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The role of homologous recombination in mitotic and meiotic double-strand break repair

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Citation

Vries, F. A. T. de. (2007, January 17). *The role of homologous recombination in mitotic and meiotic double-strand break repair*. Retrieved from <https://hdl.handle.net/1887/8784>

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Note: To cite this publication please use the final published version (if applicable).

The role of homologous recombination in mitotic and meiotic double-strand break repair

The role of homologous recombination in mitotic and meiotic double-strand break repair

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 17 januari 2007
klokke 16.15 uur

door

Femke Adriana Theodora de Vries

Geboren te Nijmegen,
in 1977

Promotiecommissie

Promotor: Prof. Dr. Ir. A.A. van Zeeland

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Referent: Dr. W.M. Baarends (EUR)

Overige leden: Prof. Dr. L.H.F. Mullenders
Prof. Dr. A.K. Raap

The role of homologous recombination in mitotic and meiotic double-strand break repair.

ISBN-10: 90-9021192-6

ISBN-13: 978-90-9021192-3

Printed by Gildeprint Drukkerijen B.V., Enschede

The printing of this thesis was financially supported by:
Makelaarskantoor Rijnland B.V.

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Chapter 1

General Introduction

1.1 Introduction

The induction of damage in DNA is a threat to all living organisms. First, the presence of damage in DNA may hamper proper functioning of a cell by blockage or impediment of transcription and indirectly of translation. Second, the occurrence of DNA damage may interfere with replication and faithful transmission of genetic information to daughter cells. Accumulation of damage in DNA can lead to programmed cell death (apoptosis) and the formation of mutations and chromosomal aberrations, which ultimately may lead to hereditary diseases and the onset of cancer. Signalling of DNA damage and subsequent repair is therefore of vital importance for each organism. To deal properly with lesions in DNA, various damage signalling and repair pathways evolved in prokaryotic and eukaryotic cells. The significance of proficient damage signalling and repair mechanisms is apparent from the cancer predisposition of individuals carrying mutations in genes involved in cellular responses to DNA damage.

One of the most severe genotoxic lesions is a DNA double-strand break (DSB), as both DNA strands of the double helix are affected. DSBs can be induced by ionizing radiation and other exogenous DNA-damaging agents, free radicals generated during cellular metabolic processes and collapsed replication forks (Cox, 2002). DSBs also arise as intermediates in several normal physiological processes such as the initiation of recombination during meiosis, mating-type switching in yeast and the assembly of immunoglobulin and T-cell receptor genes from variable (V), diversity (D), and joining (J) gene segments (V(D)J rearrangement). Persisting or incorrectly repaired DSBs in DNA can result in chromosomal rearrangements (including chromosomal fragmentation, translocations and deletions). In multicellular organisms chromosomal rearrangements contribute to carcinogenesis by activation of oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity (Ferguson and Alt, 2001). The importance of correct DSB-repair is underlined by the evolution of several DSB-repair pathways. The two major pathways in eukaryotes are non-homologous endjoining (NHEJ) and homologous recombination (HR). NHEJ is based on a religation of the ends irrespective of the presence of sequence repeats surrounding the break. HR, in which damaged chromosomes are repaired using homologous DNA sequences as a template, allows restoration of the original DNA sequence (Pâques and Haber, 1999).

1.2 DNA double-strand break repair

The two major DSB-repair pathways, HR and NHEJ, are schematically depicted in Figure 1. In NHEJ broken ends are joined without the necessity of extensive sequence homology between the ends. Due to processing of the ends before ligation, NHEJ is frequently associated with the insertion or deletion of nucleotides. DSB-repair via NHEJ in mammals involves the DNA-PK complex, i.e. Ku70, Ku80, DNA-PKcs and Artemis, a heterodimer consisting of XRCC4 and

ligase IV and the recently identified XRCC4-like factor (XLF, also named Cernunnos) (Ahnesorg et al., 2006; Callebaut et al., 2006; reviewed in Weterings and van Gent, 2004). Homologs of Ku70, Ku80, XRCC4 and Ligase IV have been identified in *S. cerevisiae* and *S. pombe*, but not of DNA-PKcs and Artemis. In contrast to mammals and also *S. pombe*, NHEJ in *S. cerevisiae* requires the Mre11/Rad50/Xrs2 complex (Zhang and Paull, 2005).

A sub-pathway in NHEJ is based on the presence of small sequence repeats (1-10 nucleotides) flanking the break and is called microhomology-mediated endjoining (μ EJ). During this type of endjoining, that is Ku70 independent, ends are resected by the action of a nuclease, creating 3' or 5'-overhangs, prior to annealing of the overhangs and removal of non-homologous single-strand DNA tails. Finally, remaining gaps are filled and nicks are ligated. This way of endjoining is necessarily error-prone and leads to deletions.

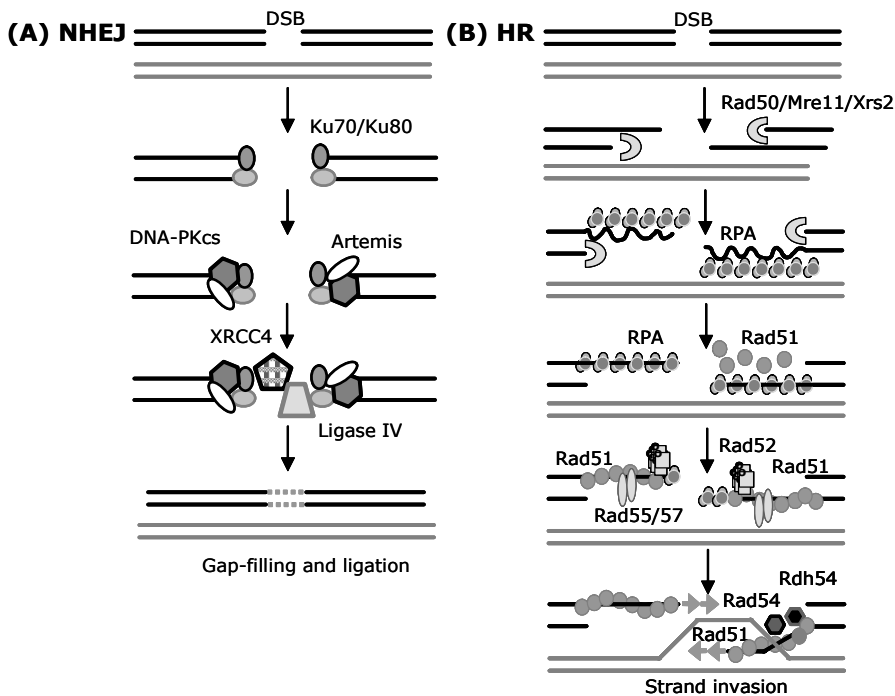


Figure 1: Repair of DSBs by non-homologous endjoining (NHEJ) in mammals and homologous recombination (HR) in *S. cerevisiae*. Details are discussed in the text. **(A)** In NHEJ, Ku70 and Ku80 bind to the double-strand DNA ends and subsequently recruit DNA-PKcs and Artemis. The ends are rejoined by the ligase IV / XRCC4 complex. **(B)** The first step in HR involves processing of the 5' ends of the DSB and is dependent on the Rad50/Mre11/Xrs2(Nbs1) complex. Secondary structures in the 3' ends produced in this way are removed by the RPA protein. Strand invasion requires the formation of Rad51 filaments and the participation of a number of associated proteins, including Rad52, Rad55, Rad57, Rad54 and Rdh54/Tid1. Following DNA synthesis, the recombination intermediates are resolved and ends are ligated.

The molecular aspects of HR were extensively studied in *Saccharomyces cerevisiae* and other fungi. The analysis of radiation-sensitive (RAD) mutants has provided a wealth of information concerning the mechanistic aspects of DNA repair. Three major classes of radiation-sensitive mutants have been identified: those that are primarily UV-radiation sensitive (RAD3 group), those that are primarily sensitive to ionizing radiation (RAD52 group) and those that are sensitive to both UV- and ionizing radiation (RAD6 group). The RAD52 group of proteins are involved in DSB-repair through HR and includes the Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, Rdh54/Tid1, Mre11 and Xrs2 proteins (Krogh and Symington, 2004) (see Table 1).

The relative contribution of NHEJ and HR to the repair of DSBs depends on the species, cell type, phase of the cell cycle and structure of the break. In lower eukaryotes, HR is the main pathway for DSB-repair. NHEJ is a minor pathway that only contributes to the repair in the absence of HR (Milne et al., 1996; Siede et al., 1996). In higher eukaryotes, HR and NHEJ are both important for the repair of DSBs but their relative contribution is dependent on cell type and phase of the cell cycle. Before replication, during G1 and early S, NHEJ is the principal DSB-repair mechanism. During S and G2 stages of the cell cycle, when sister chromatids can be effectively used as templates, HR is the predominant pathway of repair, although NHEJ also functions during S and G2 (Yamamoto et al., 1996; Takata et al., 1998; Kim et al., 2005). HR involves the exchange and transfer of genetic information between different DNAs or within the same molecule. In mitotically dividing cells HR is essential for the repair of DSBs formed during replication and collapse of replication forks. Abrogation of HR is not tolerated in mammalian cells and may lead to retarded growth in fungi. In addition, HR is involved in the processing of DSBs inflicted by exogenous and endogenous agents and in telomere maintenance in telomerase deficient cells (ALT mechanism). Breaks induced by the lymphoid specific factors RAG1 and RAG2 during V(D)J recombination are not repaired via HR but are exclusively restored by NHEJ (Lieber et al., 2004). The formation of germ cells is strictly dependent on the process of HR. Proper chromosome segregation in meiosis requires recombination between homologous chromosomes and the formation of crossovers (see section 1.4) (Smith and Nicolas, 1998). In early meiotic prophase, Ku70 is absent and DSB-repair via imprecise NHEJ, which would be highly disadvantageous, is not possible (Goedecke et al., 1999). In both yeast and *Drosophila*, deficiencies in HR result in severe defects in the formation of germ cells. Similar mutations in mammals are lethal.

Which factors in eukaryotic cells determine the choice between HR and NHEJ after the induction of DSBs is not fully comprehended. Competition between Ku70 and Rad52 as gatekeepers of NHEJ and HR for binding the same broken DNA ends is not very likely, since Ku70 preferentially binds DNA with blunt ends, while under the same conditions, Rad52 prefers binding to breaks with ssDNA ends (Ristic et al., 2003). Recent studies in yeast have shown that cyclin dependent kinase I (CDK1) is required for resection of DNA ends and has a key role in regulating HR at different stages of the cell cycle (Aylon et al., 2004; Ira

et al., 2004). There are indications of repair events combining both pathways in a way that the initial steps (strand invasion and DNA synthesis) are dependent on HR but subsequent steps (the joining of DNA ends) are mediated through NHEJ (Moynahan and Jasin, 1997; Johnson and Jasin, 2000). Recently, it has been shown by Kim and co-workers, that even in S / G2 phase cells, proteins involved in NHEJ assemble at laser-induced damage sites before HR factors are recruited (Kim et al., 2005). Although high levels of laser energy were used in this study to induce damage, these data may suggest that NHEJ functions as an early repair pathway in S / G2 cells, while HR may be specifically required for the repair of persistent lesions.

1.3 Homologous recombination

Based on experiments in fungi, *Drosophila* and mammals, two models have been proposed to explain the genetic and molecular data of DSB-repair via recombination: the DSBR (double-strand break repair) and SDSA (synthesis-dependent strand annealing) models (reviewed in Haber, 2000).

Homologous recombination according to the DSBR model of Szostak and co-workers (Figure 2A) involves several stages (Orr-Weaver and Szostak, 1983; Szostak et al., 1983). During the first step, presynapsis, both ends of a DSB are processed into highly recombinogenic 3' single-strand overhangs by 5' to 3' exonuclease(s) or by the combined actions of helicase(s) coupled to endonuclease(s). The Mre11/Rad50/Nbs1 complex is directly or indirectly involved in this step. The eukaryotic single-strand binding protein RPA (replication protein A) is required to minimize secondary structures in the resected ends and facilitates the assembly of a Rad51-ssDNA nucleofilament. The Rad51 filament mediates pairing, invasion and strand-exchange with an intact template, either a sister chromatid or an homologous chromosome, resulting in the formation of a D-loop. In *S. cerevisiae*, the efficiency of Rad51 filament-mediated pairing and strand-exchange is stimulated by the Rad52 protein and the heterodimer of the Rad51-like proteins Rad55 and Rad57. In mammals this step is facilitated by Rad52 and the Rad51 paralogs, as well as the breast cancer-associated factor BRCA2 (Sung, 1997a; Sung, 1997b; Benson et al., 1998; New and Kowalczykowski, 2002; Yang et al., 2005). The invaded Rad51 filament is able to establish a modified replication fork and the start of DNA synthesis. If the replication fork encounters the other end of the DSB, an intermediate containing two Holliday junctions can be formed which can be extended by branch migration. Resolution of the Holliday junctions leads to gene conversions that can be associated with crossover and non-crossover events, theoretically in a 1:1 ratio (Figure 2A). However, mitotic gene conversions are only infrequently associated with crossing over and less than 50% of meiotic gene conversion events are associated with crossovers (Pâques and Haber, 1999). The Szostak model also predicts the formation of two regions of heteroduplex DNA to be present on different molecules. Genetic studies in yeast showed the presence of heteroduplex DNA in the recipient molecule but not in the molecule that was used as a template. The synthesis-dependent strand

annealing (SDSA) or migrating D-loop model (Figure 2B) predicts a model of DSB-repair in which newly synthesized DNA and heteroduplex DNA are only found in the recipient molecule. The SDSA model has evolved from the recombination model of Szostak and co-workers and diverges from this model in the later stages, when synthesis of DNA at the 3' terminus of the invading strand drives migration of the D-loop into the gap, instead of being extended by branch migration. After leading- and lagging strand synthesis, resolution can occur through unwinding of the strands followed by strand annealing, resulting in gene conversion events. Gene conversion associated with crossover can be explained by other versions of the SDSA model (Pâques and Haber, 1999).

Alternative (sub)pathways in homologous recombination include break-induced replication (BIR) and single-strand annealing (SSA). In *S. cerevisiae*, if there is no rejoining with the other end of the DSB, break-induced replication (BIR) will allow replication to proceed all the way to the end of the chromosome (or until it encounters a converging replication fork) resulting in long-track gene conversion events (Figure 2C). When the break is flanked by sequence repeats, exposure of homologous sequences may allow annealing of the ends (single-strand annealing, SSA). SSA results in deletion of the intervening sequences at the site of the break and is therefore intrinsically mutagenic (Figure 2D) (reviewed by Pâques and Haber, 1999; Krogh and Symington, 2004).

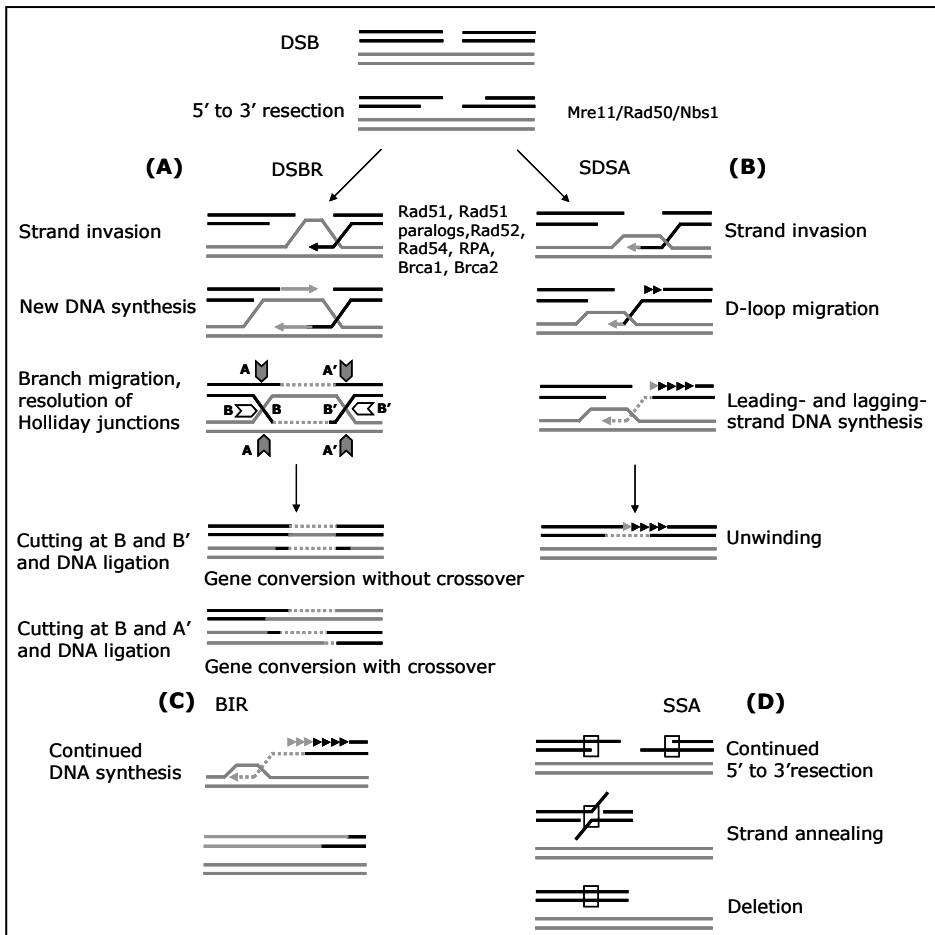


Figure 2: Models for DSB-repair through homologous recombination. **(A)** Repair according to the original Szostak double-strand break repair (DSBR) model. After the induction of a DSB, the 5' ends are resected and one of the single-strand 3' DNA ends invades an homologous molecule. After extension by DNA synthesis and branch migration, the two Holliday junctions are resolved, resulting in gene conversion without crossover (when cut at positions B and B') or gene conversion with crossover (when cut at positions B and A'). **(B)** Repair according to the synthesis-dependent strand annealing (SDSA) model. After resection of the 5' ends and invasion, DNA synthesis at the 3' terminus of the invading strand drives migration of the D-loop. After leading- and lagging-strand synthesis, the D-loop is resolved by unwinding followed by strand annealing resulting in gene conversion without crossover. **(C)** The process of break-induced replication (BIR) occurs when the two ends of the DSB fail to engage and replication can proceed all the way to the end of the chromosome (or until it encounters a converging replication fork). **(D)** Single-strand annealing (SSA) is dependent on the presence of sequence repeats (black box) on both sites of the break. Repair by SSA involves resection of the 5' ends followed by pairing of homologous sequences. After removal of non-homologous 3' DNA tails, the broken ends are rejoined resulting in deletions. Adapted from Haber (Haber, 2000).

1.3.1 Mitotic recombinational repair proteins

Genes involved in HR belong to the RAD52 epistasis group and are well characterised in eukaryotes (Table 1). In comparison with *S. cerevisiae*, the number of RAD52 group genes in mammals is larger. Besides Rad51, five Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3) have been identified in somatic cells. In addition several other factors have been identified which are required for HR, including RPA, the breast cancer-associated factors BRCA1 and BRCA2 and the DNA helicases Bloom's (homologous to Sgs1 in yeast) and Srs2 (Petrini et al., 1997; van Gent et al., 2001).

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are both ascomycetes, but are only distantly related and are as different from each other as either is from mammals (Sipiczki, 1995; Sipiczki, 2000). The study of *S. pombe* mutants, in parallel with *S. cerevisiae* homologues, can provide important insights in the function of genes and proteins in higher eukaryotes. The RAD52 group in *S. pombe* consists of *rad32*⁺, *rad50*⁺, *nbs1*⁺, *rhp51*⁺, *rad22A*⁺, *rad22B*⁺, *rhp54*⁺, *rdh54*⁺, *rhp55*⁺ and *rhp57*⁺ genes (*rad* homologue of *S. pombe*) (van den Bosch et al., 2002a).

1.3.2 Mre11, Rad50 and Nbs1/Xrs2

S. cerevisiae mre11, *rad50* and *xrs2* mutants display a spontaneous mitotic hyperrecombination phenotype, sensitivity to DSB-inducing agents, impairment in meiosis and compromised vegetative growth. The mutants also exhibit telomere shortening, increased levels of chromosomal rearrangements, reduced NHEJ and defective checkpoint activation after DNA damage. On the other hand, mating-type switching, gene conversion, SSA and telomere position effects are not affected in any of these mutants (reviewed in Symington, 2002). Through interaction with Rad50, Xrs2 and itself, Mre11 forms a stable Mre11/Rad50/Xrs2 complex. An analogous Mre11/Rad50/Nbs1 complex has been identified in mammals. In unirradiated mammalian cells these proteins are randomly distributed throughout the nucleus, but rapidly assemble into nuclear foci at the site of damage after exposure to irradiation (van den Bosch et al., 2003). The Mre11/Rad50/Nbs1 proteins are among the first factors recruited to the DSBs and most likely are involved in "sensing" of the damage. Treatment with DSB-inducing agents results in the immediate phosphorylation of the histone protein H2AX in yeast and mammalian cells. In response to DNA damage, phosphorylated H2AX (γ -H2AX) forms foci at the site of the DSB that colocalize with Rad50 or Rad51. The product of the tumor suppressor gene BRCA1 also colocalizes with γ -H2AX and is recruited to these sites before Rad50 or Rad51 (Kastan and Bartek, 2004; Kitagawa et al., 2004).

Inactivation of *MRE11*, *RAD50* and *NBS1* is not tolerated in mouse and chicken cells (Luo et al., 1999; Yamaguchi-Iwai et al., 1999; Zhu et al., 2001).

