during population growth) from somatic events. We tested at which stage of population growth a germline deletion should occur for us to identify it in a PCR-based approach. To this end, we started populations with a single wild type Bristol N2 hermaphrodite worm (P0), allowed the populations to expand and mixed in one worm heterozygous for a previously identified G4 DNA deletion when the population consisted of either predominantly F1 (~300) or F2 worms (>10,000). We grew all cultures until starvation, isolated DNA and inspected it by nested PCRs for the presence of the added deletion allele. We were able to identify such a deletion allele when we added the worm to populations that consisted of more than 10,000 worms (Figure 1C).

We then used the following scheme (Figure 2A) to obtain deletion alleles in many genes from *dog-1* mutant populations: we cloned out 96 progeny worms from a single mother and let these grow until the population ran out of food. We rinsed half of each population off the plates and used these worms for DNA isolation. To distinguish germline from somatic deletions, we split these rinsed-off populations in two (or four) before DNA isolation: germline deletions will be represented in all pools whereas somatic deletions will be in one. We performed deletion PCRs on these samples for various G4 DNA sites that were in close proximity to open reading frames. We analyzed the PCR products on 1% agarose gels in which we loaded duplicates (thus DNA derived from the same population) next to each other. If both duplicates contained a deletion product of similar size, we marked that population positive for a candidate germline deletion event (Figure 2B). We then used a sib selection protocol to isolate a worm that contains the deletion from the remaining half of the population, by fractionating the

population and searching for a positive subfraction. We did this in three rounds: in the first round, we divided positive populations into 12 subpopulations and we analyzed these again for presence of the deletion by PCR. From the positives, we then picked 48 pools of 10 worms, and from positive pools we picked 48 single worms. In 9 out of the 11 deletions that we followed, this directly led to isolation of the deletion allele. In two cases, we included one or more extra steps (that is, increasing the number of pools) because the deletion was not recovered after the second or third round, but we were always able to retrieve the deletion. We recommend to include the original positive DNA sample from the previous round as a reference on the gel when analyzing a population.

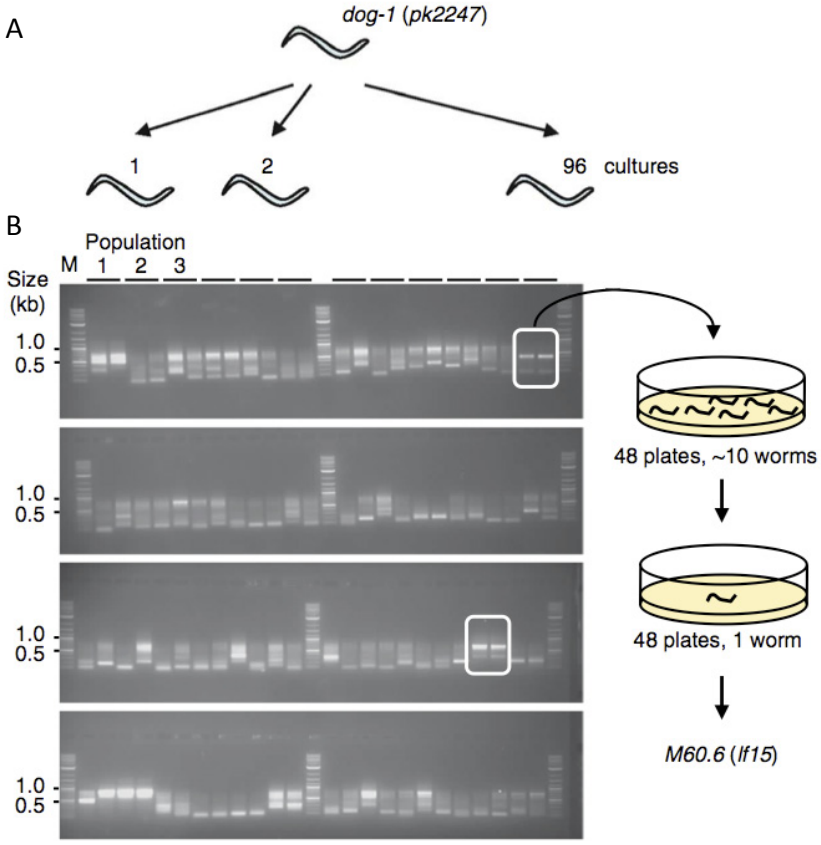

Figure 2 | Sib-selection strategy to isolate deletion alleles. (A) Schematic illustration of the first step in the procedure to isolate deletion alleles in *C. elegans dog-1* mutants (allele pk2247 contains a nonsense mutation in the *dog-1* gene): 96 progeny worms from a single P0 mother were grown clonally until the populations ran out of food. DNA was then isolated from these 96 populations in 96-well plates and subjected to PCR analysis to identify deletions **(B)** Example of the isolation of a deletion allele of *qua1442*. Each gel shows PCR products from 12 duplicates; one of the two indicated positive (i.e. PCR products of similar size are present in both duplicates) populations was followed to isolate allele *lf15* in gene M60.6 via the sib selection procedure depicted in the cartoon.

Altogether, we isolated deletion alleles for 100% of the loci investigated. On average, it took us less than 4 weeks to obtain to a strain homozygous for a deletion in the gene of interest; we recommend to outcross the strain to remove the *dog-1* allele and possible secondary mutations.

We obtained these results using standard PCR conditions, but it may be advantageous for some G4 DNA sites to use a PCR protocol that includes extra enrichment for deletion products (Edgley et al., 2002; Gengyo-Ando and Mitani, 2000) (Supplemental Figure 1). We identified 11 deletion alleles in 10 genes (Figure 3). We targeted these genes primarily to provide proof of principle, and their selection was based on otherwise *ad hoc* criteria. We sequenced the deletions, and all matched the previously

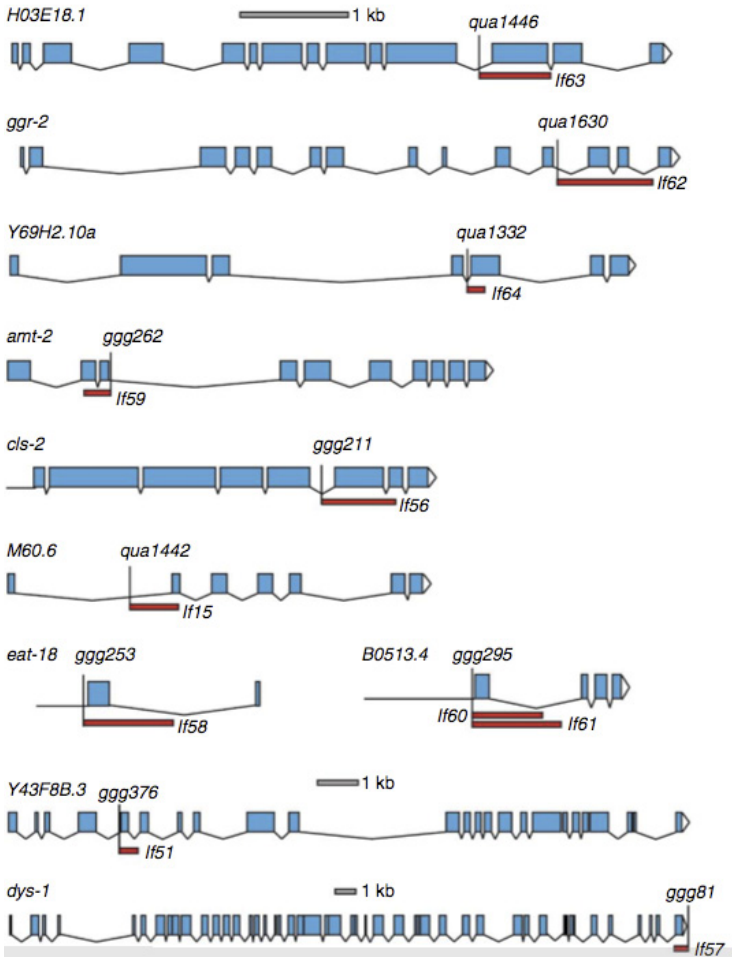

Figure 3 | Schematic illustration of 11 ORF-disrupting *C. elegans* deletion alleles that were generated by G4 DNA–mediated mutagenesis. Gene names are stated at the top left corner of each panel. The deletions (red) are according to the scale depicted in the uppermost gene, unless otherwise indicated.

established *dog-1* signature regarding orientation and breakpoint (Supplemental Table 1; (Kruisselbrink et al., 2008)), although they were generally larger than those that are found using unbiased approaches. This is primarily because we selected for open reading frame–disrupting mutations with the PCR screening strategy. G4 DNA–induced deletions are highly nonsymmetrical: although they always start at the 3'-side of the G4 DNA sequence, they end at various locations up to several kilobases upstream (Kruisselbrink et al., 2008). This feature can be exploited to generate deletions that reach a specific genomic location of interest, are of extensive length (Supplemental Figure 2) or to generate an allelic series, simply by adjusting the location of the 5'-primer and selecting PCR products by size. We provide a list of all genes for which coding sequences are 3'-flanked by one or more G4 DNA sites (Supplemental Table 2 online). There are 1,642 genes located 5' of the 2,907 G4 DNA sites, of which ~73% have no described mutant alleles in WormBase.

In conclusion, we describe a fast and simple strategy to isolate deletion alleles of many *C. elegans* genes, making use of the mutagenic potential of G4 DNA. We were able to isolate a deletion allele for all of the genes analyzed here, resulting in a success rate of 100%. We thus predict that this method will work efficiently for any other mutagenic G4 DNA site. To facilitate the use of this application for the *C. elegans* community, we included G4 DNA sites in WormBase. G4 DNA motifs are also frequently found in other genomes; for instance, there are >300,000 such sites in the mammalian genome. Our methodology thus has a broader reach and could be especially useful in model systems such as fish, flies and rats, which like nematodes lack straightforward homologous targeting technology.

MATERIALS AND METHODS

Deletion PCR analysis on *C. elegans* individuals or populations. Populations were rinsed off plates in duplicate in M9 buffer and pellets were resuspended in 50 µl (or single worms were picked directly into 10 µl) lysis buffer (50 mM KCl, 2.5% mM $MgCl_2$, 10mM Tris-HCl (pH 8), 0.45% Nonidet P40, 0.45% Tween-20, 0.01% gelatin and 200 µg ml^{-1} proteinase K). Lysis was performed for 60 min at 60 °C, followed by 15 min at 95 °C to inactivate the proteinase K. Two rounds of (nested) PCRs were performed on 1 µl lysis mix in a total volume of 10 ml containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 1.5 mM $MgCl_2$), 0.5 mM dNTPs, 0.5 µM of each primer and 0.025 U $µl^{-1}$ Taq polymerase. PCR program for the first as well as the second (nested) PCR was as follows: 1 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 40 s at 72 °C followed by 3 min at 72 °C. Approximately 0.2 µl of the first PCR was transferred to the second PCR reaction by hatching. 5 µl of each sample was run on a 1% agarose gel, where duplicates ran next to each other. The remaining 5 µl can be used for sequencing if a deletion is found.

To more specifically select for deletions, a protocol based Gengyo-Ando *et al*, 2000, can be used; by using a very low concentration of dNTPs and a short extension time, this protocol should not detect the wild type (full-length) product but selectively amplify deletions when these are present in a sample. Conditions are as follows: for the first (external) PCR, use 1× PCR buffer, 1.5 mM $MgCl_2$, 10 mM dNTPs, 0.2 mM of each primer and 0.025 U $µl^{-1}$ Taq polymerase. PCR

program for the first (external) PCR is: 1 min at 95 °C; 35 cycles of 10 s at 95 °C, 10 s at 58 °C, 10–30 s (depending on PCR product size) at 72 °C; followed by 2 min at 72 °C. Optimal extension time should be determined experimentally. The PCR product from this first reaction should not be visible on an ethidium bromide gel. The second (internal) PCR contains 1× PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.2 mM of each primer and 0.025 U ml^{-1} Taq polymerase, added to 1 ml reaction mixture from the external PCR. PCR program is similar to the program of the external PCR, but extension time should be doubled.

ACKNOWLEDGEMENTS

We thank members of the Tijsterman lab for discussions, R. Plasterk for generous support and WormBase curators for building the platform that allows *C. elegans* genomic research. This work was funded by a Horizon grant from the Netherlands Genomics Initiative and a Vidi grant from ZonMw to M. Tijsterman.

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SUPPLEMENTAL TABLES AND FIGURES

Table S1 | G4 DNA-induced deletion alleles

LG	Fragile site	5'-junction [deleted sequence] 3'-junction	ins	del. size	affected ORF
I	ggg81	taaatttaaa[tccccccccc-//-ttttgctgaa]acaggaaaaa	-	589	dys-1
I	ggg253	tcccccaata[catacccccc-//-ttatgagcat]acaacaaact	-	751	eat-18
III	ggg211	ggatttcgaa[ccccccccccc-//-ccaaagcaag]cttggttctg	tttttt	678	cls-2
IV	ggg262	tccgtttccc[agtcccccccc-//-gagtctccga]atgttagacc	-	251	amt-2
IV	ggg295	ttttctgtat[ttcgccccccc-//-ctggttttta]ccggaaaaac	-	653	B0513.4
IV	ggg295	tatttcgccc[ccccccccccc-//-aaccgagttt]tctgtgcact	-	830	B0513.4
V	ggg376	atccccccccc[ccccccccccc-//-aatcaataaa]tttccagaaa	attttttttt	533	Y43F8B.3
V	qua1332	cgtgagttcc[ccccccccccc-//-cctgcacgtc]aggatgtgcc	-	161	Y69H2.10A
X	qua1442	tcaaattcac[ccccccctcc-//-agaagggcgg]ttatcggaaa	t	443	M60.6
X	qua1446	aagcctcgca[ccccccccccc-//-caagttacca]tcaacttttt	-	714	H03E18.1
X	qua1630	agaacccccc[cccccctcc-//-tttcccggat]tgcagtttat	-	839	ggr-2

Table S2 | *C. elegans* endogenous G4 sites. Table S2, listing all endogenous G4 sites near ~2000 *C. elegans* ORFs, is available online at www.nature.com/nmeth

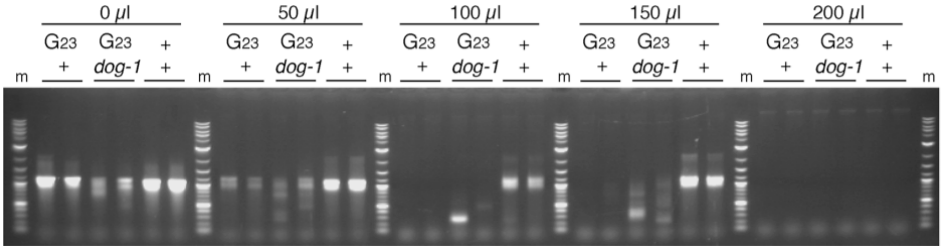
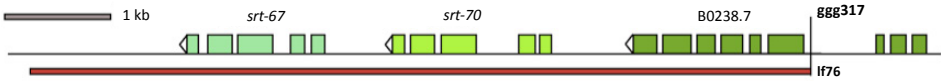


Figure S1 | KCl stabilizes G4 DNA structures *in vitro*. Increasing concentrations of KCl in the PCR reaction preferentially inhibit the amplification of G4 DNA containing amplicons. This allows for better detection of deletion products that have lost the G4 DNA sequence. The gel image displays PCR analysis with indicated concentrations of KCl on DNA isolated from 100 wild type (+) or *dog-1* deficient (*dog-1*) animals. The amplicon is a ~ 1.3 kb segment of the endogenous *unc-22* locus, which we manipulated to harbor an in frame homoguanine stretch of 23 guanines (strain construction will be described in detail elsewhere), indicated by G_{23}. Its absence is indicated by a + because here the amplicon is the wild type (+) *unc-22* locus.

A



B

Target	Size (nt)	5' flank	Deletion	3' flank	Comments
dys-1	2918	tttttcagcatatttta	[gctttaaaaattaaata... agaggtgggggggggggg]	gggggggatttaaatt	
dys-1	3131	tgccgagaccgttagaa	[aatggaaagaaaagtgg... gtgggggggggggggggg]	gggggatttaaatttaa	
dys-1	2956	tcgatatttttggttaa	[ttttttgatttttcagc... ggggggggggggggattta]	aatttaaattgcttaaa	
dys-1	3070	tgagaaatagaagagac	[gcagagggccagagaaa... gtgggggggggggggggg]	ggggatttaaatttaaa	Insertion AT
dys-1	3044	cagagaaaaccgttttt	[ctcccatcccttcttct... gtgggggggggggggggg]	ggggatttaaatttaaa	

Figure S2A | Deletions of larger size. (A) Schematic illustration of a 7.3 kb *C. elegans* deletion allele generated by G4 DNA-mediated mutagenesis that disrupts three ORFs. The deletions (in red) are according to the scale depicted in the upper left panel. **(B)** Example of deletions that were identified with a screen where the 5' primers were located 5 kb upstream of G4 DNA fragile site ggg81.