<i>S. cerevisiae</i>	<i>S. pombe</i>	Humans	Biochemical activity/function
Rad50	Rad50	Rad50	ATP-dependent DNA-binding activity; ATPase activity
Mre11	Rad32	Mre11	3' to 5' dsDNA exonuclease and ssDNA endonuclease activities; DNA-binding activity; ssDNA annealing activity; DNA duplex unwinding and hairpin cleavage activities
Xrs2	Nbs1	Nbs1	regulates DNA duplex unwinding and nuclease activities of the Mre11/Rad50/Xrs2(Nbs1) complex; recruits the Mre11/Rad50/Xrs2(Nbs1) complex to the DNA damage
Rad51	Rhp51	Rad51	ATP-dependent DNA-binding activity; ATP-dependent homologous pairing and DNA strand-exchange activities
–		Rad51B	DNA-binding activity (enhanced in complex with Rad51C); protein kinase activity; DNA-stimulated ATPase activity
–		Rad51C	DNA-binding activity (enhanced in complex with Rad51B or XRCC3); DNA-stimulated ATPase activity; resolvase activity; DNA strand-exchange and homologous pairing activity (also in complex with XRCC3); forms filamentous structures in complex with XRCC3
–		Rad51D	DNA-binding activity (also in complex with XRCC2); DNA-stimulated ATPase activity; homologous pairing activity in complex with XRCC2; forms filamentous structures in complex with XRCC2
Rad55*	Rhp55	XRCC2*	yeast: ATPase activity; human: DNA-binding activity (also in complex with Rad51D); homologous pairing activity in complex with Rad51D; forms filamentous structures in complex with Rad51D
Rad57*	Rhp57	XRCC3*	yeast: ATPase activity; human: DNA-binding activity (also in complex with Rad51C); homologous pairing activity (also in complex with Rad51C); forms filamentous structures in complex with Rad51C
Rad52	Rad22A	Rad52	DNA-binding activity; ssDNA annealing activity; weak DNA strand-exchange activity; weak homologous pairing activity
–	Rad22B		DNA-binding activity; ssDNA annealing activity
Rad59	–	–	DNA-binding activity; ssDNA annealing activity; homologous to the N-terminus of Rad52
Rad54	Rhp54	Rad54	DNA-binding activity; dsDNA-dependent ATPase activity; dsDNA unwinding activity; dsDNA topology-modifying activity
Rdh54/Tid1	Rdh54	Rad54B**	DNA-binding activity; dsDNA-dependent ATPase activity; dsDNA topology-modifying activity

Table 1: RAD52 group homologs in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and humans. * In yeast, two Rad51 paralogs, Rad55 and Rad57, have been identified and in mammals five (Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3). Although these paralogs resemble each other, it is not known at this moment whether XRCC2 and

XRCC3 are the functional homologs of Rad55 and Rad57, respectively. ** Although Rad54B shares significant similarity with yeast Rdh54/Tid1, Rad54B is probably not the functional homologue of Rdh54/Tid1.

In humans, hypomorphic mutations in the *NBS1* and *MRE11* genes are associated with Nijmegen breakage syndrome (NBS) and Ataxia telangiectasia-like disorder (ATLD) respectively (Carney et al., 1998; Varon et al., 1998; Matsuura et al., 1998; Stewart et al., 1999). Both *nbs1* and *mre11* mutant cells display chromosomal instability, increased radiation sensitivity, abnormal cell cycle regulation and impaired Rad50 foci formation after irradiation. Hypomorphic *rad50* mutant mice (*rad50^{S/S}* mice) have been shown to be predisposed to cancer (Bender et al., 2002). Mutant mouse cells also exhibit increased sensitivity to IR, DSB-repair defects and impaired checkpoint control. Similar results have been obtained for conditional *mre11*, *rad50* and *nbs1* mutant murine and chicken cells (Xiao and Weaver, 1997; Yamaguchi-Iwai et al., 1999; Luo et al., 1999; Zhu et al., 2001). Mice carrying a deletion of exons 2 and 3 of the *NBS1* gene are viable, but retarded in growth, impaired in lymphocyte development and oogenesis, hypersensitive to IR and prone to lymphoid tumors (Kang et al., 2002).

The Mre11 protein is the catalytic subunit of the Mre11/Rad50/Xrs2(Nbs1) complex. Mre11 can form dimers and multimers and has 3' to 5' dsDNA exonuclease and ssDNA endonuclease activities (Furuse et al., 1998; Usui et al., 1998; Chamankhah and Xiao, 1999; D'Amours and Jackson, 2002). In HR, the nuclease activity of the Mre11/Rad50/Xrs2(Nbs1) complex is required for processing of broken DNA ends (directly or indirectly) and the two DNA binding sites may mediate sister chromatid interactions, as well as initiating and stabilizing of D-loop formation (Hopfner et al., 2001; Connelly and Leach, 2002; Hopfner et al., 2002).

Human Mre11 binds both ssDNA and dsDNA, with a preference for ssDNA, and does not require DNA termini for efficient binding (Sharples and Leach, 1995; Paull and Gellert, 1998; Trujillo et al., 1998; Trujillo and Sung, 2001). Mre11 in man also displays activities that are not seen without Nbs1, including partial unwinding of a DNA duplex and efficient hairpin cleavage (Paull and Gellert, 1999). Moreover, Mre11 mediates the annealing of complementary ssDNA molecules but, in contrast to human Rad52, this activity is abrogated by the single-strand binding protein RPA (de Jager et al., 2001a). Rad50 belongs to the family of structural maintenance of chromosome (SMC) proteins and consists of globular N- and C-termini separated by long coiled-coil regions. Electron microscopy studies indicated the formation of intramolecular coiled-coil structures with a globular head containing Walker A and B ATPase domains (Hopfner et al., 2001). Based on studies of Mre11 and Rad50 of *Pyrococcus furiosus*, the stoichiometry of the Mre11/Rad50 complex has been determined. The complex consists of two Rad50 and two Mre11 molecules. The Mre11 dimer binds to the coiled-coils of two Rad50 molecules adjacent to the ATPase domain. The globular head is presumably responsible for the DNA-binding and/or DNA end processing activities of the complex. The Nbs1 protein most likely also binds

to the globular Mre11/Rad50 head, but the precise location has not been revealed. At the apex of the intramolecular coiled-coil structure a conserved zinc-binding Cys-X-X-Cys motif has been identified which allows dimerization of two Rad50 molecules (de Jager et al., 2001b; Assenmacher and Hopfner, 2004). A role for tethering of DNA ends and sister chromatids for the Rad50 complex is supported by genetic studies in *S. cerevisiae* and *S. pombe* showing increased levels of recombination between homologous chromosomes in *rad50* mutant strains (Bressan et al., 1999; Hartsuiker et al., 2001).

Nbs1 is likely to represent a regulatory subunit of the Mre11/Rad50/Xrs2(Nbs1) complex, which is involved in its nuclear localization, signal transduction and catalytic activation. It is also a regulator of the ATP-driven unwinding and nuclease activities of the Mre11/Rad50/ Nbs1 complex (Dong et al., 1999; Petrini, 1999; Desai-Mehta et al., 2001; Mirzoeva and Petrini, 2001).

1.3.3 Rad51 and Rad51 paralogs

Rad51 is the key protein in HR and is homologous to the bacterial RecA protein. In yeast, *rad51* null mutants exhibit a drastic increase in radiation sensitivity and severe defects in mitotic and meiotic recombination. In meiotic cells, an accumulation of DSBs at recombination hotspots, a reduced formation of recombination intermediates and reduced spore viability were observed (Shinohara et al., 1992; Donovan et al., 1994; Rattray and Symington, 1995; Fasullo et al., 2001). Inactivation of *RAD51* in mice leads to embryonic lethality (Tsuzuki et al., 1996). Chicken DT40 cells depleted of Rad51 gain massive chromosome abnormalities and accumulate in the G2/M phase of the cell cycle before dying (Sonoda et al., 1998). Rad51 contains the conserved Walker A and B motifs and has an ATP-dependent DNA-binding activity (both ssDNA and dsDNA) (Benson et al., 1994; Sung, 1994). Polymerization of Rad51 on ss- and dsDNA results in the formation of nucleoprotein filaments closely resembling those formed by the RecA protein. Rad51 filaments mediate pairing with a template DNA molecule and strand-exchange. Depending on the presence of 5'- or 3'- single-strand ends, Rad51 promotes strand-exchange in the 3'-to-5' or 5'-to-3' direction (Ogawa et al., 1993; Sung, 1994; Sung and Robberson, 1995; Baumann et al., 1996). The efficiency of the strand-exchange reaction by Rad51 is rather limited in comparison with RecA. The Rad51-mediated DNA strand-exchange is stimulated by RPA, but only when RPA is added to the reaction after Rad51. In contrast, RPA inhibits Rad51 nucleoprotein complex formation when added before Rad51 by prior binding to ssDNA, thereby preventing the formation of Rad51-ssDNA filaments (Namsaraev and Berg, 1997; Sugiyama et al., 1997). The inhibitory effect of RPA can be overcome by addition of the Rad52 protein or the *S. cerevisiae* Rad55-Rad57 heterodimer, suggesting that these proteins mediate RPA displacement by the Rad51 protein (Sung, 1997b; Sung, 1997a; New et al., 1998; Sugiyama and Kowalczykowski, 2002). The mammalian Rad52 also stimulates the pairing and strand-exchange of Rad51, although RPA was not included in these studies (Benson et al., 1998; Baumann and West, 1999). Rad55 and Rad57 in yeast have limited homology with Rad51 and contain

Walker A and B motifs. Rad55 interacts with Rad51, but not with RPA, suggesting that the role of both Rad51 paralogs differs from that of Rad52. As overexpression of Rad51 can partially complement the X-ray sensitivity of *rad55* or *rad57* mutant strains, the Rad55-Rad57 heterodimer most likely stabilizes the Rad51 nucleoprotein filament (Sung, 1997b; Song and Sung, 2000). Rad51-mediated joint molecule formation is also enhanced by Rad54 and Rdh54/Tid1 in both yeast and mammals (Golub et al., 1997; Petukhova et al., 1998; Van Komen et al., 2000; Van Komen et al., 2002).

In mammals five Rad51 paralogues have been identified: XRCC2, XRCC3, Rad51L1 (Rad51B), Rad51L2 (Rad51C) and Rad51L3 (Rad51D). Sequence homology between Rad51 and the Rad51-like proteins is limited (around 20%) and mainly restricted to regions containing the Walker A and B domains. Rad51 paralogues have been suggested to function as the vertebrate equivalents of the yeast proteins Rad55 and Rad57 (Symington, 2002; Thacker and Zdzienicka, 2003). Hamster and chicken DT40 cells containing (hypomorphic) mutations in one of these genes display chromosomal instability, a moderately increased sensitivity to ionizing radiation and a strongly increased sensitivity to cross-linking agents (Cui et al., 1999; Godthelp et al., 2002; Takata et al., 2000; reviewed by Thacker and Zdzienicka, 2003). Inactivation of Rad51B, Rad51D and XRCC2 in mice results in embryonic lethality (Shu et al., 1999; Deans et al., 2000; Pittman and Schimenti, 2000; Deans et al., 2003). Protein-protein interaction studies showed the presence of two complexes in mammalian cells, one containing Rad51B, Rad51C, Rad51D and XRCC2 and the other consisting of Rad51C and XRCC3 (Braybrooke et al., 2000; Schild et al., 2000; Lio et al., 2003). A complex consisting of Rad51B and Rad51C mediates Rad51-dependent pairing and strand-exchange, like has been shown for Rad55 and Rad57 in yeast (Sigurdsson et al., 2001; Miller et al., 2002; Lio et al., 2003). Homologous pairing activities have also been shown for the Rad51B-Rad51C-Rad51D-XRCC2 and Rad51C-XRCC3 complexes (Liu et al., 2002; Lio et al., 2003; Miller et al., 2004; Yonetani et al., 2005; Rodrigue et al., 2006). The complex containing Rad51C and XRCC3 has also been implicated in late steps in homologous recombination in the processing of Holliday junctions. The functional interaction of XRCC3 with Bloom's helicase is in agreement with this (Brenneman et al., 2002; Braybrooke et al., 2003; Yokoyama et al., 2003; Liu et al., 2004).

1.3.4 Rad52

The Rad52 protein in *S. cerevisiae* is required for almost all types of homology dependent DSB-repair, including mitotic and meiotic recombination events, SSA and BIR (Rattray and Symington, 1994; Zou and Rothstein, 1997; Sugawara and Haber, 1992a). *S. cerevisiae rad52* null-mutants are extremely sensitive to ionizing radiation and methylmethane sulfonate (MMS), defective in mitotic recombination and completely deficient in meiosis. In contrast, Rad52 deficient mouse ES and chicken DT40 cells have only mild defects in homologous recombination, but do not show hypersensitivity to DSB inducing agents (Rijkers

et al., 1998; Yamaguchi-Iwai et al., 1998). In addition, null mutant mice are normally viable and fertile and show no abnormal development of the lymphoid system. The mild phenotype of *rad52* mutant mice and chicken cells suggests the existence of additional mammalian proteins functionally redundant with Rad52. Interestingly, inactivation of *XRCC3* in *rad52*^{-/-} chicken cells is not tolerated. Conditional mutants deficient in both Rad52 and XRCC3 exhibited extensive chromosomal breaks, suggesting an overlapping role for Rad52 and XRCC3 in HR (Fujimori et al., 2001). In *Drosophila* and *C. elegans*, no Rad52 homologue was discovered, indicating that the role of Rad52 is compensated for by other factors. In *U. maydis*, a *rad52* deletion does not affect DSB-repair, similar to mammalian cells. Purified Brh2, the *U. maydis* homologue of mammalian BRCA2, stimulates Rad51-mediated filament formation and strand-exchange (Yang et al., 2005). Similar results have recently been obtained for a purified peptide encompassing the BRC3/4 motifs and the DNA binding domain of human BRCA2 (Filippo et al., 2006). Presumably, BRCA2 can functionally compensate for Rad52.

In fission yeast *S. pombe*, two *RAD52* homologs, *rad22A*⁺ and *rad22B*⁺, are present (Ostermann et al., 1993; Suto et al., 1999; van den Bosch et al., 2001a). A *rad22A* deletion mutant is hypersensitive to ionizing radiation and defective in recombination, indicating that Rad22A is crucial in recombination and repair in vegetative *S. pombe* cells (van den Bosch et al., 2001a). Inactivation of *rad22B*⁺ does not lead to a decrease in homologous recombination or enhanced radiation sensitivity. In vegetatively dividing cells, the expression level of *rad22B*⁺ is very low and most likely Rad22B has an auxiliary role in DSB-repair. In contrast to *rad22A*⁺, the expression of *rad22B*⁺ during meiosis is strongly enhanced, indicating a more pronounced role for Rad22B in meiosis (van den Bosch et al., 2001a; van den Bosch et al., 2002b). *RAD59*, a gene homologous to *RAD52*, has been found in *S. cerevisiae* (Bai and Symington, 1996) and the closely related yeast *Kluyveromyces lactis* (van den Bosch et al., 2001b), but not in *S. pombe*. *rad59* mutants exhibit reduced intrachromosomal recombination rates, show intermediate γ -irradiation sensitivity and are only slightly defective in meiosis.

During Rad51-mediated repair, Rad52 binding to Rad51 has been shown to overcome the inhibitory effect of RPA on filament formation (Sung, 1997a; New et al., 1998; Shinohara and Ogawa, 1998; Sugiyama et al., 1998; New and Kowalczykowski, 2002). Rather than binding to RPA, Rad52 loads Rad51 on the single-strand tail, thereby replacing RPA. A stable complex of Rad51 and Rad52 has been shown *in vivo* by immunoprecipitation (Sung, 1997a; Song and Sung, 2000). *In vitro* data suggest that in the presence of Rad52, strand invasion by the Rad51-ssDNA complex is more efficient. Three distinct roles for Rad52 have been suggested: a presynaptic role necessary for Rad51 assembly, a synaptic role with Rad51 filaments, and a postsynaptic role after Rad51 dissociates (Miyazaki et al., 2004). The yeast and human Rad52 proteins have Rad51-independent annealing activity for short complementary single-stranded DNAs that is specifically enhanced by RPA (Mortensen et al., 1996; Shinohara et al., 1998; Benson et al., 1998). Both human and yeast Rad52 form heptameric ring-

like structures on both ss- and dsDNA (Shinohara et al., 1998; Van Dyck et al., 1998). The N-terminal part of Rad52 has a role in DNA binding, but is also responsible for the homologous pairing activities and homodimerization of Rad52 (Shen et al., 1996c; Krejci et al., 2000; Kagawa et al., 2001; Kagawa et al., 2002). The crystal structure of the homologous-pairing domain (the N-terminal amino-acid residues 1-212 of human Rad52) reveals a closed undecameric ring with an rotational axis running down the center. The C-terminal region of Rad52 plays essential roles in interactions with Rad51 and RPA. Homologs of Rad52 with reduced length as Rad59 and Rad52 splice variants in mammals, which still exhibit strand pairing activities, may exist as undecameric rings, while the more widely spaced heptameric form might be more efficient in Rad51 filament formation and/or strand invasion (Kagawa et al., 2002).

Rad59 is structurally and functionally highly conserved between *S. cerevisiae* and *K. lactis*. Rad52 and Rad59 are suggested to have partly overlapping functions in recombination and in the single-strand annealing pathway (Bai et al., 1999). Like Rad52, Rad59 binds to ssDNA and stimulates annealing of complementary DNA strands. However, DNA annealing by Rad59, unlike Rad52, is not stimulated by the ssDNA binding protein RPA (Petukhova et al., 1999a).

S. pombe Rad22A and Rad22B proteins resemble the *in vitro* functions of Rad52. Both proteins preferentially bind ssDNA and are capable of annealing complementary oligo's. Rad22A and Rad22B are self-interacting proteins and form multimeric structures (see Chapter 2).

1.3.5 Rad54

Rad54 is a member of the Swi2/Snf2 family of chromatin remodeling proteins which have been implicated in DNA repair, transcription and recombination (Eisen et al., 1995). *S. cerevisiae rad54* mutants are viable but show enhanced sensitivity to ionizing radiation and cross-linking agents (Shinohara et al., 1997). Mutations in *RDH54/TID1*, which is homologous to *RAD54*, result in less severe defects in repair and recombination as compared to *rad54* mutations. Increased sensitivity to MMS can only be detected in diploid and not in haploid cells. Rad54 is required for both inter- and intrachromosomal recombination between sister chromatids, while *Rdh54/Tid1* functions specifically in interchromosomal recombination and BIR. Both *rad54* and *rdh54/tid1* single mutants show reduced sporulation efficiency and spore viability. However, the *rad54-rdh54/tid1* double mutant is completely defective in meiosis (Klein, 1997; Shinohara et al., 1997).

Rad54 deficient mouse cells are sensitive to ionizing radiation, methylmethane sulfonate (MMS) and the cross-linking agent mitomycin C (MMC), but not to ultraviolet light (Essers et al., 1997). *rad54*^{-/-} mice are viable and show normal development of the immune system. Interestingly, *rad54*^{-/-} mice are hypersensitive to ionizing radiation only at the embryonic stage, but regardless of their developmental stage they are hypersensitive to MMC (Essers et al., 1997; Essers et al., 2000). Ablation of a second Rad54 homologue in mammals, Rad54B, leads to increased sensitivity to ionizing radiation and mitomycin C. Mice lacking both Rad54 and Rad54B are extremely sensitive to MMC as

compared with both single mutants and at cellular levels, the efficiency of plasmid integration is drastically reduced. The absence of Rad54 affects the distribution of Rad51 in meiotic chromosomes, but fertility is not reduced in single or double mutant animals (Wesoly et al., 2006).

Also in *S. pombe* two Rad54 homologs have been identified, Rhp54 and Rdh54, which are the homologs of Rad54 and Rdh54/Tid1 in *S. cerevisiae*, respectively. Rhp54 deletion strains are extremely sensitive to ionizing radiation and slightly to ultraviolet light (Muris et al., 1996). Homologous recombination as measured by plasmid integration is also severely affected in mutant cells. Rhp54 seems less important in meiosis as spore viability is only slightly reduced. In these respects, *rhp54* mutants resemble *rad54* mutant strains in *S. cerevisiae*. Rdh54 in *S. pombe* is only expressed during meiosis and is specifically required for the repair of meiotic DSBs (Catlett and Forsburg, 2003).

Swi1/Snf2 family proteins to which Rad54 homologs belong, contain Walker A and B ATPase domains and seven motifs characteristic for RNA/DNA helicases. The Rad54 protein is a DNA-stimulated ATPase required for the formation of the D-loop, a joint molecule between the broken DNA and the intact template (Petukhova et al., 1999b) and chromatin remodeling (Alexiadis and Kadonaga, 2002; Jaskelioff et al., 2003; Alexeev et al., 2003). It is able to transiently unwind duplex DNA by introducing positive and negative supercoils and promotes homologous DNA pairing via a specific interaction with the eukaryotic recombinase Rad51 (Petukhova et al., 1998; Mazin et al., 2000; Mazin et al., 2003; Van Komen et al., 2000; Essers et al., 2002). The chromatin remodeling and DNA-stimulated ATPase activities of Rad54 are significantly enhanced by Rad51. Deletion of an N-terminal motif of Rad54, which is identical in *Drosophila*, mice, and humans, results in a complete loss of chromatin remodeling and strand pairing activities and partial inhibition of the ATPase activity of Rad54. The N-terminal region has no apparent effect on the ability of DNA to promote ATPase activity of Rad51 augmented DNA-stimulated ATPase activity (Alexiadis et al., 2004).

Rdh54/Tid1 protein has DNA binding properties, cooperates in Rad51-mediated D-loop formation and can introduce both positive and negative supercoils in DNA in the presence of ATP, as demonstrated for Rad54. (Dresser et al., 1997; Petukhova et al., 2000). Rad54B shows amino acid sequence similarity to Rdh54/Tid1, but the interactions with DNA and Rad51 are similar to those of Rad54. Rad54B also interacts with Rad51 and a meiosis-specific Rad51 homologue, Dmc1 (Tanaka et al., 2000; Miyagawa et al., 2002; Tanaka et al., 2002; Bross et al., 2003; Wesoly et al., 2006).

1.3.6 Other proteins involved in HR

Homologous recombination involves factors belonging to the RAD52 group, which are implicated in the early stages of recombination, but also proteins functioning during DNA synthesis, branch migration, resolution of Holliday junctions, ligation etc. Factors belonging to the RecQ family of 3'-5' DNA helicases have also been implicated in HR. In man, five RecQ-like helicases have

been identified: BLM, WRN, RecQL, RecQ4 and RecQ5. Mutations in *BLM*, *WRN* and *RECQ4* cause Bloom's syndrome, Werner's syndrome and Rothmund-Thompson syndrome, respectively. Hallmarks of these three autosomal recessive diseases are increased genomic instability and cancer predisposition (Bachrati and Hickson, 2003). Individuals suffering from Werner's or Rothmund-Thompson syndrome are also characterized by premature ageing. At the cellular level Bloom's cells display an elevated level of sister chromatid exchanges (SCEs) and other chromosomal aberrations, including quadriradials and translocations. *S. cerevisiae* and *S. pombe* contain only one RecQ homologue, Sgs1 and Rqh1, respectively. Mutant strains exhibit a pleiotropic phenotype associated with increased sensitivity to DNA-damaging agents, enhanced chromosome loss, increased number of meiotic crossovers and a hyper-recombination phenotype in vegetative cells (Myung et al., 2001; Doe and Whitby, 2004; Win et al., 2005; Azam et al., 2006). The hyper-recombination phenotype of *sgs1* strains can be suppressed by deleting genes of the RAD52 group genes (Gangloff et al., 2000). Recently it has been shown (Wu and Hickson, 2003) that a complex consisting of the Bloom's protein and Topoisomerase III α can resolve synthetic double Holliday junctions without crossing over of genetic material (dissolution). The authors propose branch migration mediated by the Bloom's protein, and the formation of hemicatenates and decatination through the action of TOPIII α . Sgs1 and Top3 in *S. cerevisiae* also act in the same pathway to prevent the formation of crossovers as a consequence of replication restart in proliferating cells, which depends on recombinational repair mechanisms (Ira et al., 2003). Mutations in another helicase in *S. cerevisiae*, *SRS2*, also result in increased crossover frequencies. However, the anti-recombinogenic properties of Srs2 are based on disruption of Rad51 protein filaments (Ira et al., 2003; Macris and Sung, 2005; Paffett et al., 2005). Inactivation of both pathways in *srs2-sgs1* double mutants is not tolerated.