CHAPTER 7

GENERAL DISCUSSION

C. elegans as a model for double-strand break repair

DNA double-strand break (DSB) repair is very complex. Many distinct pathways and genes act on DSBs, depending on the circumstances in which the DSB arose and on the nature of the DSB. Defective repair leads to increased mutations at sites of DSBs, and accumulation of these mutations can lead to cancer development in humans. It is clear that many genes still remain to be discovered; despite extensive studies on DSB repair over many years, novel components and pathways continue to be identified on a frequent basis (Corneo et al., 2007; Richard et al., 2008; Yan et al., 2007), and for many cancers the underlying molecular cause remains unclear.

For the study of their repair, DSBs can be introduced by a wide variety of sources. First, ionizing radiation causes DSBs, but introduces predominantly single-strand breaks, and many lesions have "dirty" ends, i.e. ends other than free 3'-OH groups (Henner et al., 1982). Certain chemicals are more specific in inducing DSBs, e.g. the topoisomerase inhibitor camptothecin (Ryan et al., 1991). Another way to introduce DSBs is by the expression of rare-cutting endonucleases, such as HO and I-SceI, that leave a "clean" DSB, often with a small overhang. A major advantage of this method is that the location of the DSB in the genome is known, allowing for detailed repair analysis. This method has been applied in mammalian cell culture (Johnson and Jasin, 2001), yeast (Yu and Gabriel, 1999) and in *Drosophila* (Rong and Golic, 2003), each system having their specific advantages. In cell culture, clones that have repaired the DSB by a specific pathway can be sorted by FACS, clonally expanded and analyzed in a quantitative manner, but analyses of mutant genetic backgrounds or epistasis is complicated, because mutant cell lines are not always available. Genetic analysis is very powerful in yeast, yet this model lacks several genes that are important for repair in mammals, such as the Fanconi Anemia genes (except FANCM), p53 and the BRCA genes. *Drosophila melanogaster* is the first multicellular organism in which I-SceI was successfully expressed, and a forward genetic screen uncovered several novel DSB repair mutations (Wei and Rong, 2007).

We have implemented this technology to create localized DSBs in the genome of *C. elegans,* a genetically tractable model organism, and combined this with a colorimetric readout that can be monitored throughout development of the animal. Advantages of studying DSB repair in *C. elegans* include the possibility to study repair in the context of developmental status, the availability of an RNAi library that covers 80% of the genome, and the great ease with which knock-outs of different genes can be obtained and combined through genetic crosses. Our double transgenic setup also allows us to test the involvement of novel candidate genes in DSB repair and thus opens possibilities for forward and reverse genetic screening.

A novel DSB repair pathway: alternative end-joining

In agreement with previous studies, the data presented in this thesis show that NHEJ is strongly conserved in *C. elegans* and plays an important role in the repair of somatic

DSBs (Clejan et al., 2006; Morton et al., 2006; Pontier and Tijsterman, 2009). However, in **Chapter 2** we identify abundant homology-based repair, which acts independently of non-homologous end-joining (NHEJ) and single-strand annealing (SSA) genes, and we term this activity alternative end-joining (alt-EJ). We use the following characteristics to define the alt-EJ pathway: First, its action is restrained by classical NHEJ; its activity was only detected in the absence of canonical NHEJ-factors *cku-80* or *lig-4*. Second, it is characterized by frequent homology-mediated repair and can operate on homologies as diverse as 5 to up to 251 nucleotides, which partly overlaps with the spectrum of SSA. Third, the genetic requirements of alt-EJ are different from SSA, since it does not require *xpf-1*. We use these definitions to distinguish alt-EJ from XPF-dependent repair and from common microhomology-mediated joining.

Evolutionary perspectives for alt-EJ

The finding that we observe alt-EJ events only in the absence of canonical NHEJ raises the question as to when this pathway is biologically relevant for an organism. Whereas activity of NHEJ, SSA and HR can all be readily demonstrated in wild type background, although often specifically in particular cell types or at particular stages of the cell cycle, alt-EJ appears to be an exception. Several reasons for this seemingly very limited activity can be envisaged. First, alt-EJ may operate alongside canonical NHEJ under yet unidentified circumstances that could not be detected in this study, e.g. in a particular uncommon cell type. The frequency of canonical NHEJ may be sufficiently high in wild type cells to mask the detection of any potential alt-EJ events here, thereby allowing for detection of alt-EJ only in its absence. Second, alt-EJ may have evolved as a backup for cases of NHEJ deficiency. Since this occurs very rarely in entire individuals, such a deficiency can be better envisaged on a cellular basis: wild type cells may encounter circumstances where classic NHEJ can not be used. Speculatively, such conditions may arise in meiotic cells that are beyond the stage of default HR. In *C. elegans*, it has been shown that there are two distinct modes of HR in meiosis, where cells in one stage (pachytene) depend on RAD-50 to accumulate repair marker foci at sites of ionizing radiation (IR) induced DSBs, whereas cells in another stage (premeiotic and late pachytene) do not need RAD-50 to establish these foci (Hayashi et al., 2007). Also, NHEJ has a negligible role in these cells that only surfaces in complex genetic mutant backgrounds (Martin et al., 2005). This means that both HR and NHEJ are compromised in at least some stages of meiosis, potentially allowing for a role for alt-EJ.

Another major unsolved issue regarding alt-EJ is that no genes have been identified that operate in this pathway. We found no evidence for involvement of the candidate gene PARP1, which is involved in a pathway that resembles alt-EJ in rodent cells (Audebert et al., 2004). Also, *mre-11*, which is implicated in end resection for a particular form of homology-mediated repair in yeast (Lee and Lee, 2007; Ma et al., 2003), had no effect on alt-EJ, nor SSA levels in *lig-4* mutant background (**Chapter 3**). Exo1, an exonuclease that is partially redundant with *mre-11* also had no effect on ORF restoration by SSA in our reporter transgene (B. Lemmens, unpublished data), but a role in alt-EJ remains

to be investigated. All these genes are implicated in pathways that resemble alt-EJ in other systems, yet we fail to observe an effect on alt-EJ in *C. elegans*. This could either be due to functional redundancy, or suggests that these are functionally distinct pathways. However, there is no consensus about the definition, mechanism and genetic requirements in other systems either, and further study is required to determine how these alternative pathways are related (McVey and Lee, 2008).

Further candidate approaches and unbiased genome-wide screens to identify genetic components of the alt-EJ pathway have not given satisfactory results. Possibly, this is because current screening efforts have been undertaken using RNAi: gene knockdown, even when substantial, may often allow sufficient remaining gene function. Moreover, genes that cause lethality can not easily be identified this way. Although we do not expect lethality upon elimination of alt-EJ because of its limited activity, alt-EJ genes may be lethal if they have a shared role in other, essential pathways. One expected phenotype for alt-EJ genes would be diminished LacZ expression in *lig-4* mutants, because we attribute LacZ expression in these mutants to alt-EJ. However, such a phenotype was not observed for any of the candidate genes we tested. An unbiased and stronger approach to identify (non-lethal) alt-EJ genes would be through genetic screens; random mutagenesis by EMS followed by identification of F2 clones that lack ORF restoration in *lig-4* or *lig-4;xpf-1* mutant background.

In addition to a screening effort to identify alt-EJ genes, several candidate genes remain to be tested. First, another PARP family member, PARP2, displays high sequence homology to PARP-1. PARP-2 is thought to be responsible for the remaining poly (ADP-ribose) synthesis in PARP-1 mutant cells, although their divergent DNA binding domains suggest a different substrate specificity (Amé et al., 1999). The *C. elegans* ortholog of PARP2, *pme-2*, has been identified (Gagnon et al., 2002). Interestingly, whereas general PARP inhibitors caused sensitivity to ionizing radiation (IR) in *C. elegans* (Dequen et al., 2005), we found that *pme-1* mutants are not sensitive to IR (unpublished data). Therefore, *pme-2* is a possible cause of this phenotype and a candidate gene for alt-EJ repair.

A second candidate is BRCA2 (*brc-2* in *C. elegans*). It has been shown that *brc-2*, besides its well-defined role in HR, may also function in SSA: *rad-51;lig-4*[RNAi] animals are still capable of repairing SPO-11 induced DSBs in a *brc-2* dependent manner (Martin et al., 2005). Because *rad-51;lig-4*[RNAi] animals are impaired in both NHEJ and HR, it was suggested that the remaining repair activity should be due to SSA. In yeast, Rad52 is required for efficient single-strand annealing (West, 2003). However, the genomes of *D. melanogaster*, *A. thalania* and *C. elegans* lack an obvious Rad52 homolog. It has therefore been suggested that *brc-2* took over the role of Rad52 in SSA to explain the remaining repair activity in *rad-51;lig-4*[RNAi] animals through this mechanism. However, the identification of the alt-EJ pathway and our preliminary data raise the possibility that the remaining *brc-2* dependent activity instead reflects alt-EJ. Our unpublished data show that *brc-2* [RNAi] does not inhibit SSA, but instead results

in increased ORF restoration. Altogether, this makes *brc-2* a possible candidate for the alt-EJ pathway, but its dual role in HR and associated lethality complicate the analysis of this gene.