The formation of SCEs is most likely not harmful, but the use of sequences on the homologous chromosome or at an ectopic site and the formation of exchanges, may have deleterious consequences for the organism. Genomic instability and cancer predisposition associated with defects in RecQ homologs underscore the importance of preventing hazardous recombination events during restart of DNA synthesis after collapse of replication.

A direct relation between defects in HR and increased tumorigenesis has come from the analysis of the tumor suppressor genes *BRCA1* and *BRCA2*. Germline mutations in one of these two genes, predispose women to an early onset of breast cancer. Both *BRCA1* and *BRCA2* encode large proteins which interact with each other and are involved in multiple processes mediating the cellular responses to DNA damage and include checkpoint activation, transcription responses and DNA repair. Hypomorphic mutations result in increased radiation sensitivity and defects in HR (Scully and Livingston, 2000; Moynahan et al., 1999; Moynahan et al., 2001a; Moynahan et al., 2001b) implying a crucial role for BRCA1 and BRCA2 in DSB-repair. Immunofluorescence experiments indicate that both BRCA1 and BRCA2 rapidly assemble at the site of damage and at the

synaptonemal complex during early stages in meiosis (Scully et al., 1997a; Scully et al., 1997b; Chen et al., 1998; Zhong et al., 1999; Bhattacharyya et al., 2000). BRCA1 interacts with a wide variety of proteins engaged in sensing of DSBs and mediating damage signals (BARD1, BACH1, ATM, Chk2, Cdk2, the Rad50/Mre11/Nbs1 complex etc.) and is rapidly redistributed after imposing DSBs (reviewed by Narod and Foulkes, 2004). Evidence obtained by several groups suggests the formation of a complex at the site of the damage in which BRCA1 acts as a scaffold and integrates the activities of various factors required for proper cellular responses to damage.

The BRCA2 protein directly interacts with Rad51 through its BRC repeats and based on recent experiments in *U. maydis* and man, most likely mediates the formation of Rad51 nucleoprotein filaments. As *rad52* mutant strains of *U. maydis* hardly show defects in DSB-repair, BRCA2 may functionally compensate for loss of Rad52 in addition to its role in other processes related to damage responses (Yang et al., 2005). Although BRCA2 acts as a tumor suppressor for breast cancer, biallelic mutations have recently been linked to Fanconi anemia (group D1), a rare disorder in man characterized by cancer susceptibility and an increased sensitivity to cross-linking agents at the cellular level (Howlett et al., 2002).

1.4 Recombination during meiosis

1.4.1 Meiosis

During S-phase of both mitotic and meiotic cell cycles DNA is replicated and each chromosome becomes duplicated, producing two sister chromatids connected by cohesin proteins (Uhlmann and Nasmyth, 1998). In meiosis, two rounds of chromosome segregation follow one round of chromosome replication, yielding haploid gametes. The first meiotic division, meiosis I, is reductional and bivalents migrate to opposite poles. In prophase I, replicated homologous chromosomes, each consisting of two chromatids, undergo synapsis. Crossovers are induced between non-sister chromatids (at least one per pair of chromosomes) leading to the formation of chiasmata. The paired chromosomes, named bivalents, align on the metaphase plate. At anaphase I, the connection between homologs is lost and the chromosomes of each homologous pair move to separate poles. The nucleus divides, creating daughter nuclei in which every chromosome consists of two chromatids. The second division, meiosis II, separates the sister chromatids yielding four haploid daughter cells. In some fungi, e.g. *Neurospora* species (*N. crassa* and *N. sitophila*), eight haploid spores are formed after meiosis followed by a mitotic division. Sexual reproduction combines genes from two different parents to form genetically diverse offspring and contributes to genetic variation in a population through independent assortment of chromosomes during meiosis I, crossing over between homologous chromosomes during meiosis I, and random fertilization of ova by sperm. Sexual reproduction and mutations generate genetic variation among individuals, which is the starting material for evolution by natural selection.

1.4.2 Meiotic recombination

Homologous recombination during meiosis is essential for generating genetically distinct gametes and for correct segregation of homologous chromosomes at the first meiotic division. In most organisms recombination between homologs is accompanied by synapsis, the side-by-side association of homologous paternal and maternal chromosomes during the first prophase of meiosis, leading to the formation of a proteinaceous structure, the synaptonemal complex (SC, Figure 3) (Moses, 1956; reviewed by Page and Hawley, 2004). The SC starts to appear during the leptotene stage of the first meiotic prophase by the formation of two proteinaceous axial cores (axial elements, AEs) along the two sister chromatids of each chromosome, held together in part by meiosis-specific cohesins. The mammalian axial elements contain at least two SC specific proteins, Scp2 and 3 (synaptonemal complex proteins 2 and 3) (Schalk et al., 1998). Following homologue recognition and alignment, during zygotene and pachytene stages of prophase I, the axial elements become closely connected by the transverse filaments (TFs), a process called synapsis. The SC can be considered as a framework to allow recombination between non-sister chromatids to lock the homologs and consists of two homologous AEs (called lateral elements (LEs) in the context of the SC) and numerous TFs (Heyting, 2005). Synapsis is complete in pachytene and the SC is disassembled again during diplotene and diakinesis stages of meiosis I.

Major TF components have been identified in mammals (Sycp1) (Meuwissen et al., 1992), *S. cerevisiae* (Zip1) (Sym et al., 1993), *D. melanogaster* (C(3)G) (Page and Hawley, 2001) and recently in *C. elegans* (Syp1 and Syp2) (MacQueen et al., 2002; Colaiácovo et al., 2003). Although these proteins hardly bear any sequence similarity, they share with Sycp1 the presence of a long central coiled-coil and globular ends. Within the SC, the TF proteins form parallel coiled-coil homodimers, with their C-termini embedded in the AEs and the N-termini of TF proteins from opposite AEs overlapping in the narrow central region between the AEs of the two homologous chromosomes (Sym and Roeder, 1995; Liu et al., 1996; Schmekel et al., 1996; Yuan et al., 1996; Dong and Roeder, 2000). The presence of Sycp1 can be detected until late pachytene and is lost from SCs coordinately with homologue-separation at diplotene. Scp3 remains a component of the AE of post-pachytene chromosomes until metaphase I and has a role in the reductional separation of sister centromeres until anaphase II (Moens and Spyropoulos, 1995). Scp2 is associated with the axial elements until metaphase I (Offenberg et al., 1998; Schalk et al., 1998).

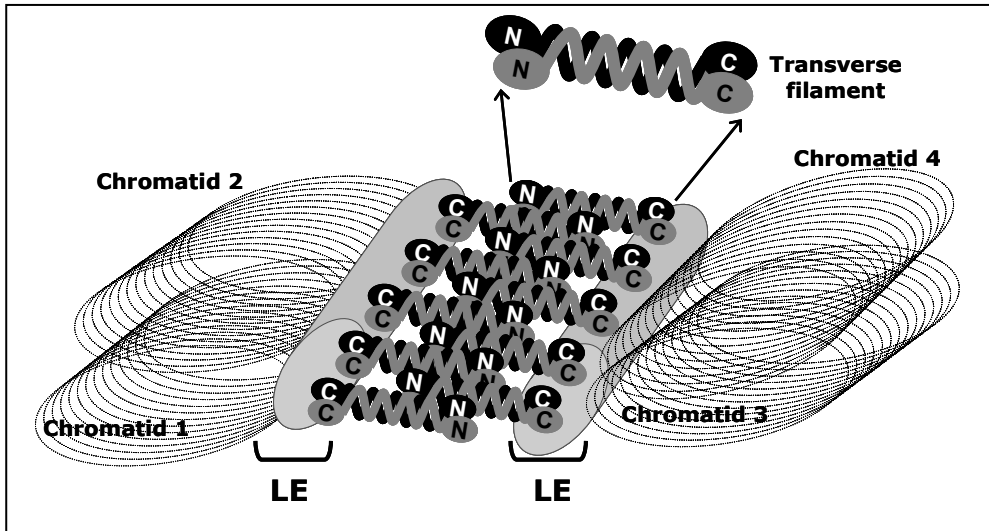


Figure 3: Model of the synaptonemal complex structure. The synaptonemal complex (SC) is formed by proteinaceous axial cores (called lateral elements (LE) in the context of the SC) along the sister chromatids and numerous transverse filaments. The LE comprises cohesins and the structural proteins Scp2 and Scp3. The transverse filaments are formed by the Sycp1 proteins. Adapted from Page & Hawley (Page and Hawley, 2004).

In all species except in *Drosophila* and *Caenorhabditis*, the recognition and pairing of homologous chromosomes requires initiation of homologous recombination by the formation of meiosis specific DSBs. The connection between homologs requires crossover activities between homologous chromosomes in all organisms, including *Drosophila* (in females) and *Caenorhabditis*.

Mutations compromising the assembly of SCs have hardly been studied in mammals, in contrast to lower eukaryotes. Disruption of *SCP3* in mice results in failure of homologue synapsis in prophase I, extended meiotic chromosomes, male sterility and the absence of axial chromosome core components (SCP2) (Yuan et al., 2000). *scp3*^{-/-} females are fertile, although a reduction in litter size has been observed as a consequence of increased aneuploidy in mutant oocytes (Yuan et al., 2002). Inactivation of the *SYCP1* analog *ZIP1* in yeast has a strong effect on spore viability and results in a decreased frequency of exchanges. Surprisingly the presence of residual crossing overs does not reduce the probability of another crossing over nearby (an event named interference) (Sym et al., 1993; Storlazzi et al., 1996; Tung and Roeder, 1998; Börner et al., 2004). Similarly, mutations in the TF proteins C(3)G and Syp1 in *D. melanogaster* and *C. elegans*, respectively, completely abrogate exchanges (Page and Hawley, 2001; MacQueen et al., 2002). In *sycp1*^{-/-} mice spermatocytes form normal AEs, which align, but synapsis was not observed and the

formation of crossovers was rare. *sycp1*^{-/-} spermatocytes also did not form XY bodies (see Chapter 5).

1.4.3 Meiotic recombinational repair proteins

Meiotic recombination in *S. cerevisiae* and also in other organisms is initiated by the formation of site-specific double-strand breaks by Spo11, a member of the type II-like topoisomerase family of proteins (Keeney et al., 1997). Spo11 expression in mice is essentially confined to reproductive tissues and is essential for fertility (Romanienko and Camerini-Otero, 1999; Baudat et al., 2000; Romanienko and Camerini-Otero, 2000). In mammals, more than 300 DSBs are induced per meiotic cell. Most of these breaks result in non-crossover events (gene conversions). Only a minor fraction leads to chiasmata and crossovers. Junctions between two homologous chromosomes can only result from recombination between non-sister chromatids during meiosis. However, in mitosis, the sister chromatid is the favourable template for repair. Specific meiotic proteins involved in homologous recombination are necessary to enhance non-sister chromatid recombination rather than recombination between sister chromatids. In *S. cerevisiae* and mice, recognition and pairing of homologous chromosomes is not possible in the absence of Spo11, in contrast to *C. elegans* and *D. melanogaster* (Heyting, 2005). In addition to Spo11, the formation and resection of breaks in *S. cerevisiae* requires factors belonging to the RAD52 group, including the Mre11/Rad50/Xrs2 complex and a number of other meiosis-specific recombination proteins (Pâques and Haber, 1999). In *rad50* null mutants in yeast, no DSBs are induced during meiosis. In hypomorphic *rad50*^{S/S} mutants, breaks are made but resection does not occur (reviewed in de Jager and Kanaar, 2002).

Besides Rad51, a second meiosis-specific RecA homologue, Dmc1, has been identified. Rad51 and Dmc1 have both distinct and cooperative roles in meiotic recombination. Compared with *rad51* mutant strains, the phenotype of *dmc1* mutants in yeast is even more severe in terms of spore viability and recombination defects. *dmc1*^{-/-} mice are infertile and although axial elements assemble on spermatocyte chromosomes, mature synaptonemal complexes are not observed in the majority of mutant cells. Presumably, Dmc1 is required for the establishment of proper interhomologue connections, since synapsis between non-homologous chromosomes is observed in mutant animals (Pittman et al., 1998; Tarsounas et al., 1999; reviewed by Shinohara and Shinohara, 2004). Immunofluorescence experiments revealed the formation of Rad51/Dmc1 foci in SCs from leptotene to late pachytene. In the absence of Spo11, these foci are not formed (Romanienko and Camerini-Otero, 2000).

As discussed before, the assembly of Rad51 filaments in yeast is promoted by RPA, Rad52, Rad55-57, Rad54 and Rdh54. Less is known about factors that stimulate Dmc1 filament formation. Recently, the involvement of a meiosis-specific heterodimer consisting of Mei5 and Sae3 in this process has been revealed (Hayase et al., 2004; Tsubouchi and Roeder, 2004). Homologs of Mei5 and Sae3 have also been identified in *S. pombe* and mammals.

Synapsis between homologous chromosomes in yeast and mice is also dependent on the Hop2 and Mnd1 proteins. Probably the Hop2-Mnd1 heterodimer is required for correct pairing and synapsis of homologs (Gerton and Hawley, 2005). Data of Petukhova and co-workers imply that Hop2, Mnd1, Rad51 and Dmc1 act coordinately in the formation of the first recombination intermediates after strand invasion (Petukhova et al., 2005).

RPA is also a component of synaptonemal complexes during late zygotene/early pachytene in mice, following the initiation of homologue synapsis (Plug et al., 1997; Raderschall et al., 1999; Walpita et al., 1999; Moens et al., 2002). RPA foci coincide with those of Rad51/Dmc1, but they appear somewhat later in time than Rad51 foci and persist longer, suggesting that RPA replaces Rad51 foci in maturing recombination nodules (Ashley et al., 1995; Plug et al., 1998; Moens et al., 2002). In pachytene when Rad51/Dmc1 foci disappear, RPA colocalizes with Msh4 and the Bloom's protein. During meicyte DSB-repair, Rad51/Dmc1 complexes are involved in synapsis of homologous chromosomes but most of these early joint molecules are later resolved by the anti-recombinogenic activities of the RPA/Msh4/BLM-topoisomerase complex, thereby preventing the formation of superfluous reciprocal recombinant events (Moens et al., 2002).

The meiosis-specific MutS homologs, Msh4 and Msh5 (MutS homologue 4 and 5) are members of the DNA mismatch repair (MMR) family but do not appear to function in classic MMR processes. The heterodimer of Msh4 and Msh5 is most likely required for recognition and stabilization of meiotic recombination intermediates (Snowden et al., 2004). Msh4 has an essential role in the control of male and female meiosis (Kneitz et al., 2000). The Msh4 protein is present in the nuclei of spermatocytes in early prophase I, forming discrete foci along meiotic chromosomes during zygotene and pachytene and localizing to early recombination nodules. After initiation of meiosis, homologous chromosomes fail to undergo normal pairing in *msh4*^{-/-} mice, indicating that the presence of Msh4 during the early stages of meiosis is essential for normal chromosome synapsis in prophase I (reviewed by Richardson et al., 2004). Also Msh5 influences chromosome pairing in prophase I. Inactivation of *MSH5* in mice leads to meiotic failure, which results in a size reduction of male and female reproductive organs (Edelmann et al., 1999).

Late steps in recombination are marked by Mlh1 and Mlh3 foci, which appear in mid-pachytene. Mlh1 and Mlh3 are also members of the MMR family and promote crossing over in an Msh4-Msh5-dependent manner and are essential for normal meiotic progression in both yeast and mice (Marcon and Moens, 2003; reviewed by Kolas and Cohen, 2004). Msh4 and Mlh1 physically interact and colocalize during pachytene progression to promote meiotic crossing over (Hunter and Borts, 1997; Santucci-Darmanin et al., 2000).

1.5 Protein modification

The properties of many proteins in eukaryotic cells are modulated by post-translational modifications. Protein modification may involve conjugation of small moieties as phosphate or acetyl groups or small proteins. The best known peptide modifications are ubiquitin and ubiquitin-like (UBL) proteins including SUMO, RUB/Nedd8, Atg12-Atg8, ISG15, FAT10 and Hub1/UBL5 (reviewed in Wilkinson, 2004).

1.5.1 Ubiquitin and other modifiers

Ubiquitin conjugates to target proteins by the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ϵ -amino group of a lysine residue in the target molecule. Ubiquitin is best known for its role in protein degradation, but ubiquitination is responsible for regulation of virtually all cellular processes, including cell division, growth, communication/signalling, movement and apoptosis. The attachment of ubiquitin to a specific target occurs in three enzymatic steps. In the first step, ubiquitin is activated by a ubiquitin-activating enzyme (E1) forming an E1-ubiquitin thioester in an ATP-dependent reaction. In the second step, the activated ubiquitin is transferred to a member of the ubiquitin-conjugating enzyme (E2) family. Finally, assisted by a ubiquitin-protein ligase (E3), which binds to specific substrates, ubiquitin is covalently attached to the target protein. Ubiquitin itself can also be modified by ubiquitination to form a polyubiquitin chain. Ubiquitin chain elongation can occur through lysine 6, 11, 27, 29, 33, 48 or 63 and depending on the lysine that is polyubiquitinated, the binding of many ubiquitin molecules to the same target protein is the triggering signal ("kiss of death") that leads to degradation of the protein in the 26S proteasome (a large ATP-dependent protease complex that consists of a 20S catalytic core and two 19S regulatory complexes) (Hershko and Ciechanover, 1998). Ubiquitination can be reversed by members of a large family of enzymes known as isopeptidases or deubiquitination enzymes (reviewed by Sun and Chen, 2004).

Ubiquitin-like (UBL) proteins are believed to act in a manner analogous to ubiquitin. UBLs can alter the conformation of the substrate or convey a new surface to their target allowing interaction with new partners. As demonstrated for ubiquitin, also modification by UBL proteins is controlled by the opposing actions of conjugating and deconjugating factors. SUMO has a wide variety of target proteins involved in transcription, nucleo-cytoplasmic transport, maintenance of chromosome structure and DNA repair. SUMO modification can have both stimulatory or negative effects on its targets. Another related-to-ubiquitin (RUB) protein, RUB/Nedd8, is post-translationally conjugated to the cullin subunits of E3 ubiquitin ligases. Neddylation regulates the function of these E3s. Organisms with lesions in the neddylation process exhibit severe growth defects (reviewed by Parry and Estelle, 2004). Atg12 and Atg8 are involved in the starvation process in yeast. ISG15 functions in immune responses. FAT10 has a putative role in apoptosis and cell cycle control.

Hub1/UBL5 is a relative newcomer to the UBL scene whose function remains to be elucidated. Additional ubiquitin-like proteins are likely to be discovered in the future (Wilkinson, 2004).

1.5.2 SUMO

Post-translational modification by the ubiquitin-related protein SUMO (small ubiquitin-like modifier) is a highly conserved process from yeast to humans and plays important regulatory roles in many cellular processes. In the budding yeast *S. cerevisiae* one SUMO gene, *SMT3*, has been identified that is essential for viability. *S. pombe* mutants lacking the SUMO gene, *pmt3*⁺, are barely viable and display serious defects in genome maintenance. In mammals, three different SUMO proteins are known: SUMO-1, SUMO-2 and SUMO-3. The *SUMO-2* and *SUMO-3* genes are closely related (86% sequence identity), while *SUMO-1* is more distantly related (50% sequence identity with *SUMO-2* and *SUMO-3*). These SUMO proteins have distinct functions. SUMO-1 conjugates to proteins as a monomer, while SUMO-2 and SUMO-3 are conjugated to proteins as higher molecular weight polymers with SUMO-1 terminating further SUMO addition (Tatham et al., 2001; Su and Li, 2002; reviewed in Johnson, 2004).

Sumoylation may affect biological processes in various ways. Consequences of SUMO modification are diverse and include transcription factor activity, differential protein-protein interactions, subnuclear localization, higher-order chromatin structure, regulation of DNA binding, integrity of PML (promyelocytic leukaemia) nuclear bodies, nuclear pore complex shuttling, and antagonism of ubiquitination (Schwartz and Hochstrasser, 2003; Seeler and Dejean, 2003; Verger et al., 2003; Girdwood et al., 2004; Johnson, 2004; Müller et al., 2004; Watts, 2004).

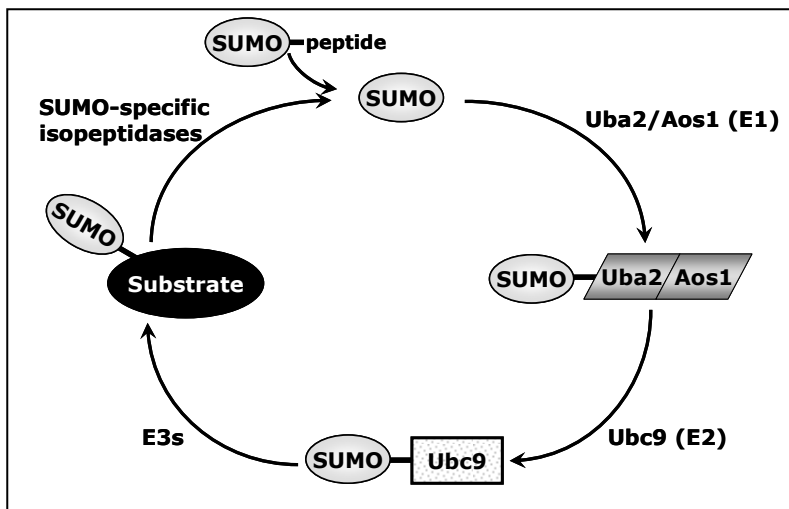


Figure 4: The SUMO conjugation pathway. SUMO is synthesized as an inactive precursor. First SUMO is matured by specific hydrolases that cleave off the SUMO carboxy-terminal,

exposing two conserved glycine residues, essential for the formation of an isopeptide bond with a target protein. The mature SUMO is then activated through an ATP-dependent transesterification process by SUMO activating enzyme (E1), a heterodimer of Aos1 and Uba2. Activated SUMO is transferred to a SUMO-conjugating enzyme (E2) called Ubc9. Subsequently, SUMO is transferred from Ubc9 to the substrate with the assistance of one of several SUMO-protein ligases (E3s), forming an isopeptide bond between the C-terminal glycine of SUMO and a lysine residue in the target protein. This residue is usually found within the SUMO ψ KxE consensus motif (where ψ is a large hydrophobic residue and x is any residue). Both Ubc9 and E3s contribute to substrate specificity. Sumoylation is reversible and removal of SUMO is carried out by the same hydrolases that mature the SUMO precursor and cleave at the C-terminus of SUMO.

SUMO is synthesized as a precursor and processed by hydrolases. It is subsequently conjugated to proteins by means of E1 activating, E2 conjugating and E3 ligating enzymes (Figure 4). E2 catalyses the formation of an isopeptide bond between a SUMO glycine and a target lysine residue. This residue is usually found within the SUMO ψ KxE consensus motif, where ψ is a hydrophobic amino acid, K the modified lysine, x any amino acid and E a glutamate. The E3-like proteins might serve to increase the affinity between E2 and the substrates by bringing them into close proximity in catalytically favourable orientations, allowing sumoylation to occur more efficiently. The resulting isopeptide bond is stable and its disruption requires a desumoylating enzyme (Schmidt and Muller, 2003; Johnson, 2004). The SUMO-activating E1 enzyme consists of a heterodimer of Aos1 (Sae1) and Uba2 (Sae2). Ubc9 is the SUMO-conjugating E2 enzyme. In most organisms the *AOS1*, *UBA2* and *UBC9* genes are essential. *S. pombe* strains mutated in homologs of *AOS1* (*fub2⁺*), *UBA2* (*rad31⁺*) and *UBC9* (*hus5⁺*) are viable, but have severe defects including slow growth, aberrant mitosis, high-frequency loss of minichromosomes and increased sensitivity to DNA-damaging agents (ionizing radiation, UV and the DNA synthesis inhibitor hydroxyurea) (al Khodairy et al., 1995; Shayeghi et al., 1997; Tanaka et al., 1999; Ho and Watts, 2003).

The E3 SUMO ligases are members of three distinct protein families. The first includes E3 ligases of the PIAS family. PIAS family members were originally discovered as protein inhibitors of activated STAT transcription factors (Chung et al., 1997; Liu et al., 1998; Schmidt and Muller, 2003). PIAS family proteins contain a chromatin binding SAP domain and a zinc-binding SP-RING domain characteristic for E3 ligases. PIAS proteins interact with and modulate activities of various transcription factors and the PIAS/SUMO pathway is important for transcriptional regulation of various important cellular processes. Different PIAS proteins may direct sumoylation to diverse substrates or may support SUMO modification by the various SUMO isoforms in mammals (Schmidt and Muller, 2003). Three *S. cerevisiae* PIAS family proteins have been identified, Siz1/Ull1, Siz2/Nfi1 and Mms21 (Johnson and Gupta, 2001; Takahashi et al., 2001; Hoege et al., 2002; Takahashi et al., 2003; Zhao and Blobel, 2005). Siz1/Ull1 was found to be an E3 ligase specific for septin components. Although Siz2/Nfi1 promotes sumoylation of septin components *in vitro*, the *in vivo* substrates of

Siz2/Nfi1 remain to be elucidated. Siz2/Nfi1, like PIAS, not only stimulates sumoylation, it is also a good substrate for SUMO modification itself (Takahashi et al., 2003). In *Drosophila melanogaster*, the *SU(VAR)2-10* gene was identified as a member of the PIAS family of proteins. Su(Var)2-10 is required for viability and regulates chromosome organization in the nucleus. Heterozygous *su(var)2-10* mutants show defects in chromosome inheritance and contain abnormal chromosome structures (Hari et al., 2001).

A second type of SUMO E3 ligases was discovered within RanBP2/Nup358 (Wu et al., 1995; Yokoyama et al., 1995; Pichler et al., 2002). A region within this nuclear pore protein is found to stimulate sumoylation of the nuclear import factor RanGAP1, the nuclear body protein Sp100 and the HDAC4 histone deacetylase. *In vitro*, RanGAP1, Sp100 and HDAC4 cannot be stimulated by PIAS proteins. The E3 ligase RanBP2 coordinates the positioning of the SUMO E2 intermediate optimal for substrate conjugation (Reverter and Lima, 2005).

The third SUMO E3 ligase is the polycomb group protein Pc2. Pc2 stimulates SUMO modification of the transcriptional repressor CtBP, that can also be sumoylated by PIAS proteins (Lin et al., 2003; Kagey et al., 2003; Kagey et al., 2005).

1.5.3 SUMO modification of DSB-repair proteins

PML nuclear bodies were first discovered in promyelocytic leukaemia and represent ring-shaped nuclear substructures associated with the regulation of transcription, replication, cell growth, apoptosis and repair processes and are characterized by the presence of the PML protein. The DNA damage induced activities of PML as a tumor suppressor and apoptosis inducing factor are exerted through the numerous proteins present in the PML bodies, including the tumor suppressor p53. SUMO modification of PML is not necessary for the nuclear bodies to form, but may affect the recruitment of proteins that interact with PML (reviewed by Johnson, 2004).

In the last few years it became clear that SUMO (and also ubiquitin) play important roles in the error-prone and error-free bypass of lesions in DNA (post-replication repair) in *S. cerevisiae*. Following treatment of yeast cells with DNA-damaging agents, the lysine 164 residue of the proliferating cell nuclear antigen (PCNA) becomes monoubiquitinated in a Rad6-Rad18-dependent way, promoting error-prone DNA repair by the translesion polymerases ζ and η . PCNA may also be polyubiquitinated at residue 164 via a lysine 63-linked ubiquitin chain in a reaction dependent on Mms2, Ubc13 and Rad5. Polyubiquitination promotes error-free replicational bypass of lesions. Interestingly, the same lysine residue that undergoes both monoubiquitination and Ub^{Lys63}-polyubiquitination is also sumoylated. In yeast PCNA, SUMO can also be linked to a specific second lysine residue at position 127. Lysine 127 lies among residues that mediate interaction with polymerases, suggesting that sumoylation at this site might interfere with polymerase binding, although interference with other proteins cannot be excluded (Hoegge et al., 2002). Sumoylation of PCNA at lysine 164 occurs in both normal and damaged cells during S-phase and blocks the activities of PCNA

invoked by ubiquitination (Matunis, 2002; Stelter and Ulrich, 2003). Moreover, SUMO modification of PCNA stimulates binding of Srs2, preventing homologous recombination by disrupting Rad51 nucleoprotein filaments. Srs2 recruitment through specific interaction with SUMO-modified PCNA may prevent unwanted recombination events of replicating chromosomes during S-phase (Papouli et al., 2005; Pfander et al., 2005; Watts, 2006).

The two components of HR in mammals, Rad51 and Rad52 have been shown to interact with Ubc9 and SUMO-1 in yeast two-hybrid analyses (Shen et al., 1996a; Shen et al., 1996b; Li et al., 2000). Although covalent modification of mammalian Rad51 and Rad52 could not be detected, the existence of a Rad51-Rad52-SUMO complex has been demonstrated (Li et al., 2000). The *S. pombe* Rad22A protein has been shown to be a target for SUMO modification (Ho et al., 2001). Overexpression of SUMO-1 down-regulates HR in CHO (Chinese Hamster Ovary) cells and results in increased ionizing radiation sensitivity (Li et al., 2000). While most homologous recombination products in mammalian cells arise by gene conversion, overexpression of SUMO-1 reduces the fraction of bidirectional gene conversion products, suggesting a regulatory role for SUMO-1 in homologous recombination (Li et al., 2000). The SUMO pathway may modulate the intracellular trafficking of Rad51 to the site of DNA damage during Rad51-mediated HR (Saitoh et al., 2002). Rad51 foci, hallmarks of functional association of Rad51 with damaged DNA, did not form efficiently when the nuclear localization of Ubc9 fails. This emphasizes the role of Ubc9 in the Rad51-mediated HR pathway, possibly because Rad51 is less efficiently targeted to the site of DNA damage when the SUMO pathway is impaired (Saitoh et al., 2002). Other components that colocalize with Rad51 foci upon DNA damage, e.g. the RecQ helicases WRN and BLM, are also sumoylation targets (Kawabe et al., 2000; Suzuki et al., 2001; Woods et al., 2004). Normally BLM is localized to PML nuclear bodies and will migrate from the PML bodies to Rad51 foci in response to DNA damage, particularly during S phase. Without sumoylation, BLM fails to localize to PML bodies. Even in the absence of DNA damage, foci are formed containing BLM and other factors implicated in DSB-repair e.g. BRCA2. These data imply a role for SUMO in regulating intra-nuclear redistribution of BLM. Redistribution of topoisomerases 1 and 2 within the nucleus and the distribution of the NHEJ factor XRCC4 in the cell is also regulated by SUMO (Rallabhandi et al., 2002; Yurchenko et al., 2006).

Müller *et al.* propose a model in which SUMO has a role in mediating the formation of DNA repair complexes. Upon DNA damage, sumoylation of recombinational repair proteins may direct the repair components towards DNA damage foci or sequester them within the foci (Figure 5) (Müller et al., 2004). The effect of sumoylation on DSB-repair can also be indirect. Defects in SUMO modification may alter proper chromosome structures and organization, since reduced levels of SUMO E3s in *Drosophila* and *S. cerevisiae* lead to defects in the regulation of chromosome structure and functioning (see Chapter 4) (Hari et al., 2001; Zhao and Blobel, 2005).

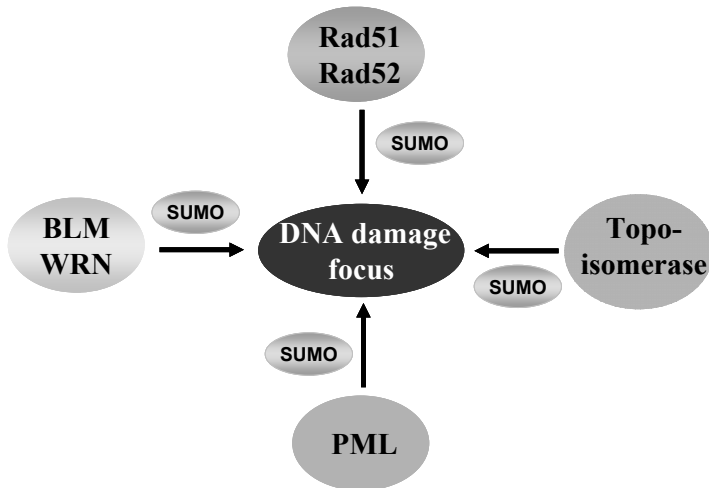


Figure 5: Putative role of SUMO in the assembly of DNA damage foci. Possibly, the subnuclear trafficking of recombinational repair proteins is regulated by SUMO. Sumoylation may target repair protein towards repair complexes or sequester them within these complexes. Adapted from Müller et al. (Müller et al., 2004).

1.6 Outline of the thesis

Homologous recombination in meiosis allows proper chromosome segregation and is responsible for genetic variation in a population and the origin of evolution by natural selection. In mitotic cells, HR is required to complete replication of the genome. Restart of replication after stalling at sites of damage, secondary structures or natural pause sites etc. in the DNA requires recombination proteins. Inactivation of factors essential in recombination is not tolerated in mammals and leads to growth defects and chromosome loss in yeast. Hypomorphic mutations lead to severe syndromes in man associated with increased cancer susceptibility. The complexity and the intricate regulation of DSB signalling and repair pathways in lower and higher eukaryotes emphasize the significance of accurate repair of DSBs.

Saccharomyces cerevisiae, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* each have two Rad52-like proteins (Bai and Symington, 1996; Suto et al., 1999; van den Bosch et al., 2001a; van den Bosch et al., 2001b). In *S. cerevisiae*, Rad52 is essential for DSB-repair and Rad59 plays an auxiliary role (Bai and Symington, 1996; Bai et al., 1999). Rad59 is about half the length of Rad52 in *S. cerevisiae* and *K. lactis* and is homologous to the N-terminal region of Rad52 proteins. In *S. pombe* the two Rad52-like proteins, Rad22A and Rad22B, display homology to the N- and C-terminal regions of Rad52. The two fission yeast proteins show a division of labor with Rad22A playing the major and Rad22B the minor role in DSB-repair, but synergistic defects in radiation sensitivity and meiosis are found in *rad22A rad22B* double mutants (Suto et al., 1999; van den Bosch et al., 2001a; van den Bosch et al., 2002b). To examine whether the diverse phenotypes of *rad22A* and *rad22B* mutants can be explained by a difference in biochemical properties, Rad22A and Rad22B proteins were purified after expression in *E. coli*. Analysis of Rad22A and Rad22B demonstrated that both proteins have very similar biochemical characteristics. Both have DNA binding properties and promote annealing of single-strand complementary DNA fragments and form multimeric structures (see Chapter 2). Rad52 and Rad54 in *S. cerevisiae* belong to the same epistasis group, i.e. single and double mutants display equal sensitivities to DNA-damaging agents. Synergistic effects relative to single mutants, indicating functions in different subpathways, were suggested for Rad22A and Rhp54 in *S. pombe* (Khasanov et al., 1999). To investigate if Rad52 and Rad54 in mammals function in the same pathway or have a role in different subpathways in HR, mice deficient for both Rad52 and Rad54 were generated and tested. In addition *rad22A*, *rad22B* and *rhp54* single, double and triple mutants in *S. pombe*, were constructed. In *rad52^{-/-} rad54^{-/-}* double mutant mice, an aggravation of mutant phenotypes was observed. However, Rad22A, Rad22B and Rhp54, like their homologs in *S. cerevisiae*, function in the same epistasis group for recombination repair of DSBs induced by DNA-damaging agents (see Chapter 3).

In a hunt for novel factors in HR, we screened *Drosophila* and *S. pombe* two-hybrid libraries with *Drosophila* Rad51 and *S. pombe* Rad22A and Rad22B. Both screens resulted in the identification of factors involved in SUMO modification.

The outcome of the two-hybrid screens indicate an important role for SUMO in facilitating repair of DSBs through HR in a direct or indirect way (see Chapter 4). Homologous recombination and crossover formation are crucial steps in the formation of germ cells. In mitotically dividing cells, HR occurs within pairs of sister chromatids which are held together by the cohesin complex. HR in meiosis is tightly connected with the formation of the synaptonemal complex. To investigate the role of Sycp1, one of the structural components of the transverse filaments in the SC, mice deficient for the *SYCP1* gene were generated. The results presented in Chapter 5 indicate that HR is initiated normally in the absence of Sycp1, but cannot be completed.

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Chapter 2

Schizosaccharomyces pombe Rad22A and Rad22B
have similar biochemical properties and form
multimeric structures.

accepted for publication in
Mutation Research

2 *Schizosaccharomyces pombe* Rad22A and Rad22B have similar biochemical properties and form multimeric structures

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Abstract

The *Saccharomyces cerevisiae* Rad52 protein has a crucial role in the repair of DNA double-strand breaks (DSBs) by homologous recombination (HR). *In vitro*, Rad52 displays DNA binding and strand annealing activities and promotes Rad51-mediated strand exchange. In *Schizosaccharomyces pombe* two Rad52 homologs have been identified, Rad22A and Rad22B. Here, we report the purification of both proteins to near homogeneity. Using gel retardation and filter binding assays, binding of Rad22A and Rad22B to short single-stranded DNAs was demonstrated. Rad22A does not bind to double-stranded oligonucleotides or linearized plasmid molecules containing blunt ends or short single-strand overhangs. Rad22B also does not bind efficiently to short duplex oligonucleotides but binds readily to DNA fragments containing 3' overhangs. Rad22A as well as Rad22B efficiently promote annealing of complementary single-strand DNAs. In the presence of Rad22A annealing of complementary DNAs is almost 90%. Whereas in reactions containing Rad22B the maximum level of annealing is 60% due to inhibition of the reaction by duplex DNA. In annealing reactions containing both proteins, the presence of Rad22A can overcome the inhibitory effect of duplex DNA on the annealing activity of Rad22B. Gel filtration experiments and electron microscopic analyses indicate self-association of Rad22A and Rad22B and the formation of multimeric structures.

Introduction

Maintaining genome integrity in response to DNA damage is critical for proper functioning of all living organisms. Damage involving breakage of both DNA strands is particularly harmful since un-repaired double-strand breaks (DSBs) can lead to loss of large stretches of DNA. DSBs arise as a result of exposure to DNA damaging agents such as ionizing radiation or certain chemical compounds and also occur as intermediates in V(D)J recombination and meiosis and after collapse of replication forks. Repair of DSBs is mediated by two major repair pathways, homologous recombination (HR) and non-homologous endjoining (NHEJ). Non-homologous endjoining simply rejoins both DNA ends of the break and may lead to inaccurate DNA repair. During HR, sequence information

residing on the sister chromatid or the homologous chromosome is used for accurate repair of DSBs. HR has been studied extensively in *S. cerevisiae* and involves members of the RAD52 epistasis group, including Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, Rdh54/Tid1, Mre11 and Xrs2. The first step in the repair of DSBs through HR involves processing of the ends to produce 3' single-strand (ss) DNA tails. The eukaryotic single-strand binding protein RPA is required to minimize secondary structures and to facilitate the assembly of a Rad51-ssDNA nucleoprotein filament. *In vitro* the efficiency of the formation of the Rad51 filament is stimulated by the Rad52 protein and the heterodimer of Rad55 and Rad57, which displace RPA and stabilize the Rad51 filament (Sung, 1997a; Sung, 1997b; Shinohara and Ogawa, 1998; New et al., 1998; New and Kowalczykowski, 2002). The Rad51 nucleoprotein filament is able to interact with a homologous DNA molecule and to initiate joint-molecule formation and strand exchange. The Rad51-mediated pairing and strand exchange reaction is stimulated by the addition of Rad54 and Rdh54 (Petukhova et al., 1998; Petukhova et al., 1999; Petukhova et al., 2000). Inactivation of genes belonging to the RAD52 epistasis group in *S. cerevisiae* leads to variable defects in meiotic and mitotic recombination and repair of damage induced by ionizing radiation and other DSB-inducing agents. This heterogeneity among the RAD52 group mutants can be explained by the different genetic requirements of sub-pathways in HR. In contrast to the central recombination protein Rad51, Rad52 is crucial for all types of homology dependent repair, including spontaneous and induced gene conversion events (Rattray and Symington, 1994), single-strand annealing (SSA) (Sugawara and Haber, 1992) and break-induced replication (BIR) (Signon et al., 2001; reviewed in Pâques and Haber, 1999; Pastink et al., 2001; Krogh and Symington 2004).

During Rad51-mediated repair, Rad52 has been shown to overcome the inhibitory effect of RPA on the formation of Rad51 filaments. Recent data suggest that a Rad51-Rad52 complex may carry out homology search and strand invasion in a more efficient way than the Rad51 filament (Miyazaki et al., 2004). Protein-protein interaction studies indicated that the C-terminal part of the Rad52 protein is required for interaction with Rad51 and RPA, while the N-terminal part is necessary for binding to DNA and homo-dimerization (Shen et al., 1996; Hays et al., 1998; Krejci et al., 2002). Electron microscopic studies of human and yeast Rad52 showed the formation of heptameric ring-like structures with a central channel on both ss- and dsDNA (Shinohara et al., 1998; van Dyck et al., 1998). Crystallographic analysis of the N-terminal domain of human Rad52 revealed undecameric ring structures resembling a mushroom (Kagawa et al., 2002). In addition to ring-like structures higher order multimers are also formed. Purified Rad52 binds to single-stranded DNA and stimulates the annealing of short complementary oligo-nucleotides, consistent with a role in SSA (Mortensen et al., 1996; Shinohara et al., 1998; Lloyd et al., 2002).

Besides Rad52, also the Rad52 homologue Rad59 has been identified in *S. cerevisiae* (Bai and Symington, 1996; Pâques and Haber, 1999) and *K. lactis* (van den Bosch et al., 2001a). The Rad59 protein is homologous to Rad52, but lacks the C-terminal part of Rad52, that is essential for the interaction with

Rad51. A *rad59* mutation leads to defects in recombination, SSA and sporulation (Bai and Symington 1996; Jablonovich et al., 1999; Sugawara et al., 2000). Rad59 is involved in certain types of Rad51-independent recombination and the protein has DNA binding and ssDNA annealing activities (Davis and Symington, 2001).

Homologs of RAD52 epistasis group proteins have been found in *S. pombe* and two RAD52 homologs were identified, namely *rad22A*⁺ and *rad22B*⁺ (Ostermann et al., 1993; Suto et al., 1999; van den Bosch et al., 2001b; van den Bosch et al., 2002). The severe recombination and repair defects of *rad22A* mutant cells resemble the phenotype of *S. cerevisiae rad52* mutant strains. Inactivation of *rad22B*⁺ does not lead to significant defects in recombination and repair. In vegetative dividing cells, the expression level of *rad22B*⁺ is very low and most likely *rad22B*⁺ has an auxiliary role in DSB repair. In contrast to *rad22A*⁺, the expression of *rad22B*⁺ during meiosis is strongly enhanced, indicating a more pronounced role for Rad22B in meiosis (van den Bosch et al., 2001b; van den Bosch et al., 2002). The amino acid sequences of Rad22A and Rad22B display significant overall identity (38%). The homology between Rad22A and Rad22B and of both proteins with Rad52 homologs from other organisms is primarily located in the N-terminal regions.

To examine the biochemical characteristics and to study the functional properties of both Rad52 homologs from *S. pombe* in more detail, we purified Rad22A and Rad22B after overexpression in *E. coli* and determined the DNA-binding and strand-annealing activities of both proteins.

Materials and methods

Proteins

To purify *S. pombe* Rad22A and Rad22B proteins after overexpression in *E. coli*, *rad22A*⁺ and *rad22B*⁺ coding regions were subcloned in the bacterial expression vector pET30a (Novagen). The *rad22A*⁺ gene was amplified by PCR using primers sp22A5 (5'-GCA CCC GGG GAA TTC ATG TCT TTT GAG CAA AAA CAG C-3') creating *Sma*I and *Eco*RI sites flanking the start codon and sp22A6 (5'-CAG GTC GAC GCA TGC TTA TCC TTT TTT GGC TTT CTT ATC-3') introducing *Sal*I and *Sph*I sites downstream of the stop codon. The PCR fragment was cloned in PCRscript using the PCRscript Amp Cloning Kit as indicated by the manufacturer (Stratagene). The *rad22A*⁺ gene was subcloned in the pET30a vector as an *Eco*RI – *Sal*I fragment. The *rad22B*⁺ gene was isolated as an *Not*I – *Sal*I fragment from pAS-*rad22B*⁺ (van den Bosch et al., 2002) and subcloned in the pET30a vector. pET-*rad22A*⁺ and pET-*rad22B*⁺ were sequenced to ensure that no mutations were introduced.

pET-*rad22A*⁺ and pET-*rad22B*⁺ were transformed into BL21-CodonPlus (DE₃)-RIL cells (*E. coli* B F⁻ *ompT hsdS* (r_B⁻ m_B⁻) *dcm*⁺ Tet^r *gal* (λDE3) *endA* Hte (*argU ileY leuW* Cam^r) (Stratagene). Fresh transformants were grown in 250 ml of LC containing 50 µg/ml of kanamycin at 25°C (for BL21 pET-*rad22A*⁺ transformants) and 37°C (for BL21 pET-*rad22B*⁺ transformants) to a density of OD₆₀₀ = 0.5. Expression of His-tagged proteins was induced by adding IPTG to 0.5 mM. Cells

were cultured for 2 h, washed with PBS, pelleted and frozen in liquid nitrogen. Pellets were kept at -80°C until use.