The debate about the hierarchy of DSB repair pathways has not yet been settled. It has long been thought that the error-free nature of HR would result in a "preference" for this pathway above the error-prone NHEJ and SSA pathways whenever possible. However, we and others have shown that the mere availability or competition amongst proteins with varying affinities may determine the decision: elimination of one repair protein results in increased processing of the DSB by alternative pathways (Mansour et al., 2008; Pontier and Tijsterman, 2009).

In **Chapter 3**, we present our data on the genetic and cell cycle requirements for the different DSB repair pathways. Remarkably, repair flexibility was greatly reduced in non-dividing cells in adult animals. We observed only very rare LacZ ORF restoration after I-SceI induction in these young adults, even in mutants that had shown high levels when DSBs were introduced during development. We showed that adult *lig-4* mutants have reduced deletion formation, but without an accompanying increase in SSA/alt-EJ, even though these pathways are theoretically not limited to a particular stage of the cell cycle because they do not require a sister chromatid or DNA replication. Nevertheless, there is no absolute requirement to repair a DSB when cells are in a terminally differentiated state, and the failure to detect repair by any of the known pathways may indicate that a DSB can be left unrepaired and persists in NHEJ-deficient G1 cells.

Our data suggest that non-replicating cells are not proficient for homology-mediated repair by SSA or alt-EJ. Whereas resection has been shown to be restricted to G2/S-phase for endonuclease-induced DSBs in yeast (Frank-Vaillant and Marcand, 2002; Ira et al., 2004), several studies have shown that resection in G1 can occur but is inhibited by NHEJ: In *Xenopus laevis*, resection in G1 is stimulated in NHEJ mutants (Clerici et al., 2008), and in yeast, competition with NHEJ factors inhibits substantial processing of a DSB (Zierhut and Diffley, 2008). Nevertheless, IR-induced DSBs have been shown to be resected throughout the cell cycle (Barlow et al., 2008), possibly because NHEJ can not cope with a large number of DSBs. If resection is also limited by NHEJ in *C. elegans*, resection in NHEJ mutants should lead to a shift to SSA and alt-EJ even in non-dividing cells, but this is not what we observe. Possibly, the resected substrate persists until G2/S-phase arrives, presumably because proteins that are required for further processing and repair are only expressed or active at this stage.

G4 DNA: Reason or random?

DSBs can arise from exogenous sources such as ionizing radiation and certain chemical compounds, or from endogenous sources, e.g. collapsed replication forks or mutagens that arise as a byproduct of normal cell metabolism. In this thesis, we provide evidence that replication fork stalling at sites of G4 DNA, and possibly also at other lesions that pose permanent blocks to the replication machinery, can also lead to DSBs. G4 DNA, also

called quadruplex or tetraplex DNA, is a highly stable alternative DNA configuration of four stretches of guanines running in parallel or antiparallel direction to form stacked plains. These conformations can form between DNA molecules (intermolecular) or intra-molecularly in sequences that match the motif $G_3N_xG_3N_xG_3N_xG_3$. The FANCJ ortholog, *dog-1,* of *C. elegans* is involved in maintaining stability of these sequences at the replication fork (Cheung et al., 2002; Kruisselbrink et al., 2008). In *C. elegans*, exons are generally devoid of G4 sequence, but they are abundant in introns and intergenic regions. Whether there is biological function for G4 DNA remains unknown, but in mammals, three genomic regions are characterized by particularly high frequencies of G4 DNA: these are ribosomal DNA (rDNA), telomeric regions, and immunoglobulin switch regions (Maizels, 2006), all highly recombinogenic loci. Also, centromere DNA has been suggested to form G4 DNA. In contrast to mammals, *C. elegans* telomeres do not comply to the G4 sequence motif, and *C. elegans* lacks immunoglobulin switch regions and centromeres; instead, kinetochores are assembled over the full length of the chromosomes to form holocentric chromosomes (Maddox et al., 2004).

Even though in *C. elegans*, G4 DNA is absent at regions characterized by G4 DNA in other organisms, homo-guanine tracts of 9-19 nt are strongly overrepresented in the *C. elegans* genome (Figure 1). Together with the observation of specialized pathways to repair G4 DNA, this is suggestive of positive selection and biological function of G4 DNA. Although sequences of 9-14 nt are not long enough to form in*tra*molecular G4 DNA, they may be able to form in*ter*molecular G4 DNA with other molecules. One possible function that has been suggested is that intermolecular G4 DNA is involved in homolog pairing during meiosis by aligning and holding together chromosomes (Sen and Gilbert, 1988). In support of this, it has been shown that the MutSα complex (a complex of Msh2 and Msh6) binds G4 DNA *in vitro* and promotes synapsis at the G4-rich immunoglobulin switch regions in murine cells (Larson et al., 2005). Nevertheless, meiotic synapsis is normal in *msh-2* and *msh-6* deficient *C. elegans*. Furthermore, proteins that are essential and sufficient for homolog pairing and synapsis have been identified in *C. elegans*, but the sequence motifs that they bind to can not form G4 DNA (Phillips et al., 2009; Phillips and Dernburg, 2006). The G4-structure can also be adopted by RNA (Maizels, 2006). Therefore, another possibility is that G4 DNA

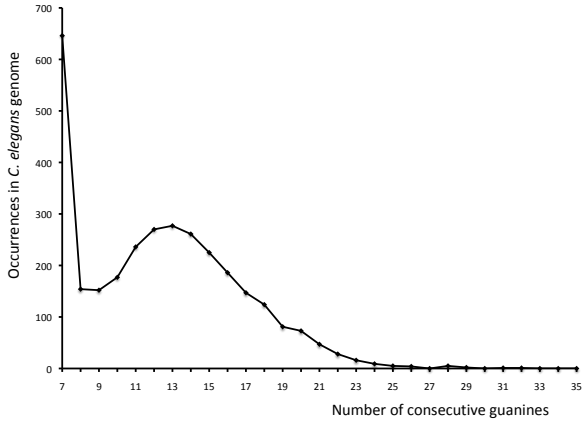


Figure 1 | Frequencies of homoguanine stretches. Homoguanine tracts of >9 bp are overrepresented in the *C. elegans* genome.

plays a role in the stability of mRNA. If this is the case, G4 should be preferentially located in the non-transcribed strand of expressed genes. However, such a bias is not observed in *C. elegans* and our preliminary data suggests that the presence of G4 DNA in a transcript does not affect transcript levels (K. Okihara, unpublished data). Altogether, there is no data to support a role for G4 DNA in synapsis of transcript stability, and the overrepresentation of homoguanine DNA in *C. elegans* can thus not easily be explained by a role for G4 DNA in these processes.

Intriguingly, the relatively high frequency of G4 DNA in the *C. elegans* genome could potentially also occur as a side effect from various repair pathways that operate on this mutagenic sequence. Besides G4-forming potential, another feature of homoguanine tracts (and of repetitive sequences in general) is also intrinsically mutagenic: sequence tracts composed of single nucleotides or small repeats will occasionally shift in length due to slippage of the replication machinery (Tijsterman et al., 2002). Whereas small loops that occur during this slippage are repaired by a complex of Msh2 and Msh6, longer loops are repaired by the Msh2/Msh3 complex (Li, 2008). However, *C. elegans* lacks an Msh3 orthologue, and no other proteins have been identified that could have taken over this function. Strikingly, *Drosophila melanogaster*, which also lacks an Msh3 ortholog, shows a similar overrepresentation of homo guanine DNA as *C. elegans*. The absence of Msh3 to repair replication slippage could thus hypothetically lead to gradual expansion of homoguanine tracts without biological function driving selection of this process.

In conclusion, whether G4 DNA has biological function or arises as a side product of replication errors remains elusive and a matter of intense debate, and further study is required. A recently developed technique called PICh (proteomics of isolated chromatin segments) showed how DNA fragments of a particular sequence can be precipitated, after which proteins that are associated with this sequence can be identified by mass spectrometry (Dejardin and Kingston, 2009). Such an approach aimed at the identification of G4 DNA binding proteins could yield valuable insight into G4 DNA biology.

G4 DNA induced deletions: Give it a break!

In *dog-1* mutants, deletions frequently arise at sites of G4 DNA. These deletions span on average ~200 bp, always initiate at the 3'-side of the G4 sequence and end at various distances upstream (Cheung et al., 2002; Kruisselbrink et al., 2008). In **Chapter 5** of this thesis, we present data that point towards a DSB intermediate in deletion formation. In this model, incision (or breakage) would precede ligation of the stalled fragment to a downstream sequence. The following findings support this model: First, spontaneous RAD-51 foci, a marker of DSBs, are increased in mitotic germline cells of *dog-1* mutants. Second, germline repair footprints at G4 deletions resemble sites of transposon excision. Third, homology-mediated deletion of G4 DNA on transgenes depends on the SSA gene XPF-1, similar to repair of I-SceI induced DSBs. Fourth,

homology near the DSB is never used for repair, suggesting that the sequence near the G4 DNA is excised. A final argument in favor of the DSB model stems from literature, where DSBs at difficult to replicate regions have been described previously. For example, hypomorphic mutations in Dna2, a helicase/nuclease involved in the maturation of Okazaki fragments (Waga and Stillman, 1998), also lead to replication fork stalling, and these mutants show spontaneous DSBs at difficult to replicate regions (Weitao et al., 2003). A similar situation is observed at common fragile sites; these are sequences that are intrinsically hard to replicate for unknown reasons. Deletions and genome rearrangements occur more frequently at these loci, and instability is further increased when the Fanconi anemia pathway is compromised (Chan et al., 2009; Naim and Rosselli, 2009). This leads to frequent repeat expansions or genome rearrangements at these sites (Durkin and Glover, 2007).

Despite the evidence pointing towards a DSB intermediate, we can not completely rule out the possibility that ligation precedes excision of the G4 and surrounding sequence. In this model of deletion formation, the intermediate structure consists of a loop that contains the G4 DNA (Figure 2). However, several features of this model remain unclear: First, the loop would be a hypothetical substrate for the Msh2/Msh3 complex of the mismatch repair machinery (MMR), but *C. elegans* lacks an Msh3 ortholog, and it is unclear how the loop will then be processed. Another MMR complex, the MutSα (Msh2/Msh6) complex, has been shown to bind to G4 DNA *in vitro* (Larson et al., 2005), but mutations in *msh-6*, or in the other MMR gene *mlh-1* have no effect on the frequency of deletion formation in *C. elegans* (E. Kruisselbrink, unpublished data). It is thus unclear how such a loop would be processed, and none of the MMR candidate genes tested have an effect on the number of deletions. Also, it is difficult to explain with this model how the occasional insertions of flanking sequences arise and why homology close to the G4 sequence is not used for repair.

Both the DSB and alternative model require ligase activity to join the stalled 3'-end to a downstream sequence. An interesting candidate that remains to be tested is DNA ligase I. This ligase is required for the joining of Okazaki fragments during lagging strand synthesis (Ellenberger and Tomkinson, 2008; MacNeill, 2001). In addition, this is also the ligase acting during mismatch repair (Li, 2008). Unfortunately, these very characteristics prohibit the testing of this candidate, because replication is required to investigate a role for this ligase in G4 DNA stability: *lig-1* [RNAi] confers complete sterility in wild type background (our unpublished observation). A hypomorphic mutation would be desirable to study its function, but no mutants or temperature-sensitive alleles have been identified for this gene. Whether *lig-1* has a role in the ligation of Okazaki fragments at G4-DNA stalled forks therefore remains elusive.

An alternative approach to G4 DNA resolution

Germline repair footprints were similar in *dog-1* mutants at sites of G4 DNA as at sites of transposon excision. The footprints of deletions associated with frequent insertions

are striking, because HR is the predominant repair route in germline cells. Still, our findings are reminiscent of several other studies that reported unusual HR-like repair. For example, repair of a transposon-induced DSB in *Drosophila* preferentially uses a template that is present *in cis*, even in premeiotic germ cells where HR is thought to be default (Engels et al., 1994). Also, RAD-51 *in*dependent strand invasion has been proposed to be responsible for human copy number variations (CNVs) by a break-induced replication (BIR)-like model. Here, DNA synthesis is started with minimal priming, if sufficient ssDNA is available to anneal to (Hastings et al., 2009). This can occur through multiple cycles of invasion, DNA synthesis and dissociation, and preferentially in the lagging strand, in *Drosophila* (McVey et al., 2004). A similar mechanism of invasion and synthesis could explain how insertions of both flanks arise at G4 DNA deletions, and has already been proposed for transposon footprints (Brouwer, 2009).

One possibility for why these lesions are processed by an unconventional mechanism may be that they are replication associated; in the case of G4 DNA, a sister chromatid, which is required for HR, may not have been completed when the replication fork stalls at the G4 sequence. However, it is unknown whether transposon excision is replication associated in *C. elegans*. Excision of P elements, a *Drosophila* transposon, seems to occur predominantly during G2 phase, possibly as a result of having the sister chromatid available for repair (Engels et al., 1990). Other studies have shown that

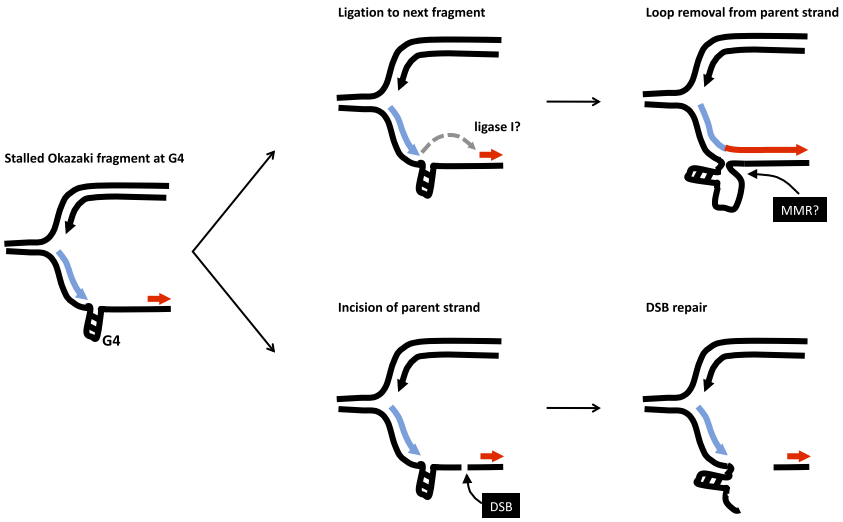


Figure 2 | Alternative model for deletion formation of sites of G4 DNA. Top: If ligation precedes incision of the damaged strand, no DSB intermediate is formed. Instead, a loop will arise that contains the G4 sequence and needs to be excised. The MMR machinery is likely candidate for removal of such a loop, but mutations in MMR genes have no effect on deletion frequencies. Whereas other repair routes may process this loop, this absence of an MMR effect, together with our data presented in Chapter 5, point towards a DSB intermediate model as a more likely explanation for deletion formation (bottom). In this model, a DSB is generated, followed by non-canonical DSB repair.

P-element excision is also possible during G1 phase (Weinert et al., 2005). Transposon excision during replication is thus not a general feature, and it is questionable whether this would be the case in *C. elegans*. Our preliminary and unpublished data show that in mutants for *mut-7* and *mut-8*, where active transposition takes place, RAD-51 foci occur in both the mitotic and in the meiotic germline compartment. However, most of these cells are in S/G2 phase. It is therefore unlikely that the similarities between transposons and G4 DNA are the result of a special form of replication-associated repair.

Speculatively, the mechanism that is responsible for G4-induced deletions in *dog-1* background could be the same as alt-EJ: The deletions that we sequenced from *dog-1* mutants were all derived from germline events, where the action of NHEJ is limited compared with the soma, and DSBs are a substrate for alt-EJ in the absence of NHEJ. Second, for both mechanisms, no genes have been identified and no known mutants in canonical repair pathways can abolish the repair activity. However, the repair footprints at G4 DNA deletions and at I-SceI-induced deletions are different, but this can be explained by their different origins: All sequenced G4 DNA deletions were from germline origin, whereas we sequenced I-SceI induced deletions from somatic tissues. Whether deletions at these two seemingly different lesions can be generated through a common mechanism thus remains to be determined.

Conclusion

The data presented in this thesis show highly robust repair of DSBs and G4 DNA by a complex network of repair proteins. The choice of repair pathway is highly regulated and subject to extreme flexibility depending on the type of DSB, developmental stage and the availability of repair proteins. This flexibility is relevant for cancer development, as error-prone pathways are more mutagenic and can initiate and accelerate tumor formation and progression. Limiting the actions of error-prone repair may thus provide a promising step towards prevention and treatment of tumorigenesis.

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CHAPTER 8

SAMENVATTING IN HET NEDERLANDS
(VOOR NIET-INGEWIJDEN)

Mutaties

In het DNA ligt alle informatie opgeslagen die nodig is voor een organisme om te functioneren. Een goede metafoor om beter te begrijpen hoe DNA werkt is door het te vergelijken met een bibliotheek. Een bibliotheek bestaat uit boeken over verschillende onderwerpen, en zo bestaat het DNA uit genen met verschillende functies. Elk gen is een handleiding voor hoe de cel een bepaald eiwit moet bouwen. En eiwitten zijn de drijvende kracht achter alles wat er in en buiten een cel gebeurt; sommige eiwitten zijn de bouwstenen van de cel zelf, andere versnellen chemische reacties, vormen antistoffen voor je immuunsysteem, of kunnen zuurstof binden in je rode bloedlichaampjes.