BL21 pET-*rad22A*⁺ cells were lysed by adding 5 ml lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 0.1% NP-40), 50 μl lysozyme (fresh stock of 10 mg/ml lysozyme) and protease inhibitors (leupeptin, pepstatin A and PMSF). The lysate was tumbled for 2 h at 4°C and sonicated afterwards on ice with an amplitude of 40-60 till the lysate was clear ($\sim 4 \times 15$ sec). The lysate was centrifuged for 20 min at 8000 rpm and 4°C . To the supernatant 18 μl 5% PEI (poly-ethyleneimine) per ml was added to remove DNA. The mix was rotated for 20 min at 4°C and DNA was removed by centrifugation for 15 min at 4°C . Supernatants were collected and applied immediately on a HiPrep 16/60 Sephacryl S-300 HR gel-filtration column equilibrated in 20 mM potassium phosphate (pH 7.4), 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 50 mM KCl. Proteins were fractionated at 0.3 ml/min for 5 h and 0.5 ml/min for 8 h using an ÄKTA purifier and UNICORN software (Amersham Biosciences). Elution of proteins was followed at 215 nm, 254 nm and 280 nm. Peak fractions containing Rad22A protein were applied on a 1 ml HiTrap Q HP anion exchange column (Amersham Biosciences) equilibrated in 20 mM potassium phosphate (pH 7.4), 10% glycerol (buffer A) containing 50 mM KCl and washed with 10 column volumes of the same buffer. Rad22A was eluted with buffer A containing 200 mM KCl. Peak fractions containing Rad22A were loaded directly on a Tricorn 5/50 column packed with 2 ml BD TALON (Clontech) resin. The TALON column was equilibrated with 5 column volumes buffer B (50 mM sodium phosphate (pH 7.0), 500 mM NaCl, 10% glycerol). After loading the column was washed with 5 column volumes buffer B containing 7.5 mM imidazole. Rad22A protein was eluted with 150 mM imidazole in buffer B, dialysed against storage buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM DTT, 10 % glycerol, 320 mM KCl), frozen in liquid nitrogen and stored at -80°C .

BL21 pET-*rad22B*⁺ transformants were lysed as described for BL21 pET-*rad22A*⁺ transformants. After lysis the supernatant was filtered through a 0.2 μm filter and Rad22B was purified through two consecutive TALON affinity chromatography steps as described for Rad22A with the exception that the TALON column was washed with buffer B containing 10 mM imidazole. Rad22B was eluted with buffer B containing 200 mM imidazole. Purified Rad22B was dialysed against storage buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 320 mM KCl), frozen in liquid nitrogen and stored at -80°C .

DNA substrates

For gel mobility shift and strand annealing assays, DNA substrates (2 pmole) were 5'-end-labelled with [γ - ^{32}P]-ATP using T4 polynucleotide kinase in a 10 μl reaction volume for 30 min at 37°C (see Table 1). The [^{32}P]-labelled probes were purified by Sephadex G-50 chromatography.

DNA binding assays

Reactions (20 μl) contained 0.05 pmole 5'- end-labelled DNA substrate in 20 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 , 4% glycerol, 120 mM KCl, 0.1 mg/ml BSA and

the indicated amounts of protein. Samples were incubated for 30 min at 30°C. Protein-DNA complexes were fixed by the addition of glutaraldehyde to 0.02% followed by 30 min incubation at 30°C. Loading buffer (40% sucrose, 0.25% bromophenolblue) was added and 10 µl of each reaction was loaded on a 0.8% agarose gel in 0.5x TBE buffer. After electrophoresis, gels were dried onto Whatman 3MM and analyzed by phosphorimaging (Cyclone, Packard Instruments) and autoradiography.

For filter binding assays, reactions were diluted in 3 ml ice-cold binding buffer and passed through a nitrocellulose filter (Millipore 0.45 µm HA). The filters were counted and the level of protein-DNA complex formation was calculated as percentage of input DNA retained on the filter (Moolenaar et al., 2001).

DNA	Length	Sequence
FV1	50bp	GGATGATTATGGTTATACGTGATTAGTAGCGACGAACATTTTGTAGCAGC
FV2	50bp	GCTGCTACAAAATGTTCTGCTCGCTACTAATCACGTATAACCATAATCATCC
FV3	61bp	GATCTGGCCTGTCTTACACAGTGGTAGTACTCCACTGTCTGGCTGTACAAAAACCCTCGGG
FV4	61bp	CCCAGAGGGTTTTGTACAGCCAGACAGTGGAGTACTACCACTGTGTGAAGACAGGCCAGATC
5'end	800bp	Double-strand <i>Sa</i> I fragment of pGAW2
3'end	239bp	Double-strand <i>Pst</i> I / <i>Bgl</i> II fragment of pBlueScript
blunt	328bp	Double-strand <i>Hpa</i> II fragment of pUC13

Table 1: DNA substrates. Oligonucleotides were purified by HPLC. Plasmid-derived DNA fragments were purified by agarose electrophoresis.

Strand annealing assays

Reactions containing 0.25 pmole FV4 DNA substrate and purified Rad22A or Rad22B protein in 500 µl annealing buffer (50 mM Hepes (pH 7.6), 10 mM KCl, 0.5 mg/ml BSA) were incubated for 5 min at 16°C in the cold room in the absence of labelled DNA. Subsequently 0.25 pmole 5'-[³²P]-end-labelled FV3 was added and the incubations were proceeded at 16°C. Samples of 20 µl were withdrawn at regular intervals and terminated by adding 5 µl stopbuffer (100 mM Tris-HCl (pH 8.0), 5% SDS, 1 mg/ml proteinase K, 250 mM EDTA). 5 µl of loading buffer was added to each sample and 2 µl was loaded on a 30 cm long non-denaturing 12% polyacrylamide gel in 0.5 x TBE buffer. After electrophoresis for 6 h at 22 mA, gels were analyzed by phosphorimaging and autoradiography.

Electron microscopy

Prior to electron microscopy, Rad22A and Rad22B protein were bound to ssDNA. Reactions (20 µl) containing 1.6 µM pUC120 ssDNA in binding buffer (20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5% glycerol) were incubated for 5 min at 30°C. 200 nM of Rad22A or Rad22B protein (diluted in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 100 mM KCl) was added and the incubation was proceeded for another 15 min at 30°C. Protein-DNA complexes were fixed with 0.2% glutaraldehyde for 15 min at 30°C. Fixed proteins were absorbed for

1 min to formvar and carbon coated electron microscopy grids that were negatively glow discharged before use. Subsequently grids were deposited on a 1% uranyl-acetate solution for 3 minutes and blotted dry. Images were recorded at a magnification of 39,000x on a Philips CM10 Electron microscope operating at 80 kV and digitized on an NIKON Coolscan 8000 ED scanner at 2000 dpi.

Results and discussion

Binding of Rad22A and Rad22B to DNA

Rad22A and Rad22B were overexpressed in *E. coli* as His-tagged proteins and purified to near homogeneity through column chromatography (see Figure 1). As was observed previously for Rad52, the apparent molecular weights of both proteins (60 kD and 56 kD, respectively) determined by SDS electrophoresis were somewhat higher than the calculated molecular weights (56.8 kD and 46.7 kD, respectively) (Benson et al., 1998; Shinohara et al., 1998).

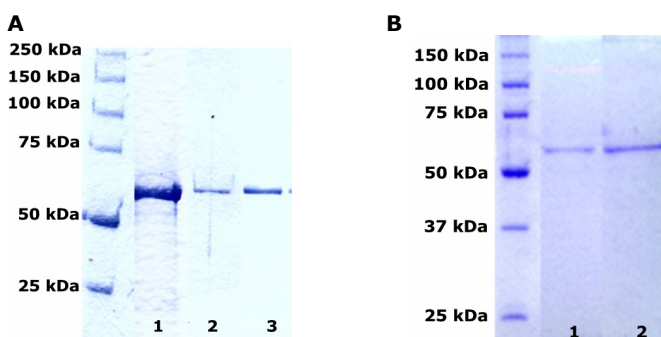


Figure 1: Purification of Rad22A and Rad22B. SDS-PAGE analysis of column fractions obtained during purification of Rad22A (**A**) and Rad22B (**B**). Numbers to the left indicate the size and position of marker proteins. (**A**). Lane 1: molecular weight marker; lane 2: Sephacryl S-300 HR fraction; lane 3: first peak fraction after ion-exchange chromatography on HiTrap Q; lane 4: purified Rad22A protein after TALON affinity chromatography and dialysis against storage buffer. (**B**) Lane 1: molecular weight marker; lane 2: Rad22B eluate after first TALON metal affinity purification; lane 3: Rad22B eluate after second TALON metal affinity purification and dialysis against storage buffer.

The DNA binding properties of Rad22A and Rad22B were first analyzed by gel retardation assays. Incubation of single-strand (ss) substrate FV1 (see Table 1) with increasing amounts of Rad22A protein resulted in the formation of protein-ssDNA complexes which could not enter the agarose gel (see Figure 2A). The failure of Rad22A-ssDNA complexes to enter the gel has also been observed for human Rad52 (van Dyck et al., 1998) and is most likely due to the formation of large DNA-protein aggregates.

Incubation of FV1 with Rad22B resulted in the formation of protein-ssDNA complexes that exhibited reduced mobility (Figure 2B). In contrast to Rad22A, large aggregates trapped in the well of the gel were not formed.

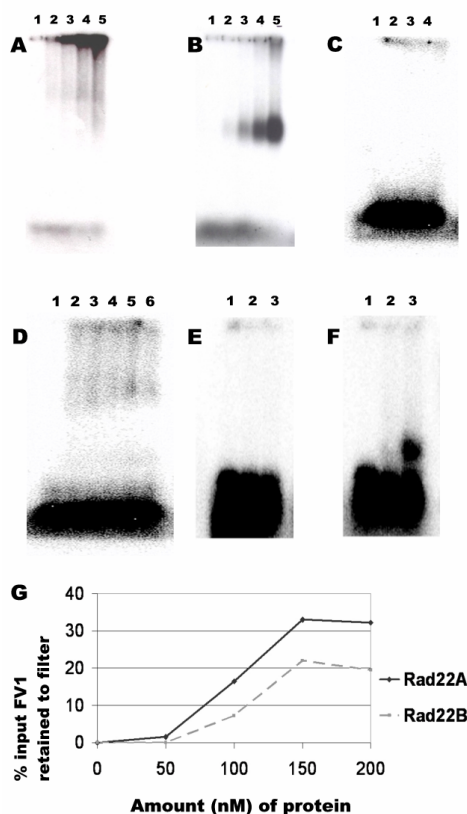


Figure 2. DNA binding properties of Rad22A and Rad22B. ^{32} P-labelled single- and double-stranded DNAs were incubated with purified protein fractions and analyzed by electrophoresis on 0.8% agarose gels (A-F) or filter binding (G). (A) Binding of Rad22A to 50-mer ssDNA substrate FV1. Lane 1: 0 nM; lane 2: 12.5 nM, lane 3: 25 nM; lane 4: 50 nM; lane 5: 100 nM Rad22A protein. (B) Binding of Rad22B to 50-mer ssDNA substrate FV1. Lane 1: 0 nM; lane 2: 12.5 nM, lane 3: 25 nM; lane 4: 50 nM; lane 5: 100 nM Rad22B protein. (C) Binding of Rad22A to FV1-FV2 duplex DNA. Lane 1: 0 nM; lane 2: 100 nM, lane 3: 150 nM; lane 4: 250 nM Rad22A protein. (D) Binding of Rad22B to FV1-FV2 dsDNA. Lane 1: 0 nM; lane 2: 100 nM, lane 3: 150 nM; lane 4: 250 nM; lane 5: 375 nM; lane 6: 500 nM Rad22B protein. (E-F) Binding of Rad22A (E) and Rad22B (F) to *Pst*I / *Bgl*II fragment of pBlueScript. Lane 1: 0 nM; lane 2: 25 nM; lane 3: 50 nM protein. (G) Filter binding assay using increasing amounts of Rad22A and Rad22B protein. Protein-DNA complex formation was calculated as percentage of input FV1 DNA retained on the filter.

To confirm the DNA-binding properties of Rad22A and Rad22B filter binding assays were performed. In the presence of increasing amounts of Rad22A and Rad22B (0 to 200 nM) retention of the labelled ssDNA (2.5 nM) on the filter was detected (see Figure 2G). At lower protein concentrations (up to 150 nM) a proportional increase in retention of ssDNA was observed. Above 150 nM the binding of Rad22A or Rad22B to 2.5 nM ssDNA becomes saturated. Both types of

assays indicate that Rad22A and Rad22B have very similar ssDNA binding properties and strongly resemble Rad52 from *S. cerevisiae* and humans in this respect (Mortensen et al., 1996; Shinohara et al., 1998; van Dyck et al., 1998; van Dyck et al., 1999; Parsons et al., 2000; van Dyck et al., 2001).

Next we tested binding of Rad22A and Rad22B protein to double-stranded (ds) DNA. Binding of Rad22A protein to the annealing product of complementary oligo's FV1 and FV2 could not be detected (Figure 2C). In the presence of Rad22B a weak binding was detected, but the formation of a specific retardation product was not observed (Figure 2D). At all protein-substrate ratios tested, a smearing in the gel was seen. In this respect Rad22A and Rad22B differ from Rad52 which readily complexes with dsDNA (Shinohara et al., 1998; van Dyck et al., 1998; van Dyck et al., 1999).

Incubation of Rad22A with linear plasmid substrates, that were either blunt or had 5'-overhangs, did not result in a shift in mobility (results not shown). Rad22A protein also did not bind to DNA fragments containing 3'-overhangs (see Figure 2E). However, Rad22B-DNA complexes were formed with dsDNA containing 3'-overhangs (see Figure 2F).

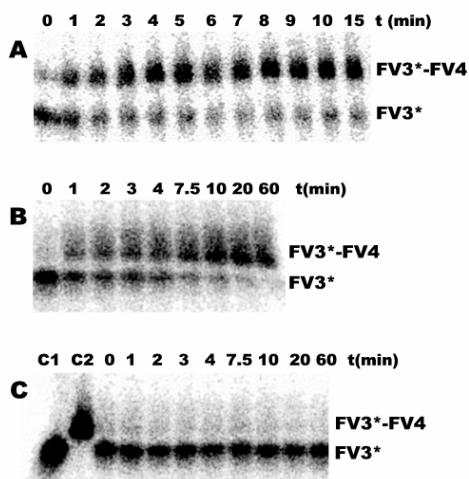


Figure 3: Strand annealing properties of Rad22A and Rad22B. Single-strand oligonucleotides FV3 and FV4 were incubated with purified Rad22A (**A**) and Rad22B (**B**). Reactions contained 4 nM protein and 0.5 nM of [^{32} P] labelled-FV3 and 0.5 nM of FV4. At regular intervals after starting the annealing reaction, samples were withdrawn, terminated and analyzed by electrophoresis on native 12% polyacrylamide gels. The degree of annealing was calculated after phosphorimaging or autoradiography. Timepoints are indicated. (**C**) Strand annealing reaction in the absence of protein. Lane 1: 5'-[^{32} P]-end-labelled FV3 DNA (C1). Lane 2: annealing product of [^{32} P]-labelled FV3 DNA and FV4 DNA (C2). Duplex DNA was obtained by heating both DNA substrates to 98°C and allowing them to cool down overnight to room temperature.

Rad22A and Rad22B mediate annealing of complementary ssDNA

Strand annealing properties of Rad22A and Rad22B were investigated by incubation of complementary ssDNA oligonucleotides with purified proteins. 0.5 nM 5'-[³²P]-end-labelled FV3 was annealed with 0.5 nM of FV4 DNA substrate. To avoid spontaneous renaturation between the ssDNA substrates, the 61-mer oligo's FV3 and FV4 were used and the assays were carried out at low temperature (16°C). Samples were taken at regular intervals and reaction products were analyzed by non-denaturing polyacrylamide gel electrophoresis (see Materials and methods). In the absence of Rad22A and Rad22B no renaturation was detected (Figure 3C). In the presence of 4 nM of Rad22A or Rad22B a robust annealing activity was detected, like has been observed for Rad52 (Mortensen et al., 1996; Shinohara et al., 1998; Ranatunga et al., 2001; Lloyd et al., 2002). Within the first minute after the initiation of the reactions the conversion of ssDNA into linear duplex DNA could be detected. After five minutes 80% of the ssDNA had annealed in the presence of Rad22A (Figure 3A). Addition of Rad22B protein resulted in the conversion of 60% of the ssDNA into dsDNA (Figure 3B).

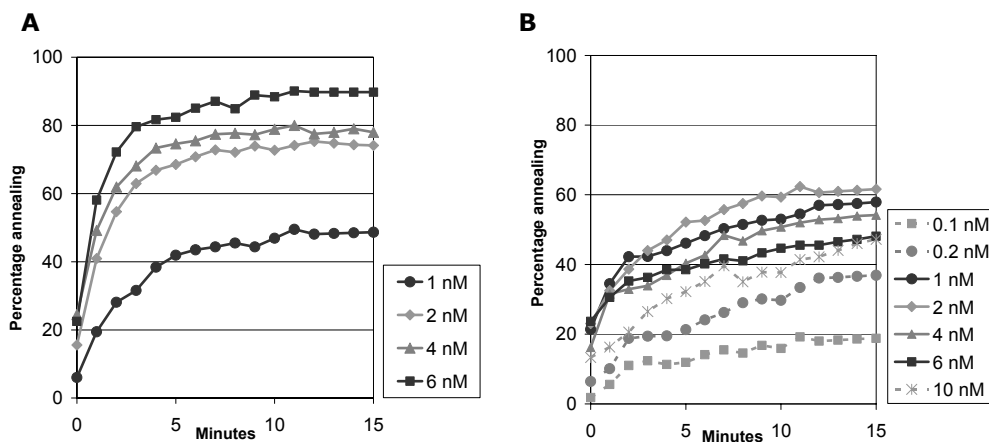


Figure 4: Kinetics of strand annealing. DNA strand annealing was performed in the presence of 0.5 nM of [³²P labelled]-FV3 and 0.5 nM of FV4 and increasing concentrations of Rad22A (A) and Rad22B (B). Time-course experiments were performed by taking samples every minute during 15 minutes after initiation of the strand annealing reaction and terminated immediately by adding stopbuffer. After electrophoresis the percentage of duplex DNA formation was calculated using PhosphoImager quantification software.

To examine the kinetics of Rad22A- and Rad22B-mediated strand annealing, time-course experiments were performed in the presence of increasing amounts of protein. Samples were drawn every minute during 15 minutes. By using PhosphoImager software (ImageQuant), the formation of dsDNA was quantified. Increasing amounts of Rad22A protein, resulted in an increase in the rate and level of annealing. In the presence of 6 nM Rad22A 90% of the ssDNA was converted into duplex molecules (Figure 4A). At higher concentrations of

Rad22A, the level of annealing was no further increased or decreased. Compared with Rad22A, annealing of FV3 and FV4 by Rad22B occurred at a much lower rate. A maximum level of annealing of 60% was observed at 2 nM Rad22B. At higher concentrations of Rad22B, the maximum level of annealing started to decrease. This was most apparent at the highest concentration (10 nM) used (see Figure 4B). Calculation of the trendlines for the first three minutes of the annealing of FV3 and FV4 confirmed that the highest rate of RAD22A-mediated annealing is reached at 6 nM protein. Trendlines for the annealing promoted by Rad22B protein show that the maximum rate of annealing rate during the first three minutes was achieved at approximately 1 nM of Rad22B protein (data not shown).

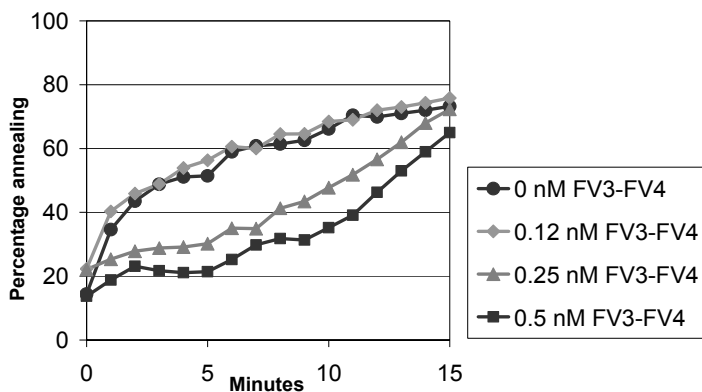
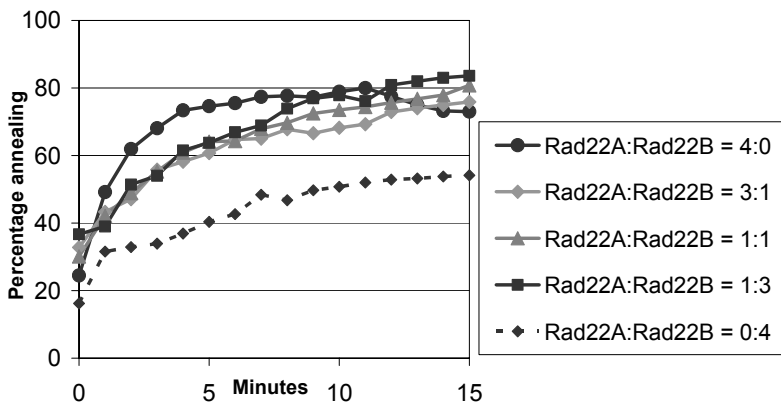
A**B**

Figure 5: Analysis of strand annealing by Rad22B. Annealing reactions contained 0.5 nM of [^{32} P labelled]-FV3 and 0.5 nM of FV4. **(A)** Strand annealing in the presence of 2 nM Rad22B protein and increasing amounts of unlabelled FV3-FV4 duplex DNA. **(B)** Strand annealing in the presence of both Rad22A and Rad22B. Different ratios of Rad22A and Rad22B were tested as indicated. The total concentration of protein in the reactions was kept constant at 4 nM.

Since the maximum level of annealing of oligo's FV3 and FV4 in the presence of Rad22B is 60% we hypothesised that Rad22B promotes annealing of complementary ssDNAs but also the formation of ssDNA from duplex DNA. However, incubation of 0.5 nM [^{32} P labelled]-FV3-FV4 duplex DNA with 2 nM Rad22B protein did not result in the formation of ssDNA (results not shown). Maybe, Rad22B-mediated annealing of ssDNA is inhibited by duplex DNA present in the reaction. Addition of increasing amounts of unlabelled ds FV3-FV4 DNA to the reaction, resulted in a decreased annealing of [^{32} P labelled]-FV3 and FV4, especially during the first 10 min of the reaction (see Figure 5A). Strand annealing studies using Rad52 did not show any inhibitory effect of duplex DNA molecules and approximately 90% annealing could be obtained as was observed for Rad22A (Mortensen et al., 1996).