Stel nou dat je een bladzijde (of een heel katern) uit een bibliotheekboek scheurt. Of je maakt hem onleesbaar door er met een dikke zwarte viltstift in te krassen. Dit zorgt ervoor dat de informatie in dit boek niet meer goed leesbaar is, en dat de processen die in dat boek beschreven staan niet meer goed kunnen worden uitgevoerd. In een cel kan een vergelijkbare situatie ontstaan. Door beschadiging van het DNA kan een gen kwijtraken, of onleesbaar worden door veranderingen in de genetische code. In DNA heten zulke veranderingen **mutaties**. Er zijn verschillende soorten mutaties: Het kan gaan om een kleine verandering in maar 1 woord van het boek: Een **puntmutatie**. Dit heeft niet per se grote gevolgen; het hangt ervan af of de mutatie in een essentieel woord van de boodschap plaatsvond, maar het kan ook best zijn dat de rest van het boek nog perfect leesbaar blijft met zo'n mutatie. In een ander type mutatie kan er een stuk van een boek, of zelfs een heel boek of meer verdwijnen. In dat geval spreken we van een **deletie**. Dit heeft meestal grotere gevolgen omdat er nu echt informatie ontbreekt. Tot slot kunnen er ook **inserties** optreden; hierbij wordt nieuwe (vaak betekenisloze) tekst toegevoegd aan een boek. Behalve door het verdwijnen of toevoegen van informatie, kunnen deleties en inserties ook om het volgende desastreus uitpakken: Stel je voor dat je op een willekeurige plek in een boek nieuwe tekst plakt, maar voor iedere letter die je toevoegt schuif je ook alle spaties tussen de woorden die erna komen 1 positie op; spaties tussen woorden komen dan ineens middenin bepaalde woorden te liggen. Bij een deletie kan dit ook; je haalt tekst weg, maar voor iedere letter die je weghaalt schuif je de spaties in de woorden erna ook een positie terug. Door het verschuiven van spaties worden de woorden betekenisloos. Dit kan ook op het niveau van een gen gebeuren; in het geval van een deletie verdwijnt niet alleen (een deel van) het gen, maar het deel na de deletie wordt vaak ook nog eens onleesbaar door verschuiven van de spaties, en bij een insertie kunnen de spaties ook verschuiven. In beide gevallen is er grote kans dat het gen onleesbaar en onbruikbaar wordt, en deleties en inserties zijn dan ook de meest catastrofale mutaties die kunnen plaatsvinden in het DNA.

Mutaties kunnen door enorm veel factoren veroorzaakt worden en zijn aan de orde van de dag. Chemische reacties die nodig zijn om je voedsel te verteren produceren bijvoorbeeld bijproducten die mutaties kunnen veroorzaken. Verder wordt het DNA iedere keer gekopieerd als een cel deelt, zodat beide nieuwe cellen een kopie van het DNA hebben, maar bij dit kopieerproces wordt spontaan weleens een klein foutje

gemaakt. Op mutaties die op die manieren ontstaan kun je weinig invloed uitoefenen, maar op sommige andere mutaties kan dat wel: mutaties ontstaan bijvoorbeeld ook door UV-licht van de zon, of door sigarettenrook. De meeste mutaties leveren geen grote problemen op, maar het kan bij toeval weleens gebeuren dat een mutatie ontstaat in een boek waarin heel belangrijke processen staan beschreven voor het voortbestaan en overleven van de bibliotheek (of cel). Gelukkig kan dit niet zo makkelijk gebeuren. Er staan namelijk ook heel veel boeken in de bibliotheek waarin staat beschreven hoe een kapot boek gerepareerd moet worden. Een bladzijde uit een boek scheuren heeft doorgaans geen grote gevolgen, zo lang je het boek met reparatie-instructies nog maar hebt. Met die instructies kun je kapotte en beschadigde boeken weer als nieuw maken, en is de kans klein dat er mutaties optreden in belangrijke boeken. Het is dus behoorlijk belangrijk dat je alle reparatieboeken goed bewaart, anders kunnen er naar hartenlust bladzijden uit alle boeken beklad en gescheurd worden zonder dat je weet hoe je de schade moet herstellen, en is het hek van de dam. Maar wat nou als je een boek met reparatie-instructies beschadigt? Dan heb je wel een groot probleem, en dit is precies wat er mis is in veel tumoren. Als een reparatiegen eenmaal uitgeschakeld is door een mutatie, kunnen er versneld nieuwe mutaties op allerlei plekken in het DNA ontstaan omdat er niet meer goed gerepareerd wordt. En veel mutaties is precies wat een cel nodig heeft om kankercel te worden.

Zo lang je de reparatie-instructies nog maar hebt…

Kanker is een groepje cellen dat door meerdere mutaties andere eigenschappen heeft gekregen dan zijn buurcellen. Versnelde celgroei is de bekendste eigenschap van kanker, maar alleen dit is niet genoeg om kanker te laten ontstaan; het levert hooguit een goedaardig gezwel op. Er zijn veel meer mutaties nodig om een kwaadaardige tumor te vormen. Een tumor moet bijvoorbeeld ook kunnen ontsnappen aan opsporing door het immuunsysteem. In tegenstelling tot wat vaak wordt gedacht, is het immuunsysteem enorm efficiënt in het opsporen en vernietigen van cellen die neigen naar kanker vorming. Om hieraan te ontkomen, is onherkenbaarheid voor het immuunsysteem dus ook een essentiële eigenschap van een "succesvolle" tumor. Die onherkenbaarheid kunnen ze ook krijgen door een mutatie. Verder kunnen normale cellen niet in je lichaam rond gaan zwerven en op andere plekken gaan groeien, iets wat bij uitzaaiingen wel gebeurt. Om kankercel te worden, moet een cel dus ook nog eens mutaties krijgen die ervoor zorgen dat dit ineens wel kan. Zo zijn er nog veel meer nieuwe eigenschappen nodig om van normale cel tot kankercel te worden, en al deze nieuwe eigenschappen worden verkregen door mutaties. Alles bij elkaar is kanker dus een heel complex proces en er zijn heel veel mutaties nodig op heel specifieke plaatsen in 1 cel om kankercel te worden. Op basis van toeval kunnen zoveel mutaties nooit allemaal op precies die plekken ontstaan. Die mutaties ontstaan echter veel makkelijker en sneller als het allemaal begint met een eerste mutatie in een reparatiegen: mutaties hopen zich nu ineens op omdat er niet meer (goed) gerepareerd wordt.

Reparatie van DNA breuken

Mutaties in reparatiegenen liggen ten grondslag aan heel veel vormen van kanker, zowel de erfelijke als de sporadische vormen. Het bekendste voorbeeld is misschien wel erfelijke borstkanker. Er zijn families waarin veel vrouwen op relatief jonge leeftijd al borstkanker ontwikkelen. In een aantal van deze families is bekend hoe dit komt; ze hebben een mutatie in een bepaald DNA reparatiegen geërfd. Hierbij gaat het vaak om een gen dat BRCA heet, en door een mutatie kan het BRCA gen in deze personen niet meer goed functioneren. Helaas bevat het BRCA-gen behoorlijk belangrijke reparatie-instructies: het vertelt de cel namelijk hoe een breuk in het DNA foutloos moet worden gerepareerd. Breuken komen niet heel vaak voor, maar kunnen af en toe tijdens normale celprocessen ontstaan, of door bepaalde chemicaliën. Ook ioniserende straling, wat gebruikt wordt voor röntgenfoto's, veroorzaakt DNA breuken.

Nu wordt de metafoor met de bieb wat moeilijker, maar een breuk in het DNA zou zoiets zijn als met een enorme kettingzaag het bibliotheekgebouw in tweeën zagen. Of als het door een aardbeving in tweeën wordt gereten. Dat wordt natuurlijk een grote puinhoop, want nu is er niet één boek beschadigd, maar ligt er wel meer overhoop. De vraag is hoe het vervolgens allemaal weer netjes en op volgorde aan elkaar moet worden gezet. Met het BRCA-boek in de hand had je dat makkelijk opgelost; hierin staat namelijk hoe je toegang krijgt tot een "back-up" bibliotheek die een exacte kopie is van jouw eigen bibliotheek. Handig, dan kan je daar even spieken hoe het er precies uitzag, en het weer net zo herstellen als het was. Helaas is door de mutatie het BRCA-boek onbegrijpelijk geworden. Noodgedwongen improviseer je een beetje en zet je het op het oog weer aan elkaar, maar zonder handleiding is de kans groot dat je misschien de volgorde van sommige boeken of kasten verwisselt. Daardoor kun je de volgende keer moeilijker iets vinden. En als je met die kettingzaag ook net een boek hebt geraakt dat nu doormidden is, is dat boek ook verloren gegaan.