Next we investigated whether the Rad22A and Rad22B proteins act cooperatively in annealing of ssDNA. Annealing of FW3 and FW4 was studied in reactions containing different ratios of Rad22A and Rad22B (see Figure 5B). The total amount of protein in the reactions was kept constant at 4 nM. Substitution of Rad22B by Rad22A significantly enhances the efficiency of annealing (see Figure 5B). Even in reactions containing 1 nM Rad22A and 3 nM Rad22B 90% annealing was achieved.

Both Rad22A and Rad22B form multimeric complexes

S. cerevisiae and mammalian Rad52 are multimeric and form heptameric ring-like structures with a hollow centre and an outer diameter of 9 nm (Stasiak et al., 2000; Kagawa et al., 2002). Also super-rings with an outer diameter of 30 nm were found (Shinohara et al., 1998; van Dyck et al., 1998; Ranatunga et al., 2001; Lloyd et al., 2002). During purification of Rad22A protein, the bacterial lysates were first fractionated by gel-filtration using Sephacryl S-300. Most Rad22A protein eluted between 670-2000 kDa, indicating that Rad22A forms specific multimers (see Figure 6A). Given a monomer molecular weight of 57 kD for the His-tagged version of Rad22A, these multimeric structures contain approximately 12 to 35 subunits. Gel filtration of purified Rad22B showed a peak fraction between 670 and 2000 kDa which contained Rad22B (Figure 6B). Given a molecular weight of 47 kD for the monomer, approximately 15 to 40 subunits are present in the multimeric Rad22B particles.

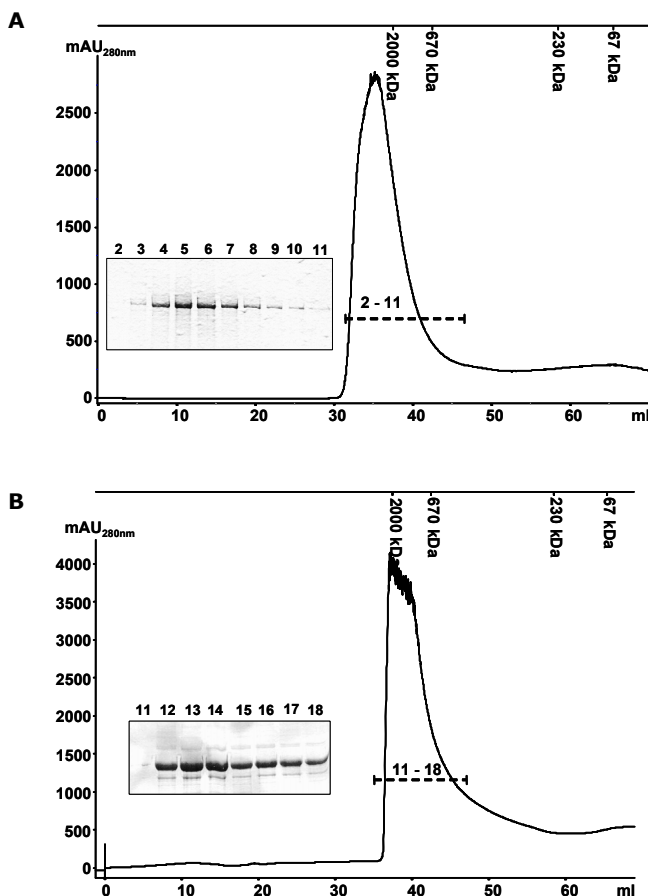


Figure 6: Gel filtration analysis of Rad22A (**A**) and Rad22B (**B**). Proteins were loaded onto a Sephacryl S-300 column and elution was followed at 280 nm. Peak fractions were analyzed by polyacrylamide electrophoresis (see inserts). Both Rad22A and Rad22B eluted between 670 and 2000 kDa indicating self-association and the formation of multimeric structures.

The self-association of Rad22A and Rad22B was also examined by electron microscopic analysis of protein-ssDNA complexes. After negative staining with uranyl-acetate Rad22A (Figure 7A) and Rad22B (Figure 7B) containing particles were observed. From three-dimensional reconstruction of the negatively stained particles it appeared that the Rad22A complexes have an apparent slightly flattened globular structure with a particle size of about 20 x 20 x 15 nm (data not shown). The Rad22B particles resemble the shape of Rad22A particles but the size and shape of the Rad22B structures were more heterogeneous and no clear three-dimensional reconstructions could be made, probably because of the formation of different sized complexes containing variable numbers of Rad22B monomers. From the negatively stained samples it appeared that neither

Rad22A nor Rad22B has clear ring-like structures with a distinct hole at the centre as was observed for Rad52 (data not shown). Possibly, the central channel is absent in case of Rad22A multimers or is much smaller. Evidence presented by Parsons *et al.* showed that the ssDNA is not bound within the central channel of the human Rad52 heptamer but lies on the surface of the protein ring (Parsons *et al.*, 2000). A central channel may therefore not strictly be required for the function of Rad22A.

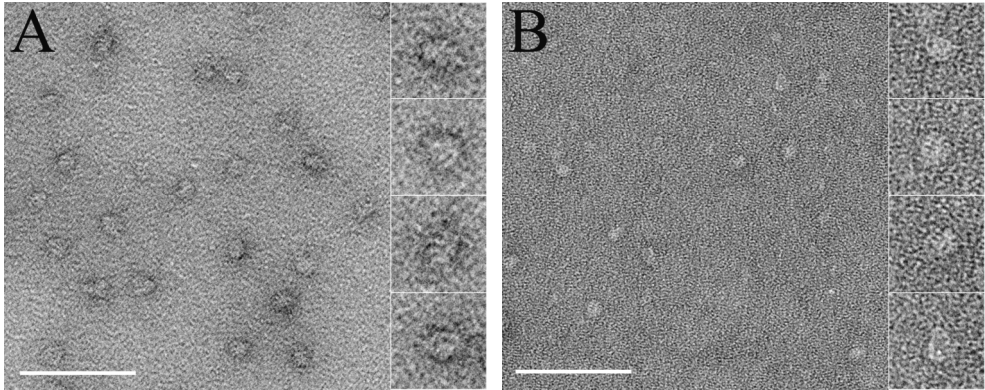


Figure 7: Negative stained electron micrographs of complexes of Rad22A **(A)** and Rad22B **(B)** on single-stranded plasmid DNA. Protein-DNA complexes were formed at a protein-DNA ratio of 1 : 160. On the right individual particles are depicted at two time increased magnifications compared to the overview. Scale bar is 100 nm.

Although Rad22A and Rad22B proteins differ to some extent in strand annealing properties, the biochemical functions studied here substantially overlap. Both proteins also interact with each other and with other factors implied in HR (e.g. Rhp51 and RPA) (van den Bosch *et al.*, 2002 and unpublished results). As *rad22B⁺* is expressed at a relatively low level in vegetative cells and is not induced after induction of DNA damage, the weak phenotype of *rad22B* mutant strains can be explained by low levels of Rad22B protein. The partial correction of a *rad22A* mutant by overexpression of *rad22B⁺* is in agreement with this. Rad59 in *S. cerevisiae* promotes strand annealing *in vitro*, but not *in vivo* in the absence of Rad52 (Davis and Symington, 2001). It has been suggested that Rad59 stimulates the strand annealing activity of Rad52, especially in case of short repeats (Krogh and Symington, 2004). Rad22B may have a similar function in *S. pombe* in augmenting the annealing properties of Rad22A in certain types of recombination. Also, a specific role for Rad22B in only certain certain types of recombination, however, cannot be excluded. As Rad22B protein levels are increased in meiotic cells, the role of Rad22B in HR may be specifically important during meiosis (van den Bosch *et al.*, 2002). It will be of interest to determine the role of Rad22A and Rad22B in Rhp51 filament formation in *S. pombe* in overcoming the inhibitory effects of RPA. Although human Rad52 promotes Rad51-mediated D-loop formation, the inhibitory effects of RPA on

nucleation of Rad51 on ssDNA cannot be overcome (P.Sung, personal comm.). Recently, it has been shown that the human BRCA2 polypeptide can act as a mediator in Rad51-mediated pairing reactions in the presence of RPA (Filippo et al., 2006). A similar recombination mediator function has been demonstrated for the *U. maydis* BRCA2 homologue Brh2 (Yang et al., 2005). As *rad22A* mutants in *S. pombe* strongly resemble *rad52* mutants in *S. cerevisiae*, it is tempting to speculate that Rad22A has a similar mediator function in recombination as Rad52 in *S. cerevisiae*. Additional genetic and biochemical experiments will be required to shed more light on this.

Acknowledgements

The authors would like to thank Dr. Leon Mullenders for critical reading of the manuscript. This work was supported by the EU (RISC-RAD / FI6R-CT-2003-508842).

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Chapter 3

Inactivation of *RAD52* aggravates *RAD54* defects in mice but not in *Schizosaccharomyces pombe*.

adapted from
DNA repair, 2005, 4 (10): 1121-1128

3 Inactivation of *RAD52* aggravates *RAD54* defects in mice but not in *Schizosaccharomyces pombe*

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Abstract

RAD52 and *RAD54* genes from *S. cerevisiae* are required for double-strand break repair through homologous recombination and show epistatic interactions *i.e.* single and double mutant strains are equally sensitive to DNA damaging agents. In here we combined mutations in *RAD52* and *RAD54* homologs in *Schizosaccharomyces pombe* and mice. The analysis of mutant strains in *S. pombe* demonstrated nearly identical sensitivities of *rhp54*, *rad22A* and *rad22B* double and triple mutants to X-rays, cis-diamminedichloroplatinum and hydroxyurea. In this respect, the fission yeast homologs of *RAD54* and *RAD52* closely resemble their counterparts in *S. cerevisiae*. To verify if inactivation of *RAD52* affects the DNA damage sensitivities of *RAD54* deficient mice, several endpoints were studied in double mutant mice and in bone marrow cells derived from these animals. Haemopoietic depression in bone marrow and the formation of micronuclei after *in vivo* exposure to mitomycin C (MMC), was not increased in either single or double mutant mice in comparison to wildtype animals. The induction of sister chromatid exchanges in splenocytes was slightly reduced in the *RAD54* mutant. A similar reduction was detected in the double mutant. However, a deficiency of *RAD52* exacerbates the MMC survival of *RAD54* mutant mice and also has a distinct effect on the survival of bone marrow cells after exposure to ionizing radiation. These findings may be explained by additive defects in HR in the double mutant but may also indicate a more prominent role for single-strand annealing in the absence of Rad54.

Introduction

DNA double-strand breaks (DSBs) are among the most genotoxic DNA lesions and can be induced by ionizing radiation and other DNA damaging agents but also arise as intermediates in several cellular processes such as V(D)J rearrangement, meiosis and normal replication. The repair of DSBs in DNA is crucial to prevent chromosomal fragmentation, translocations and deletions.

Genetic instability resulting from unrepaired DSBs can lead to cell death and in a multicellular organism to cancer. The importance of DSB repair is underlined by the evolution of several DSB repair pathways. The two major pathways are non-homologous endjoining (NHEJ) and homologous recombination (HR) (van Gent et al., 2001; Valerie and Povirk, 2003; Liu and West, 2004). Repair through HR requires an undamaged homologous DNA sequence on the allelic chromosome or the sister chromatid, to retrieve sequence information lost at the site of the break. In addition to the major pathway in HR leading to gene conversion and crossover formation, at least two sub-pathways, break-induced replication (BIR) and single-strand annealing (SSA), are known in homology-dependent repair of DSBs (Pâques and Haber, 1999; Symington, 2002). In BIR invasion of a template molecule is followed by replication to the end of the template. This process may be important for telomere maintenance. SSA depends on the presence of two direct repeats flanking the break. Exposure of regions of homology during specific resection of the 5'-ends at the site of the break allows formation of joined molecules. DSB repair through SSA results in loss of one of the repeats and of the region in between the direct repeats and is therefore inherently mutagenic.

Genes involved in HR belong to the RAD52 epistasis group and were first characterized in *S. cerevisiae*. Later on homologs were identified in other organisms including *S. pombe* and mammals. Genetic studies, as well as biochemical experiments using purified proteins, indicated that Rad51, Rad52 and Rad54 proteins are crucial factors in the repair of DSBs through HR (Dudas and Chovanec, 2004). The Rad51 protein assembles on single-strand DNA ends after specific resection of the 5'-ends and catalyses invasion of homologous DNA molecules and strand exchange. Rad52 and Rad54 are essential accessory proteins in this process. Inactivation of *RAD51* in *S. cerevisiae* and of *rhp51*⁺ (*rad* homologue *S. pombe*) in *S. pombe* results in severe defects in recombination, reduced spore viability and increased sensitivity to various DNA damaging agents *i.e.* X-rays, methyl methanesulphonate (MMS) and crosslinking agents (van den Bosch et al., 2002a). In vertebrates disruption of *RAD51* is not tolerated and leads to lethality after 7-8 days of embryonic development (Tsuzuki et al., 1996; Sonoda et al., 1998). In *S. cerevisiae* the Rad52 protein is required for all forms of homology dependent DSB repair and recombination (Game, 1993) including induced somatic recombination events (Rattray and Symington, 1994), the formation of joint molecules (Zou and Rothstein, 1997), SSA (Sugawara and Haber, 1992b) and BIR (Malkova et al., 1996). *Rad52* null mutants are extremely sensitive to ionizing radiation and completely deficient in meiosis. In the fission yeast *S. pombe*, two *RAD52* homologs, *rad22A*⁺ and *rad22B*⁺, are present (Ostermann et al., 1993; van den Bosch et al., 2001; Suto et al., 1999). The phenotype of *rad22A* mutant strains resembles that of *S. cerevisiae rad52* mutants. Inactivation of *rad22B*⁺ does not lead to obvious defects in recombination and repair. In vegetative dividing cells, the expression level of *rad22B*⁺ is very low and most likely *rad22B*⁺ has an auxiliary role in DSB repair (van den Bosch et al., 2001). Surprisingly, disruption of *RAD52* in mouse and chicken cells only results in very minor defects in recombination. Increased

sensitivity to agents that induce DSBs was not observed (Rijkers et al., 1998; Yamaguchi-Iwai et al., 1998). In addition, null mutant mice are normally viable and fertile and show no abnormalities in development of the lymphoid system. The Rad54 protein belongs to the SNF2/SWI2 family and changes DNA topology in an ATP-dependent manner. It is required for heteroduplex DNA formation (Petukhova et al., 1999b) and chromatin remodeling (Alexiadis and Kadonaga, 2002; Jaskelioff et al., 2003; Alexeev et al., 2003). Ablation of *RAD54* in *S. cerevisiae* or *rhp54⁺* in *S. pombe* causes severe defects in recombination and increased sensitivity to X-rays and MMS (Muris et al., 1996; Pâques and Haber, 1999). Disruption of *RAD54* or *RAD54B*, a second *RAD54* homologue in vertebrates, leads to moderate defects in repair and recombination (Essers et al., 1997; Bezzubova et al., 1997; Miyagawa et al., 2002). Interestingly, *RAD54* mutant mice are hypersensitive to ionizing radiation at the embryonic stage but not at the adult stage. An enhanced sensitivity to the crosslinking agent MMC was seen at all developmental stages (Essers et al., 2000).

On the basis of radiation and MMS sensitivity, *RAD51*, *RAD52* and *RAD54* from *S. cerevisiae* belong to the same epistasis group *i.e.* the sensitivity of a double mutant is the same as that of the most sensitive single mutant. Additive or synergistic effects relative to the single mutants were sometimes seen in certain recombination assays, suggesting the existence of various sub-pathways in homologous recombination. Data of Khasanov and co-workers suggested that Rad22A in *S. pombe* functions in a different sub-pathway than Rhp51 and Rhp54 in the repair of radiation and MMS induced lesions (Khasanov et al., 1999). To determine if Rad52 and Rad54 function in one pathway or in (overlapping) sub-pathways in HR in mammals, mice deficient for both *RAD52* and *RAD54* were generated and tested for aggravated defects. In addition, we evaluated the proposed synergistic sensitivity to DSB inducing agents of *rad22A*, *rad22B* and *rhp54* single, double and triple deletion mutants in *S. pombe*.

Materials and methods

Mouse breeding

The generation of *RAD52* and *RAD54* mutant mice has been described (Rijkers et al., 1998; Essers et al., 1997). Mice heterozygous for mutations in both *RAD52* and *RAD54* (both strains have a C57BL/6 background) were bred to generate control, single and double mutant animals and were maintained at the animal facilities of the National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands. Genotyping was performed by PCR analysis on tail DNA as described (Essers et al., 1997; Rijkers et al., 1998). Male and female mice showed similar sensitivities and data were pooled in all experiments.

Micronucleus (MN) assay in mouse bone marrow polychromatic erythrocytes

RAD52^{-/-} / RAD54^{-/-} double mutant mice, single mutants and controls (homozygous wildtype and heterozygous animals were taken together), were injected intraperitoneally with 0.75, 1.5 or 2.5 mg/kg bodyweight mitomycine C (MMC).

24 hours after treatment, femoral bone marrow cells were extracted and smear slides were prepared. Slides were stained successively with 100% May-Grünwald and 15% Giemsa stain and analyzed for toxicity (ratio of polychromatic erythrocytes (PCEs) and normochromatic plus orthochromatic erythrocytes (NCEs plus OCEs)) and for the presence of micronucleated PCEs by scoring 1000 PCEs per femur.

In vivo mitomycine C survival

RAD52^{-/-} / *RAD54*^{-/-} double mutant mice, single mutants were injected intraperitoneally with 5, 7.5, 10 or 15 mg/kg bodyweight MMC. Control animals were only treated with the highest dose. Animals were kept in sterile isolators and monitored for 14 days. Afterwards surviving mice were euthanized.

Measurement of haemopoietic depression in bone marrow after irradiation

Wildtype mice, *RAD52*^{-/-} and *RAD54*^{-/-} single mutants and *RAD52*^{-/-} / *RAD54*^{-/-} double mutants were exposed to 1 Gy using a 225 SMART X-ray source (200 kV, 4mA, 1.0 mm Al filter and a dose rate of 0.5 Gy/min; Andrex SA, Copenhagen, Denmark). After 72 hours, bone marrow cells were processed as described for the MN assay and the ratio of PCEs and NCEs plus OCEs was determined by scoring 1000 PCEs per femur. As reported earlier, maximum haemopoietic depression occurs 60-80 hours following irradiation (van Buul et al., 1998).

Analysis of MMC-induced sister chromatid exchanges (SCEs) in mouse splenocytes

Mouse splenocytes were isolated from spleens of *RAD52*^{-/-} / *RAD54*^{-/-} double mutants, single mutants and control mice. Splenocytes were stimulated by 2.5 µg/ml ConA and cultured in the presence of 10 µg/ml BrdU. 5 hours after culture initiation, 5 ng/ml MMC was added. The MMC treatment was continued till the end of culturing. Colcemid was present during the last two hours of culturing. Metaphases were fixed 48 hours after culture initiation and slides were prepared. Preparations were stained successively with Hoechst 33258, exposed to ultraviolet light and stained with 3% Giemsa (Perry and Wolff, 1974). 25 metaphases per culture were analyzed by light microscopy for the presence of SCEs and cell cycle progression (ratio of 1st – 2nd – 3rd etc. division metaphases).

S. pombe strains and gene disruptions

The *S. pombe* strains used in this study are listed in Table 1. Growth media and culturing of cells have been described (Gutz et al., 1974). A *rhp54* deletion mutant was constructed by replacing the open reading frame with the kan^r marker essentially as described by Bähler et al. (Bähler et al., 1998). *rad22A-rhp54*, *rad22B-rhp54* and *rad22A-rad22B-rhp54* double and triple mutants were generated by standard crossings. Phenotypes of individual clones were verified by replica plating on selective media and PCR as described (van den Bosch et al., 2001; van den Bosch et al., 2002b).

Strain	Genotype	Source/reference
Y4 - wildtype	<i>h⁺ ura4-D18 leu1-32 ade6-M216</i>	A. Yasui
RGL4	<i>h⁺ rad22A::ura4⁺ ura4-D18 leu1-32 ade6-M216</i>	van den Bosch <i>et al.</i> (2001)
RGL7	<i>h⁺ rad22B::LEU2 ura4-D18 leu1-32 ade6-M216</i>	van den Bosch <i>et al.</i> (2001)
<i>rhp54</i>	<i>Smt-0 rad54::kan⁺ ura4-D18 leu1-32 ade6-M216</i>	This study
RGL11	<i>h⁺ rad22A::ura4⁺ rad22B::LEU2 ura4-D18 leu1-32 ade6-M216</i>	van den Bosch <i>et al.</i> (2001)
<i>rad22Arhp54</i>	<i>rad22A::ura4⁺ rad54::kan⁺ ura4-D18 leu1-32 ade6-M216</i>	This study
<i>rad22Brhp54</i>	<i>rad22B::LEU2 rad54::kan⁺ ura4-D18 leu1-32 ade6-M216</i>	This study
<i>rad22Arad22Brhp54</i>	<i>rad22A::ura4⁺ rad22B::LEU2 rad54::kan⁺ ura4-D18 leu1-32 ade6-M216</i>	This study
HE720	<i>h⁺ rhp51::ura4⁺ rhp54::ura4⁺ ura4-D18 leu1-32 ade6-704</i>	H. Schmidt

Table 1: *S. pombe* strains used in this study.

Survival studies of *S. pombe* mutants

To measure the sensitivity of cells to ionizing radiation, exponentially growing cells were irradiated with X-rays using a 225 SMART X-ray source (Andrex SA, Copenhagen, Denmark) at a dose rate of 10 Gy/min (200 kV, 4mA, 1 mm Al filter). Duplicates of irradiated cells and unirradiated cells were plated on YES medium (0.5% yeast extract, 3% glucose, supplemented with 80 mg/l adenine, leucine and uracil) and incubated at 30°C for 4 days before colonies were counted. To test the sensitivity to cis-diamminedichloroplatinum (cisDDP) and hydroxyurea (HU), a spot assay was employed. Briefly, 5 µl of 10-fold dilutions of log-phase cells (10^5 , 10^4 , 10^3 , 10^2 and 10^1 cells per spot) were spotted onto YES plates containing cisDDP (1-10 µg/ml) or HU (1 or 3 mM). Cell survival was determined after 3 days of incubation at 30°C. All experiments were repeated at least twice.

Results

Generation of *RAD52* / *RAD54* deficient mice

To investigate whether the repair and recombination defects of *RAD54* mutant mice are compromised by a deficiency of *RAD52*, *RAD52*^{+/-} / *RAD54*^{+/-} heterozygous animals were crossed. Among the offspring, all genotypes were obtained in accordance with normal Mendelian ratios. Homozygous double mutant mice were viable and showed no visible macroscopic abnormalities. Both *RAD52*^{-/-} / *RAD54*^{-/-} males and females were fertile (data not shown).

RAD52 / RAD54 double deficiency does not result in increased toxicity and micronuclei formation in mouse bone marrow polychromatic erythrocytes after exposure to MMC

Rapidly dividing bone marrow cells are a major target for damage induction *in vivo* by MMC. The number of surviving polychromatic erythrocytes (PCEs) in relation to the number of normo- plus orthochromatic erythrocytes (NCEs plus OCEs) is a measure for cytotoxicity after exposure to MMC. Exposure to 2.5 mg/kg MMC results in a decreased ratio of surviving PCEs and NCEs plus OCEs. However, the toxic effect of MMC was not significantly increased in *RAD52* and *RAD54* singly and doubly deficient mice compared to control mice (see Figure 1A).

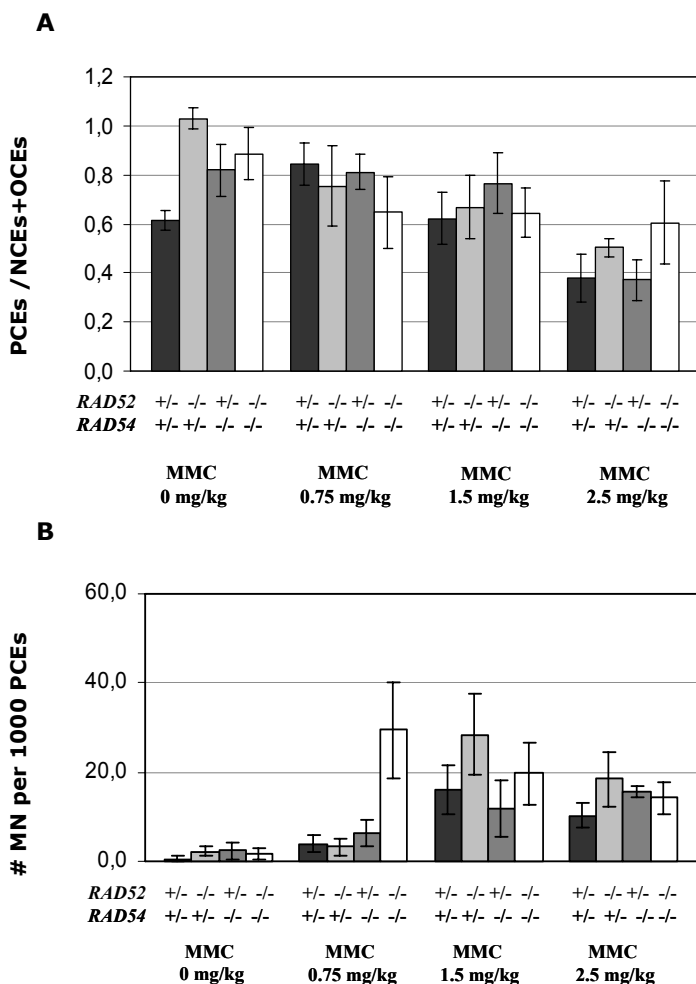


Figure 1: Sensitivity of bone marrow cells to mitomycin C. Two-month old *RAD52*^{-/-} / *RAD54*^{-/-} double mutant mice, single mutants and injected controls (homozygous wildtype and heterozygous animals were taken together) were injected intraperitoneally with 0.75, 1.5

and 2.5 mg/kg MMC. Femoral bone marrow cells were collected 24 hours afterwards and analyzed as stained smear slides. **(A)** The number of surviving polychromatic erythrocytes (PCEs) in relation to the number of normo- plus orthochromatic erythrocytes (NCEs plus OCEs). Per animal 2000 erythrocytes (PCEs, NCEs and OCEs) were scored. Each data point represents the mean of four independently treated animals. Standard errors of the mean are indicated. (T test for 2.5 mg/kg MMC versus 0 mg/kg MMC for each data point: $0.02 < p < 0.2$) **(B)** The induction of micronuclei (MN) in PCEs after MMC treatment. For each animal 1000 PCEs were analyzed for the presence of MN. Data points represent the average of four independently treated animals. Standard errors of the mean are indicated. (T test for wildtype 0.75 mg/kg MMC versus *RAD52*^{-/-} / *RAD54*^{-/-} 0.75 mg/kg MMC: $p=0.09$)

The formation of micronuclei (MN) in PCEs is an indication for the induction of chromosomal aberrations. Increasing doses of MMC led to a dose-dependent induction of MN in PCEs isolated from wildtype mice and mutant animals. Exposure to MMC did not result in significantly higher levels of MN in PCEs in bone marrow from single and double mutants as compared to wildtype mice (see Figure 1B).

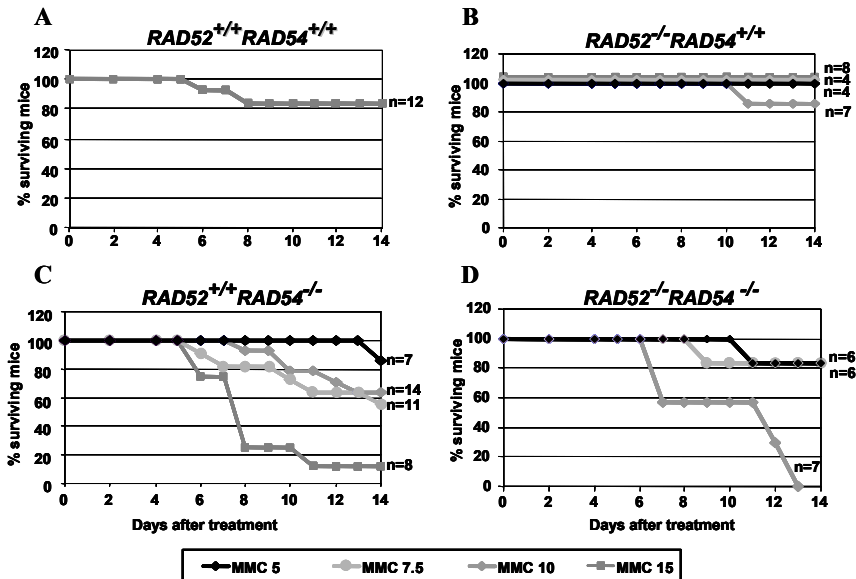


Figure 2: Survival of mice following mitomycin C treatment. Animals were injected intraperitoneally with 5, 7.5, 10 or 15 mg/kg bodyweight MMC. Total numbers of individually treated mice are indicated per dose. Control **(A)** *RAD52*^{-/-} **(B)**, *RAD54*^{-/-} **(C)** and *RAD52*^{-/-} / *RAD54*^{-/-} **(D)** mice. **See appendix for a colour version of this figure.**

*Increased MMC sensitivity of *RAD52*^{-/-} / *RAD54*^{-/-} mice*

To determine whether inactivation of *RAD52* aggravates the MMC sensitivity of adult *RAD54*^{-/-} mice, the survival of double mutant animals was compared to single mutants and control mice. *RAD52*^{-/-} animals did not show a reduced

survival after treatment in comparison with the control (Figure 2A and 2B). As previously observed, disruption of *RAD54* resulted in increased sensitivity to MMC in adult animals. After treatment with 15 mg/kg MMC, 88% of the *RAD54*^{-/-} mice die within 14 days, while a dose of 10 mg/kg MMC resulted in a lethality of 36% (Figure 2C). Treatment of *RAD52*^{-/-} / *RAD54*^{-/-} double mutants showed increased lethality in comparison with *RAD54* single mutants. At a dose of 10 mg MMC per kg body weight death occurred in all exposed animals (Figure 2D).

Severe haemopoietic depression after X-rays in a double mutant of RAD52 and RAD54

Depression of haemopoiesis in bone marrow is a very sensitive assay in mammals for the detection of cytotoxicity after exposure to ionizing radiation. Exposure to radiation leads to a reduction in the number of polychromatic erythrocytes (PCEs) in comparison to cells matured during haemopoiesis, the normo- and orthochromatic erythrocytes (NCEs and OCEs). Treatment of adult wildtype and *RAD52*^{-/-} single mutant mice with an X-ray dose of 1 Gy, did not result in a statistically significant decrease in the ratio of polychromatic erythrocytes versus matured cells in comparison with unirradiated bone marrow (Table 2). In *RAD54*^{-/-} mutant mice, a small but significant decrease in the PCEs/NCEs + OCEs ratio was seen. The *RAD52*^{-/-} / *RAD54*^{-/-} double mutant mice were hypersensitive to X-rays in comparison with *RAD54*^{-/-} single mutant mice and showed a distinct haemopoietic depression (Table 2).

Genotype	Dose (Gy)	No. of mice	PCEs/NCEs+OCEs
<i>RAD52</i> ^{+/+} / <i>RAD54</i> ^{+/+} *	0	3	0.78±0.08
	1	4	0.59±0.03
<i>RAD52</i> ^{-/-} / <i>RAD54</i> ^{+/+}	0	2	0.70±0.11
	1	4	0.54±0.03
<i>RAD52</i> ^{+/+} / <i>RAD54</i> ^{-/-}	0	2	0.79±0.03
	1	3	0.52±0.02
<i>RAD52</i> ^{-/-} / <i>RAD54</i> ^{-/-}	0	2	0.70±0.03
	1	4	0.32±0.02

Table 2: The haemopoietic depression following a dose of 1 Gy of X rays is expressed as the ratio of polychromatic erythrocytes versus normo- plus orthochromatic erythrocytes (PCEs/NCEs + OCEs). In total 1000 PCEs were scored per femur using two different slides. T-test for wildtype 1 versus 0 Gy: 0.1<p<0.2; t-test for *RAD52*^{-/-} single mutant 1 versus 0 Gy: 0.2<p<0.3; t-test for *RAD54*^{-/-} single mutant 1 versus 0 Gy: p<0.001; t-test for *RAD52*^{-/-} / *RAD54*^{-/-} mice 1 versus 0 Gy: p<0.001; t-test *RAD54*^{-/-} mice 1 Gy versus *RAD52*^{-/-} / *RAD54*^{-/-} 1 Gy: p < 0.001.

* Both *RAD52*^{+/+} / *RAD54*^{+/+} and *RAD52*^{+/+} / *RAD54*^{-/-} mice were used as controls.

MMC-induced sister chromatid exchanges (SCEs) in single and double mutant mice

To study the role of Rad52 and Rad54 in recombination between sister chromatids, we determined the levels of SCEs in single and double mutant lymphocytes. After isolation, splenocytes were treated with MMC for 48 hours. In cells from wildtype mice and single and double mutants a strong induction of SCEs was seen after exposure to MMC (Figure 3). Ablation of *RAD52* did not influence the level of SCE induction. In splenocytes obtained from *RAD54*^{-/-} animals, a small reduction in the frequency of SCEs was found. In the double mutant no further reduction in the formation of SCEs was detected (Figure 3).

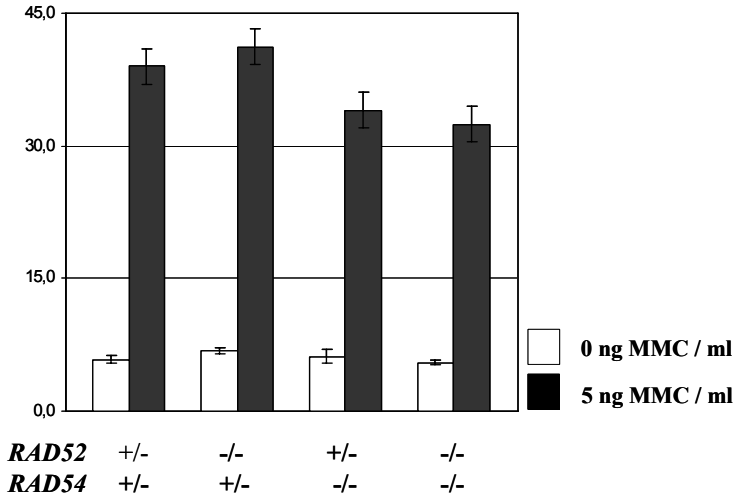


Figure 3: Induction of sister-chromatid exchanges by mitomycin C. Mouse splenocytes were isolated from spleens of three animals from each group, stimulated by 2.5 µg/ml ConA and cultured in the presence of 10 µg/ml BrdU. 5 hours after culture initiation, 5 ng/ml MMC was added to half of the cultures. Metaphases were fixed 48 hours after culture initiation and the number of SCEs present in one metaphase was determined. Per culture, 25 metaphases were analyzed. The average number of SCEs present in one metaphase after MMC treatment (■) or without MMC treatment (□) are plotted. Standard errors of the mean are indicated for every experiment. The cell cycle progression (ratio of 1st – 2nd – 3rd etc. division metaphases) was determined while analyzing the slides; no significant differences in cell cycle progression were observed between wildtype and mutant animals (data not shown). (T-test for 5 ng MMC / ml *RAD52*^{-/-} / *RAD54*^{-/-} versus 5 ng MMC / ml wildtype and 5 ng MMC / ml *RAD54*^{-/-} versus 5 ng MMC / ml wildtype: 0.02 < p < 0.16)

Epistasis of rad22A⁺, rad22B⁺ and rhp54⁺ in S. pombe

Based on the analysis of double mutant strains, *RAD52* and *RAD54* have been assigned to one epistasis group in *S. cerevisiae* and function in one pathway in the repair of damage inflicted by ionizing radiation and MMS (reviewed in Pâques and Haber, 1999). Survival studies in *S. pombe* after exposure to UV and MMS suggested that Rad22A and Rhp54 proteins function in overlapping but different

pathways in fission yeast (Khasanov et al., 1999). To extend our analysis of mouse *RAD52*^{-/-} and *RAD54*^{-/-} single and double mutants, mutations in *rad22A*⁺, *rad22B*⁺ and *rhp54*⁺ were combined to examine whether these combinations would lead to additional defects in survival. Double and triple mutants were obtained by standard crosses and tested for X-ray, hydroxyurea (HU) and cis-diamminedichloroplatinum (cisDDP) sensitivity. Since the *rad22A* mutant strain readily acquires suppressor mutations (also reported by Hartsuiker et al., 2001; Doe et al., 2004), only small and slow growing colonies were tested. As reported before, single mutants of *rad22A* and *rhp54* were extremely sensitive to X-rays, whereas the *rad22B* mutant did not display an increased sensitivity (Muris et al., 1993; Muris et al., 1996; van den Bosch et al., 2001; van den Bosch et al., 2002b). Double and triple mutants containing mutations in *rad22A*⁺, *rad22B*⁺ and *rhp54*⁺ showed similar sensitivities as the single *rad22A* and *rhp54* mutant strains (Figure 4). The sensitivity of the *rhp51* / *rhp54* double mutant is also comparable to the survival of the single mutants. At a dose of 150 Gy, part of the *rad22A* and *rad22A* / *rad22B* mutant survivors may have acquired a suppressor mutation (Figure 4). In all experiments, the introduction of a *rad22B* mutation in an *rhp54* deficient background resulted in a slightly decreased sensitivity in comparison with the *rhp54* single mutant, especially at higher doses. Possibly, in the absence of Rad22B the repair defect in Rhp54-deficient cells is partially rescued.

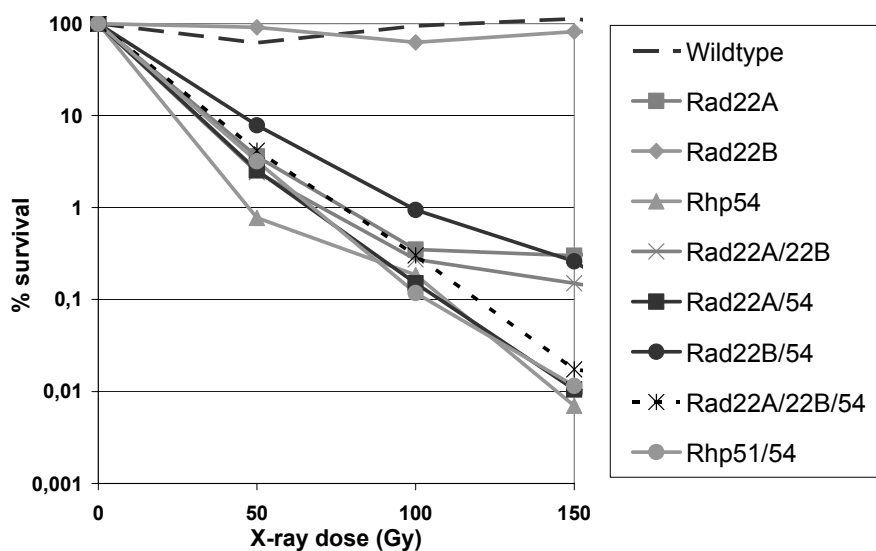


Figure 4: X-ray survival of wildtype and single, double and triple mutant *S. pombe* strains. Exponentially growing cells were harvested, irradiated and appropriate dilutions were plated in triplicate on YES media. After 3 days of incubation at 30°C the colonies were counted. Each survival experiment was repeated at least twice. **See appendix for a colour version of this figure.**

Next we tested the sensitivity to hydroxyurea (HU). Inhibition of ribonucleotide reductase by HU results in depletion of nucleotide pools and the formation of DSBs in replicating cells. With the exception of the *rad22B* mutant, all single, double and triple mutants showed a similar hypersensitivity to HU (see Figure 5). At a dose of 1 mM HU the survival of the *rad22A* mutant is increased in the absence of Rhp54 or Rad22B. This effect was not seen at a dose of 3 mM. Possibly, the defect in the *rad22A* mutant is partially rescued in the double mutants, although the induction of suppressor mutations cannot be excluded. To induce interstrand crosslinks mutant strains were treated with cisDDP. Except for the *rad22B* single mutant all single, double and triple mutant strains displayed very similar sensitivities (data not shown). Taken together the survival data imply that *rad22A*⁺, *rad22B*⁺ and *rhp54*⁺ are epistatic for the repair of DSBs in DNA. Similar data have recently been reported for *rad22A*⁺ and *rhp51*⁺ (Doe et al., 2004).

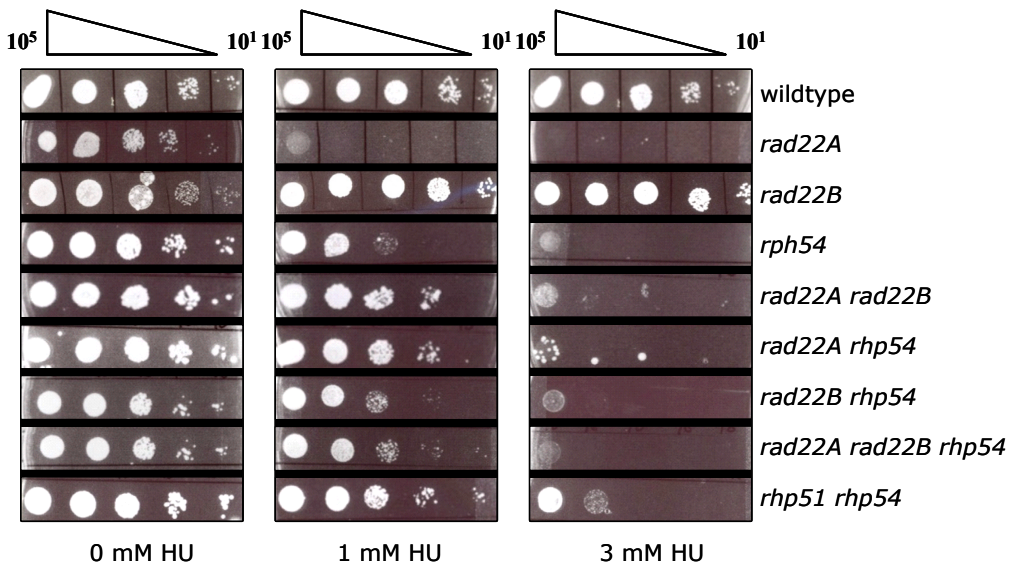


Figure 5: Hydroxyurea sensitivity of wildtype and single, double and triple mutant *S. pombe* strains. The survival of the indicated strains was tested by a spot assay (see Materials and methods). Serial dilutions of exponentially growing cells were spotted onto YES plates containing 0 mM, 1 mM or 3 mM of hydroxyurea (HU). The plates were incubated for 3 days at 30°C and photographed.

Discussion

Faithful repair of DSBs in DNA through homologous recombination (HR) is essential for each organism to maintain genomic integrity. Mutations leading to inactivation of HR are tolerated in yeast but not in mammals. Exceptions are deletion mutations in *RAD52*, *RAD54* or *RAD54B*. The critical role of Rad52 in gene conversion and crossover formation is most likely compensated for in vertebrates by the Rad51 paralogs, as inactivation of *XRCC3* in *RAD52*^{-/-} DT40 cells is lethal (Fujimori et al., 2001). To investigate the contribution of Rad52 to DSB repair *in vivo* and to determine if inactivation of *RAD52* in *RAD54* deficient mice exacerbates the mild phenotype of the *RAD54* mutant, doubly deficient mice were generated. In all experiments described here, *RAD52* single mutant cells and animals did not differ from wildtype controls. However, survival studies showed a distinct increase in sensitivity to MMC of *RAD52*^{-/-} / *RAD54*^{-/-} double mutant mice in comparison with the *RAD54*^{-/-} single mutant. The sensitivity of bone marrow cells to DNA damaging agents was determined by studying haemopoietic depression. Treatment with MMC did not reveal an increased sensitivity of single and double mutant animals but after exposure to ionizing radiation a significant decrease in the ratio of PCEs and NCEs plus OCEs was observed in the double mutant in comparison with the *RAD54*^{-/-} single mutant. The induction of SCEs in bone marrow cells after MMC treatment was slightly affected in the absence of Rad54. In the double mutant no aggravation of this phenotype was seen.

To account for the stronger phenotype of the double mutant in two of the assays performed, several explanations are possible. One is that lack of both Rad52 and Rad54 in the double mutant leads to additive defects. Although, Rad52 and Rad54 do not interact directly and act at different but overlapping stages of HR, it is conceivable that the absence of both proteins in the double mutant influences the efficiency of functional compensation. The decrease in MMC survival of double mutant mice and the increased haemopoietic depression after X-rays may also be indicative for a role of Rad52 and/or Rad54 in different sub-pathways in HR. Since SSA in *S. cerevisiae* requires Rad52 and is not dependent on the presence of Rad54, the results may suggest a role for SSA in mammals in overcoming DSBs in the *RAD54* mutant. A contribution of BIR in DSB-repair in the *RAD54* mutant cannot be excluded but does not seem very likely, as BIR is presumably important for the repair of chromosome ends (Pâques and Haber, 1999). Evidence for a role of Rad52 in SSA in mammals, has been recently obtained using *RAD52*^{-/-} ES cells containing a specific reporter construct to detect SSA events (Stark et al., 2004). At present there is no indication for a role of SSA in mammals in overcoming DSBs inflicted by exogenous agents. However, SSA may contribute to the repair of DSBs when HR is partially hampered. Mouse ES cells containing a specific recombination substrate with direct repeats displayed an increased level of SSA in the absence of Rad54 (Dronkert et al., 2000).