Als het DNA breekt, zijn de BRCA genen er om de boel te redden. De backup-bibliotheek bestaat ook op DNA niveau; je hebt namelijk van je moeder en van je vader een intacte DNA kopie geërfd, die vrijwel identiek aan elkaar zijn. Het BRCA gen zorgt ervoor dat de cel toegang krijgt tot de andere kopie om zo de breuk foutloos te herstellen. De biologische term voor dit proces is "homologe recombinatie" (HR), en dit is 1 van 3 manieren waarop een cel een breuk kan repareren. Met een mutatie in het BRCA gen gaat dit allemaal niet zo makkelijk, en worden er andere manieren gebruikt om het DNA weer aan elkaar te plakken. De tweede, en makkelijkste manier is dat als je twee losse uiteindes vindt, je deze direct weer aan elkaar plakt. Ofwel, de eerste de beste omgevallen boekenkasten die je ziet, zet je tegen elkaar, ook al weet je niet zeker of ze eigenlijk wel naast elkaar hoorden, en misschien zijn er wel boeken uitgevallen. Dit kan een cel inderdaad ook doen en dit heet "Non-homologous end-joining" (NHEJ). Hier is het probleem dat er niet wordt gecontroleerd of de betreffende eindjes wel bij elkaar horen en of er misschien een stukje DNA verloren is gegaan rondom de breuk (ofwel of er deleties zijn ontstaan). Breukherstel via NHEJ veroorzaakt daarom vaak

nieuwe mutaties op de plek waar ooit de breuk zat, en is in tegenstelling tot HR geen foutloze manier van reparatie. Reparatie kan ook nog plaatsvinden op een manier die single strand annealing (SSA) heet; hier zoekt de cel naar gelijkenissen tussen de beide uiteindes. Via een nogal ingewikkelde reactie worden de uiteindes dan aan elkaar gelijmd, maar daarbij gaat wel altijd aan beide kanten een stuk DNA verloren. Ook hier ontstaan dus nieuwe mutaties op de plek waar de breuk zat. En deze mutaties kunnen weer kankerverwekkende eigenschappen veroorzaken zoals boven beschreven. De manier waarop een breuk wordt gerepareerd kan dus grote gevolgen hebben voor kankerontwikkeling.

Dit proefschrift

In dit proefschrift proberen we onder andere beter te begrijpen hoe een cel "kiest" hoe hij de breuk gaat repareren; via HR, SSA of NHEJ? We maken gebruik van een modelorganisme om dit te onderzoeken. Het zou immers een beetje onmenselijk zijn om in patiënten allerlei genen uit te schakelen, kanker te veroorzaken en onderzoek te doen, en bij gekweekte cellen is het weer lastig om dingen aan het DNA te veranderen (genetische modificatie), iets dat we wel wilden doen om onze onderzoeksvraag te beantwoorden. We maakten daarom gebruik van *C. elegans*; een minuscuul rondwormpje van minder dan 1 millimeter lang. Voordelen hiervan zijn dat ze klein zijn (in tegenstelling tot bijvoorbeeld muizen kun je er miljoenen op het lab in kweek houden), gemakkelijk genetisch te modificeren en doorzichtig zodat we kunnen zien wat er van binnen gebeurt (onder de microscoop dan). Ondanks dat we evolutionair nogal ver van dit kleine beestje afstaan, lijken we qua DNA reparatie heel sterk op elkaar. Vrijwel alle genen die we kennen die betrokken zijn bij breukherstel, zoals het BRCA-gen, en andere typen DNA herstel zijn door de evolutie heen vrijwel onveranderd gebleven tussen mens en worm. Resultaten op dit vlak uit de worm gelden dus zeer waarschijnlijk ook voor de mens.

Hoofdstuk 1 is een algemene introductie over DNA schade en reparatie. In **hoofdstuk 2** beschrijven we dat er behalve de drie bekende manieren om een breuk te repareren nog een vierde manier mogelijk is. Deze manier lijkt wel een beetje op SSA, en ook een beetje op NHEJ, maar is dan toch net anders. We noemden deze nieuwe breukherstel route alternatieve end-joining (alt-EJ). We zagen dat als we de boeken over HR, SSA of NHEJ gelijktijdig weghalen uit de bieb, reparatie van breuken nog steeds plaatsvindt. Blijkbaar staat er nog ergens een boek in de bieb dat over herstel van breuken gaat. De bieb is echter nogal groot, en ondanks verwoedde pogingen hebben we het betreffende boek nog niet gevonden, ook al weten we zeker dat het er moet zijn. In **hoofdstuk 3** bekijken we een aantal boeken (genen) waarin we dachten dat het zou kunnen staan, maar die blijken het allemaal niet te zijn. Omdat we het boek niet kunnen vinden gingen we beter bestuderen hoe deze route werkt. We zagen toen dat alleen cellen die aan het delen zijn deze manier van repareren kunnen gebruiken. De bieb moet dus een kopie van zichzelf hebben gemaakt om deze route te gebruiken. Verder zagen we dat alt-EJ alleen gebruikt wordt in cellen waarin we NHEJ hebben uitgeschakeld. Het

lijkt dus alsof alt-EJ een soort back-up is voor NHEJ. Maar dat is gek, want NHEJ kan altijd plaatsvinden, ook in niet-delende cellen. Een goede back-up is alt-EJ dus niet, want het kan niet op elk moment inspringen. Waarom dit is weten we niet, maar deze gegevens samen betekenen dat alleen delende NHEJ-defecte cellen een breuk kunnen repareren met alt-EJ. Inderdaad zien we dat in niet-delende NHEJ-defecte cellen een breuk gewoon blijft bestaan, en niet meer gerepareerd wordt. Wonderlijk genoeg kan *C. elegans* wel gewoon groeien ondanks deze enorme schade aan het DNA.

In **hoofdstuk 4** kijken we naar breukherstel in een heel ander type cellen, namelijk in geslachtscellen (dit zijn de voorlopers van de ei- of spermacellen). Als een mutatie in een geslachtscel zit, is het probleem dat het kindje dat uit die cel gaat groeien geen enkele correcte kopie van het gemuteerde gen heeft. Dit is anders als een mutatie nieuw ontstaat in bijvoorbeeld een huidcel. Dit is geen ramp; je hebt dan nog miljoenen cellen over zonder die mutatie. Om zoveel mogelijk te voorkomen dat er mutaties ontstaan in geslachtscellen die vervolgens dus in al je lichaamscellen zouden belanden, zijn geslachtscellen normaal gesproken nog veel nauwkeuriger met het repareren van hun DNA dan "gewone" cellen. Wij laten zien dat in geslachtscellen de keuze van reparatieroute heel strak gereguleerd is en sterk afhangt van de exacte ontwikkelingsfase waarin cellen zich bevinden. Ook laten wij zien dat er in deze verschillende fasen heel anders op een breuk gereageerd wordt, en vinden we dat er wel degelijk gebruik kan worden gemaakt van andere reparatieroutes dan het foutloze HR, ook al is het in sterk verminderde mate.

Vervolgens onderzoeken we een heel ander type mutaties. In voorbereiding op een celdeling wordt het DNA altijd gekopieerd, zodat één kopie kan worden meegegeven aan elk van beide dochtercellen. Tijdens dit kopieerproces kunnen bepaalde stukken DNA een andere 3-dimensionale structuur aannemen die G4 DNA heet. Die G4 structuur maakt verder kopiëren onmogelijk. Misschien zou het een beetje lijken op dat er bij een bepaald sleutelwoord - G4 - in je boeken de bladzijden erna soms met elkaar verlijmd raken; je kan niet verder gaan met kopiëren zolang je die niet van elkaar losgeweekt hebt. Ons lab heeft eerder al laten zien dat een gen genaamd *dog-1* nodig is voor dit losweek proces: ofwel *dog-1* is nodig om alsnog langs deze G4 DNA stukken te kunnen kopiëren.

Als je *dog-1* uitschakelt gebeurt er iets geks; soms raakt dan een heel stuk DNA rondom de G4 structuur zomaar ineens kwijt; alle aan elkaar geplakte bladzijden en een willekeurig aantal bladzijden erna verdwijnen zomaar. Het kwijtraken van een stuk DNA noemen we, zoals eerder uitgelegd, een deletie. Het is belangrijk om te weten hoe die deleties ontstaan, omdat er ook patiënten zijn die een defect hebben in het *dog-1* gen (in mensen heet dit gen alleen geen *dog-1*, maar FANCJ). Deze mensen hebben allerlei aangeboren afwijkingen en een verhoogde kans op kanker, mogelijk deels als een gevolg van de deleties die rond G4 DNA ontstaan.

Hoe die deleties nou kunnen ontstaan als je geen *dog-1* hebt, was nog de vraag, maar in **hoofdstuk 5** laten we zien dat de blokkerende G4 DNA structuur eerst wordt omgezet in een DNA breuk. Als je vastloopt met kopiëren op de aan elkaar geplakte bladzijden worden dus niet die bladzijden eruit gehaald terwijl de kaft van het boek intact blijft, maar het boek wordt op die plek volledig in tweeën gehakt. De bovenstaande manieren van breukherstel (HR, SSA of NHEJ) werken echter niet, want dan ben je nog steeds niet van die verplakte bladzijden af. Inderdaad zien we dat deze breuk niet helemaal via één van de bekende routes gerepareerd wordt. Misschien dat ook hier de alt-EJ reparatieroute een rol speelt?

In het algemeen geldt dat als onderzoekers willen weten wat de functie van een bepaald gen is, dat gen wordt uitgeschakeld. Door te kijken naar wat er vervolgens mis gaat in de cel leer je iets over wat dat gen normaal allemaal doet. Maar hoe schakel je zomaar een gen uit? Wij dachten dat we misschien wel gebruik konden maken van de spontane deleties die ontstaan rondom G4 DNA als *dog-1* gemuteerd is. Als zo'n G4 sleutelwoord toevallig dichtbij een boek (gen) ligt dat jij wilt onderzoeken, dan kun je in een *dog-1* mutant wachten tot juist daar toevallig een keer een deletie optreedt. Die deletie gooit dan namelijk ook (een deel van) jouw gen eruit, waardoor het gen wordt uitgeschakeld. In **Hoofdstuk 6** laten we zien dat dit inderdaad een bruikbare methode is om genen die dichtbij G4 DNA liggen uit te schakelen. Wij hebben alle G4 sleutelwoorden in de bieb opgezocht en gekeken welke ervan in of dichtbij een belangrijk boek staan. In totaal vinden we zo'n 2000 G4 sleutelwoorden waarvoor dit geldt. Vervolgens hebben we een methode ontwikkeld waarop je deleties op die plekken kan vinden. Onderzoekers die geïnteresseerd zijn in de functie van een bepaald gen kunnen nu dus onze lijst van sleutelwoorden doorzoeken of hun gen in aanmerking komt, en de door ons beschreven methode gebruiken om hun gen van interesse uit te schakelen en de functie ervan te bestuderen.

ADDENDUM

SUMMARY
ABBREVIATIONS
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CURRICULUM VITAE
LIST OF PUBLICATIONS

SUMMARY

DNA double-strand breaks (DSBs) are highly toxic DNA lesions that can cause genome rearrangements and deletions that can ultimately result in development and progression of cancer. Pathways for repair of DSBs and other DNA lesions are described in detail in **Chapter 1**. Using the model organism *C. elegans*, we study DSB repair in the context of a developing animal and in complex genetic backgrounds. We make use of a transgenic approach where the restriction enzyme I-SceI can be expressed in an inducible manner, combined with a reporter transgene that contains the 18-nt recognition site for I-SceI in an out-of-frame LacZ gene. In **Chapter 2** of this thesis, we use this assay to reveal the activity of a novel repair pathway, which we term alternative end-joining (alt-EJ). This pathway seems to act as a backup for the canonical non-homologous end-joining (NHEJ) pathway because it predominates repair only in the absence of NHEJ, but its repair products are characterized by frequent use of homology in a way that is similar to SSA. Alt-EJ operates independently of many known repair genes and leads to very efficient DSB repair even in triple mutants that are defective for HR, SSA and NHEJ. Despite its putative function as a backup for classic NHEJ, we show in **Chapter 3** that, in contrast to NHEJ, alt-EJ only occurs in replicating cells, leading to DSB persistence in non-replicating NHEJ-deficient somatic cells.

Despite their toxicity, endogenous DSBs are introduced in a regulated manner in meiotic cells in the germline. These DSBs need to be repaired by HR to establish crossover formation between homologous chromosomes which is required for genetic diversity among the offspring and for correct chromosome segregation. In **Chapter 4** we show that besides HR, other repair pathways are also active in the germline. Moreover, the response to DSBs is highly dependent on the stage of the cell cycle at the time of DSB induction and differs between different germline zones.

Quadruplex or G4 DNA is a stable secondary ssDNA structure that can form in particular G-rich sequences during DNA replication. In mutants for the gene *dog-1* (mammalian FANCJ), spontaneous deletions arise at G4 DNA. These deletions always initiate immediately downstream of the G-rich sequence and end at various locations downstream. In **Chapter 5**, we show that these deletions are likely formed through DSB intermediates, because they resemble DSBs at other locations in many ways. Remarkably, these DSBs are not repaired by one of the canonical repair routes, but may instead be repaired by alt-EJ or another mechanism. In **Chapter 6**, we show how deletion formation in *dog-1* mutant background can be used as a tool to isolate deletion alleles of many *C. elegans* genes.

ABBREVIATIONS

alt-EJ Alternative End Joining
BER Base Excision Repair
CO Crossover
DAPI 4',6-diamidino-2-phenylindole
DDR DNA Damage Response
DIC Differential Interference Contrast
DNA Deoxyribose Nucleic Acid
DSB Double-Strand Break
HR Homologous Recombination
HS Heat Shock
ICL Interstrand Crosslink
IPTG Isopropyl β-D-1-thiogalactopyranoside
IR Ionizing Radiation
NCO Non-Crossover
NER Nucleotide Excision Repair
NHEJ Non-Homologous End Joining
ORF Open Reading Frame
PBS Phosphate-buffered Saline
PCR Polymerase Chain Reaction
RNA Ribose Nucleic Acid
RNAi RNA interference
RT Room Temperature
SC Synaptonemal Complex
SSA Single-Strand Annealing
SSB Single-Strand Break
TLS Translesion Synthesis
TUNEL Terminal dUTP Transferase Nick End Labeling
TZ Transition Zone

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Daphne

LIST OF PUBLICATIONS

Pontier D.B., Kruisselbrink E., Guryev V. and Tijsterman M. Isolation of deletion alleles by G4-induced mutagenesis.
Nature Methods Vol 6(9) p655, 2009

Pontier D.B. and Tijsterman M. A robust network of DNA repair pathways governs genome integrity during *C. elegans* development.
Current Biology Vol 19(16) p1384, 2009

Kruisselbrink E.*, Guryev V.*, Brouwer K., **Pontier D.B**., Cuppen E. and Tijsterman M. Mutagenic capacity of G4 DNA underlies genome instability in FANCJ-defective *C. elegans*.
Current Biology Vol 18(12) p900, 2008
* these authors contributed equally to this work

Pontier D.B., Van Arkel J. and Tijsterman M. Alternative end-joining of double-strand breaks is dependent on DNA replication in *C. elegans*.
Manuscript in preparation

Pontier D.B., Kruisselbrink E., Van Arkel J. and Tijsterman M. DOG-1 prevents double-strand breaks at G4 DNA-induced stalled replication forks.
Manuscript in preparation

CURRICULUM VITAE

Daphne Pontier was born on July 10, 1981 in Breukelen, The Netherlands. In 1999, she received her diploma from the Gemeentelijk Gymnasium in Hilversum. In September 2000, she started her studies Biomedical Sciences at Leiden University. Her first internship was performed at the Clinical Oncology department at the LUMC in Leiden, on "The search for an apoptotic serine protease" under supervision of Lucy Peltenburg in the group of Jan-Paul Medema. A second internship was performed on the subject of "Carotid artery bifurcation variation and the relation with atherosclerosis" in the department of Clinical and Experimental Image Processing at the LUMC in the group of Prof. Dr. Reiber. Her final research project was performed from February-September 2005 at the University of California at Berkeley, under supervision of Dr. Judith Jans in the group of Prof. Dr. Barbara Meyer to study dosage compensation in *C. elegans*. Also, a course was taken at the Karolinska Institutet in Stockholm, Sweden and she obtained a *propedeuse* in Medicine at Leiden University. The master's degree Biomedical Science was obtained in January 2006 (*cum laude*). In February of the same year, Daphne started the research described in this thesis at the Hubrecht Institute in Utrecht under supervision of Dr. Marcel Tijsterman in the group of Prof. Dr. Ronald Plasterk. In 2007, the research was continued in the group of Marcel Tijsterman at the Hubrecht Institute and at the department of Toxicogenetics at the LUMC in Leiden. Mid-2010, she will initiate her postdoctoral studies on X-chromosome inactivation in the lab of Dr. Gribnau at the Erasmus University in Rotterdam.