According to data obtained by Khasanov and co-workers Rad22A and Rhp54 in *S. pombe* function in different sub-pathways in DSB repair (Khasanov et al.,

1999). However, survival studies shown in Figure 4 and 5 of *rad22A*, *rad22B* and *rhp54* single, double and triple mutants in *S. pombe*, clearly indicated that Rad22A and Rhp54 function in the same epistasis group for the repair of X-ray and cisDDP induced DSBs and breaks that occur after inhibition of replication by HU, like has been shown for *S. cerevisiae*.

Acknowledgements

We are indebted to the Central Mouse Facility at the RIVM in Bilthoven for general mouse housing. This work was supported by the EU (RISC-RAD / FI6R-CT-2003-508842).

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Chapter 4

The role of SUMO modification in repair of
DNA double-strand breaks
in *D. melanogaster* and *S. pombe*.

4 The role of SUMO modification in repair of DNA double-strand breaks in *D. melanogaster* and *S. pombe*

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Abstract

Homologous recombination is one of the two principle pathways for the repair of double-strand breaks in DNA. To identify novel proteins involved in this pathway, *Drosophila* DmRad51 and both Rad52 homologs from *S. pombe*, Rad22A and Rad22B, were used as bait proteins in yeast two-hybrid studies. In these screens components of the SUMO conjugation pathway were identified as associating factors. DmRad51 shows complex formation with Uba2, Ubc9 and Su(Var)2-10, the SUMO E1 activating, E2 conjugating and E3 ligating enzymes in *Drosophila*, respectively. Su(Var)2-10, which was originally identified as a dominant suppressor of position-effect-variegation, is essential for viability and is involved in the regulation of chromosome structure. In heterozygous *Su(Var)2-10* flies an increased X-ray sensitivity was observed, implicating sumoylation in DNA damage responses to X-rays. Both Rad22A and Rad22B display interaction with Hus5, the SUMO E2 conjugating enzyme in *S. pombe*. However, mutations in the single SUMO consensus motif of Rad22A did not influence the complementation of *rad22A* mutant strains. Screening of the *S. pombe* database resulted in the identification of the SP-RING domain protein Pli1, which belongs to the PIAS family of SUMO ligases. Targeted disruption of *pli1*⁺ did not result in hypersensitivity to X-rays and hydroxyurea. Although, the protein-protein interaction studies suggest a connection between SUMO modification and the repair of double-strand breaks by homologous recombination, the role for SUMO in DNA repair may be rather indirect as defects in chromosome structure in *Su(Var)2-10* mutants imply an essential role for SUMO in maintaining proper nuclear organization. Subtle alterations in nuclear architecture could have serious effects on repair and other DNA metabolizing processes.

Introduction

Posttranslational modification of proteins by the ubiquitin related protein SUMO (small ubiquitin-like modifier) affects various cellular processes. SUMO modification has been shown to play an important role in regulating transcription, redistribution and transport of proteins through nuclear pores, maintaining proper chromosome organization, formation of PML nuclear bodies, activation of DNA damage responses and antagonizing ubiquitination (Verger et al., 2003; Johnson, 2004; Watts, 2004). In mammals three variants of SUMO have been identified: SUMO-1 and the closely related SUMO-2 and SUMO-3 proteins

(reviewed in Su and Li, 2002; Johnson, 2004; Watts, 2004). In *S. cerevisiae* only one SUMO gene, *SMT3*, has been identified which is essential for viability. In *S. pombe* the SUMO protein is encoded by the *pmt3*⁺ gene. Strains having a mutated allele of *pmt3*⁺, are barely viable and display serious defects in genome maintenance.

SUMO is synthesized as a precursor which is processed by hydrolytic cleavage at the C-terminal end. It is subsequently conjugated to proteins by means of E1 activating, E2 conjugating and E3 ligating enzymes. E2 catalyses the formation of an isopeptide bond between the C-terminal glycine of SUMO and a lysine residue in the target protein. This residue is usually found within the SUMO ψ KxE consensus motif (where ψ is a large hydrophobic residue and x is any residue). The E3-like proteins might serve to increase the affinity between E2 and substrate proteins by bringing them into close proximity in catalytically favourable orientations, allowing sumoylation to occur more efficiently. The resulting isopeptide bond is stable and its disruption requires the action of a desumoylating enzyme (Su and Li, 2002; Schmidt and Muller, 2003; Johnson, 2004; Watts, 2004). The SUMO activating E1 enzyme is a heterodimer and consists of AOS1 (SAE1) and UBA2 (SAE2). UBC9 is the SUMO E2 conjugating enzyme. In most organisms *AOS1*, *UBA2* and *UBC9* genes are essential. *S. pombe* strains mutated in homologs of *AOS1* (*fub2*⁺), *UBA2* (*rad31*⁺) and *UBC9* (*hus5*⁺) are not or barely viable, displaying various phenotypes including slow growth, aberrant mitosis, high-frequency loss of minichromosomes and increased sensitivity to various genotoxic agents as ionizing radiation, UV and the DNA synthesis inhibitor hydroxyurea (al Khodairy et al., 1995; Shayeghi et al. 1997; Tanaka et al., 1999; Ho and Watts, 2003).

The E3 SUMO ligases are members of three distinct protein families. The first includes E3 ligases of the PIAS family. PIAS family members were originally discovered as protein inhibitors of activated STAT transcription factors (Chung et al., 1997; Liu et al., 1998; Schmidt and Muller, 2003). PIAS proteins interact with and modulate activities of various transcription factors and the PIAS/SUMO pathway is important for transcriptional regulation of various important cellular pathways. The different PIAS proteins may direct SUMO to different substrates or support SUMO modification by different SUMO isoforms in mammals (Schmidt and Muller, 2003). In *S. cerevisiae* three PIAS family proteins have been identified, Siz1/Ull1, Siz2/Nfi1 and recently it was shown that Mms21 also has SUMO E3 activity (Johnson and Gupta, 2001; Takahashi et al., 2001; Hoege et al., 2002; Takahashi et al., 2003; Zhao and Blobel, 2005). Siz1/Ull1 was found to be an E3 ligase specific for septin components and PCNA. Although Siz2/Nfi1 promotes sumoylation of septin components *in vitro*, the *in vivo* substrates of Siz2/Nfi1 remain to be elucidated (Takahashi et al., 2003). Siz2/Nfi1 and other members of the PIAS family contain a highly conserved SP-RING domain, a nuclear localization signal (NLS) and ψ KxE consensus motifs, suggesting autosumoylation. In *Drosophila melanogaster*, the *Su(Var)2-10* gene was identified as a member of the PIAS family of proteins. Su(Var)2-10 is required for viability and regulates chromosome organisation in the nucleus. *Su(Var)2-10* mutant larvae have defects in chromosome inheritance and show abnormal

chromosome structures (Hari et al., 2001). A second type of SUMO E3 ligases was discovered within the nuclearporin factor RanBP2 /Nup358 (Wu et al., 1995; Yokoyama et al., 1995; Pichler et al., 2002). A domain within this nuclear pore protein was found to stimulate *in vitro* sumoylation of the RanGAP1, Sp100 and HDAC4 subunits of the nuclear pore complex. Enhanced sumoylation of these substrates by PIAS proteins was not observed. Most likely, the RanBP2 E3 ligase coordinates the positioning of the SUMO E2 intermediate for optimal substrate conjugation (Reverter and Lima, 2005). A third SUMO E3 ligase is the polycomb group protein Pc2. Pc2 can stimulate SUMO modification of the transcriptional repressor CtBP, that can also be sumoylated by PIAS proteins (Lin et al., 2003; Kagey et al., 2003; Kagey et al., 2005).

DNA damage can occur as a result of exposure to genotoxic agents such as ionizing radiation or certain chemical compounds. A particular harmful type of damage is the breakage of both DNA strands leading to the formation of double-strand breaks (DSBs). Repair of DSBs in eukaryotes can occur through homologous recombination (HR) or non-homologous endjoining. Rad51, one of the key proteins in HR, forms nucleoprotein filaments at the site of the break. Stimulated by among others Rad52, Rad51 mediates pairing of the nucleofilament with homologous DNA, invasion and strand exchange (Pâques and Haber, 1999). Sumoylation of proteins involved in HR has been shown for Rad22A, one of the two Rad52 homologs in *Schizosaccharomyces pombe* (Ho et al., 2001; van den Bosch et al., 2001). Interaction with the SUMO E2 enzyme Ubc9 in a yeast two-hybrid system has been shown for mammalian Rad51 and Rad52 (Shen et al., 1996). Sumoylation of Rad51 could not be detected, but the existence of a Rad51-Rad52-SUMO complex, in which neither Rad51 nor Rad52 is sumoylated, has been demonstrated (Li et al., 2000). Perturbation of Ubc9 in mammalian cells prevents the redistribution of Rad51 and the formation of foci after exposure to radiation (Saitoh et al., 2002). It is conceivable that the SUMO pathway modulates the intracellular trafficking of Rad51 and other proteins involved in HR. Upon DNA damage, SUMO modification may direct these repair factors to the DNA damage and/or sequester them into foci (Müller et al., 2004). A role for sumoylation in the control of intracellular localization of repair factors has also been shown for XRCC4 and BLM's protein (Eladad et al., 2005; Yurchenko et al., 2006).

In this work, we describe the interactions between proteins involved in HR and components of the SUMO pathway in *Drosophila melanogaster* and *Schizosaccharomyces pombe*. Heterozygosity for *Su(Var)2-10*, encoding one of the E3 ligating enzymes in *Drosophila*, results in increased X-ray sensitivity. Alteration of the unique SUMO consensus sequence of Rad22A in *S. pombe* did not result in enhanced radiation sensitivity nor did disruption of the *pli1*⁺ gene, coding for one of the PIAS E3 ligases in *S. pombe*.

Materials and Methods

Two-hybrid analysis

The single-copy two-hybrid vectors pPC97, carrying the GAL4 DNA binding domain, and pPC86, containing the GAL4 activation domain, have been described (Chevray and Nathans, 1992). The *Drosophila melanogaster* *DmRad51* gene and the *S. pombe* *rad22A*⁺, *rad22B*⁺, *rad51*⁺, *rad54*⁺, *rad55*⁺, *rad57*⁺, *ssb1*⁺, *ssb2*⁺, *hus5*⁺ and *plil1*⁺ open reading frames (ORFs) were amplified using Platinum Pfx DNA polymerase (Invitrogen) according to the manufacturers instructions and PCR primers that create 5'-*SaI*I and 3'-*Bam*HI sites flanking the start and stop codons, respectively. PCR fragments were cloned in PCRscript using the PCRscript Amp Cloning Kit (Stratagene) and subcloned in pPC97 and pPC86, as *SaI*I-*Bam*HI fragments. A *Drosophila melanogaster* matchmaker library (BD Biosciences) was screened using *DmRad51* as a bait. *S. pombe* matchmaker (BD Biosciences) and pBI771 two-hybrid libraries (Kohalmi et al., 1998) were screened using *Rad22A* and *Rad22B* as baits. For the two-hybrid screens the *S. cerevisiae* Y190 strain was used (Harper et al., 1993). Transformants were selected on YNB media containing 30 mg/l adenine and 50 mM 3-aminotriazole (Fields and Sternglanz, 1994). Protein-protein interactions were verified by a filter assay for β -galactosidase activity in permeabilized yeast cells. DNA from positive colonies was isolated using glass-beats and used for PCR, sequencing and BLAST analysis to determine the identity of the prey plasmid inserts.

GST pull down experiments

Two-hybrid interactions were confirmed by GST pull-down assays. DNA inserts from positive two-hybrid clones were isolated as *SaI*I-*Not*I fragments and subcloned in pGEX-6P-2 and pMV2-HA. GST pull-down experiments were performed essentially as described elsewhere (van den Bosch et al., 2002).

Fly strains

Flies were grown on standard sugar-agar medium at 25°C and adults were handled under CO₂ anesthesia. The *Su(Var)2-10[2]/CyO* strain was obtained from the Bloomington *Drosophila* Stock Centre. Heterozogous *Su(Var)2-10[2]/CyO* flies were maintained using the *Pm/CyO* strain, containing the second chromosome balancers *In(2LR)bw*^{V1} and SM5 (Lindsley and Zimm, 1992).

Survival studies of Su(Var)2-10 heterozygous flies

In *Drosophila*, the sensitivity to ionizing radiation is dependent on the developmental stage of the fly and therefore embryos and larvae of different stages were used for treatment. *Su(Var)2-10[2]/CyO* females and *Pm/CyO* males were crossed and 0- to 24-hr embryos, 24- to 48-hr larvae and 48- to 72-hr larvae were exposed to a single dose of 10 Gy using a 225 SMART X-ray source (200 kV, 4mA, 1.0 mm Al filter and a dose rate of ~1 Gy/min; Andrex SA, Copenhagen, Denmark). After irradiation, fly cultures were kept at 25°C, and the offspring was scored after 12 to 18 days. Untreated embryos/larvae of

the same cross were used as controls. To determine the relative sensitivity, the ratio between heterozygous *Su(Var)2-10[2]/CyO* and *Su(Var)2-10[2]/Pm* mutants and *Pm/CyO* flies was calculated. In the untreated control the ratio of the three genotypes is 1:1:1 according to Mendelian laws. If the sensitivity to DNA-damaging agents is increased, the relative number of *Su(Var)2-10[2]* heterozygotes will decrease after exposure to 10 Gy of X-rays.

Fission yeast strains

Growth media and general handling of fission yeast were essentially as described (Gutz et al., 1974). The *S. pombe rad22A*, *rad22B* and *rad22Arad22B* double mutant strains used in this study have been described (van den Bosch et al., 2001; de Vries et al., 2005). The Y4 wildtype strain was used for control studies.

Mutational analysis of the SUMO modification motif in Rad22A

An expression construct of the *rad22A*⁺ gene was made by cloning the *rad22A*⁺ ORF in pREP1 (Maundrell, 1993). The *rad22A*⁺ ORF was amplified as described before using primers containing *NdeI* and *BamHI* restriction sites, respectively. After cloning in PCRscript (Stratagene) and confirmation of the sequence, a *NdeI-BamHI* DNA fragment was subcloned in the *LEU2* containing autonomously replicating expression vector pREP1. Analysis of the amino acid sequence revealed a single sumoylation consensus sequence CKKE between residues 134 and 138. To study the role of this sequence motif, several amino acid changes were introduced: 135 K>R, 135 K>A, 136 K>R, 135,136 KK>RR. Site-directed mutagenesis was performed using the QuikChange Multi kit (Stratagene). To introduce sequence alterations, primers containing the mutations were synthesized for one strand of the pREP1/*rad22A*⁺ template. The mutagenic primers were extended by PfuTurbo enzyme, generating a strand bearing the mutation and also a unique restriction site to facilitate selection of mutant plasmids. pREP1plasmids expressing wildtype or mutant Rad22A were introduced into *rad22Arad22B* double mutant *S. pombe* strains.

Isolation of the pli1⁺ gene

BLAST searches exploiting *S. cerevisiae* and human PIAS family members led to the identification of the *S. pombe pli1*⁺ gene, which is homologous to *NFI1* from *S. cerevisiae*. The *pli1*⁺ gene was obtained by amplifying a 4142 bp region encompassing the *pli1*⁺ ORF using Pfx DNA Polymerase (Invitrogen) and primers PliFW2 (5'- CCA GGC GTT TGC AGA GAT TC - 3') and PliREV2 (5' - TCT ATG CTC GCC AGA GAC AA - 3'). To amplify the *pli1*⁺ ORF a second PCR was performed using Pfx Polymerase and primers PliSkip (5' - CGC GTC GAC CAT GAA CCA GGC GAA CTT TTT ACA GGA GCT TCC AAA TGT ACT AAA GCG ACT GGA AAC TGG TCT T - 3'), which creates a *SaI* restriction site flanking the start codon and skips the 67 bp intron and PliStop (5' - CCG GGA TCC GTT AAT CTA TAC TCT GAA A - 3') which creates a *BamHI* site downstream of the stop codon. The *pli1*⁺ cDNA was cloned in PCRscript and verified by sequencing. The *pli1*⁺ cDNA was

subsequently subcloned in pPC97 and pPC86 and used in two-hybrid studies (see elsewhere).

*Disruption of the *pli1*⁺ gene*

A *pli1* deletion mutant was constructed by replacing the open reading frame with a kan^r marker by PCR-based gene disruption (Wach et al., 1994). PliS1 primer (5' – ATG AAC CAG GCG AAC TTT TTA CAG GAG CTT CCA GTA AGC CCA ATA ATA AGA TAT TTA CAA GTT AAC TAA CAA AAT GAA TGC GTA CGC TGC AGG TCG AC – 3') and PliS2 primer (5' – TTA ATC TAT ACT CTG AAA AGT GTT TCC CGT TCC TTC AAA TCC GGA ATT ATG ATA GGA GTT ATT TGA CTG TAA CTC AGA ATT CCA TCG ATG AAT TCG AGC TCG – 3') were used in combination with plasmid pFA6a-*kanMX4* to create a kan^r fragment with arms flanking the *pli1*⁺ gene (the nucleotide sequences underlined overlap with the *pli1*⁺ DNA). The *S. pombe* diploid sp.101 strain (Murray et al., 1991) was transformed with 5 µg of PCR product according to the LiAc method (Keeney and Boeke, 1994). G418-resistant colonies were screened by PCR using primers flanking the *pli1*⁺ gene in combination with primers specific for the kan^r cassette and targeted disruption was confirmed by Southern blot analysis. An h⁹⁰/h⁺ derivative was selected and used to isolate haploid G418-resistant cells. Both Southern blot and PCR analysis were performed to assure correct integration of the kan^R cassette.

S. pombe survival studies

Survival studies to measure the sensitivity of *S. pombe* cells to ionizing radiation, hydroxyurea (HU) and methyl methanesulphonate (MMS) were performed as described before (de Vries et al., 2005). To test for sensitivity to MMS, 5 µl of 10-fold dilutions of log-phase cells were spotted onto YES plates containing 0.0001-0.0005% MMS. Cell survival was determined after 3 days of incubation at 30°C. All experiments were repeated at least twice.

Results

Two-hybrid interactions of recombinational repair proteins and components of the SUMO pathway in flies and fission yeast

To identify new partners of proteins involved in homologous recombination (HR), *Drosophila* and *S. pombe* two-hybrid libraries were screened using *Drosophila* Rad51 and the two Rad52 homologs from *S. pombe*, Rad22A and Rad22B, as bait proteins. Yeast transformants were selected for histidine prototrophy and subsequently screened for β-galactosidase activity. Screening of 10⁶ transformants with DmRad51 resulted in 41 positive clones. Among the proteins that display interaction with DmRad51 were Uba2, homologous to the mammalian SUMO E1 activating enzyme, Ubc9, homologous to the mammalian SUMO E2 conjugating enzyme and Su(Var)2-10, homologous to mammalian SUMO E3 ligases belonging to the PIAS family. Analysis of Gal4 binding domain and activation domain fusions of Uba2, Ubc9 and Su(Var)2-10 also showed interactions among the E1, E2 and E3 enzymes of the SUMO conjugation pathway (see Figure 2A). Among the other clones identified in the screen with

DmRad51, were Tosca and PCNA. Tosca belongs to the RAD2 family of nucleases involved in DNA repair. Within this group of proteins, Tosca is closely related to Exo1, a 5'-3' double-stranded DNA exonuclease from *S. pombe*. In *Drosophila* Tosca is specifically expressed in female germ cells (Digilio et al., 1996). The expression pattern may suggest a role for Tosca in the specific resection of 5'-ends of DSBs during meiotic recombination. Screening of two different *S. pombe* libraries (10^6 transformants in total) with Rad22A resulted in the isolation of 28 clones and with Rad22B in the recovery of 17 clones. Sequence analysis identified Hus5, homologous to the mammalian SUMO E2 conjugating enzyme, as one of the proteins interacting with both Rad22A and Rad22B.

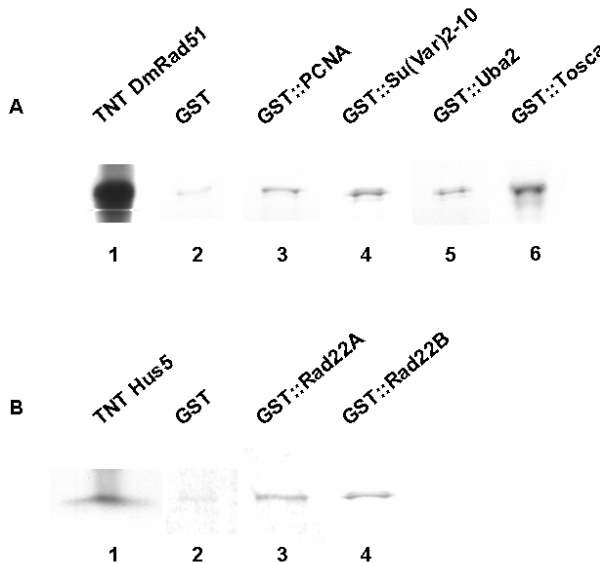


Figure 1: Protein-protein interactions investigated by GST pull down assays.

(A) 3 μ l of [^{35} S]methionine labelled DmRad51 (lane 1); 15 μ l of [^{35} S]methionine labelled DmRad51 was mixed with glutathione-Sepharose beads loaded with 2.5 μ g of GST (lane 2), 2.5 μ g of GST::PCNA (lane 3), 2.5 μ g of GST::Su(Var)2-10 (lane 4), 2.5 μ g of GST::Uba2 (lane 5), 2.5 μ g of GST::Tosca (lane 6). **(B)** 2 μ l of [^{35} S]methionine labelled Hus5 (lane 1); 15 μ l of [^{35} S]methionine labelled Hus5 was mixed with glutathione-Sepharose beads loaded with 2.5 μ g of GST (lane 2), 2.5 μ g of GST::Rad22A (lane 3), 2.5 μ g of GST::Rad22B (lane 4).

The associations detected by two-hybrid screens were validated by reciprocal combinations of Gal4 binding domain and activator domain fusions and confirmed by GST pull-down assays. Full length PCNA, Su(Var)2-10 and Tosca proteins were expressed in *E. coli* as glutathione S-transferase (GST) fusions. GST-fusion proteins were bound to glutathione-Sepharose beads and incubated with [^{35} S]methionine labelled DmRad51. After washing, proteins bound to the beads were detected by electrophoresis on polyacrylamide gels. The results shown in Figure 1 indicate *in vitro* interaction of DmRad51 with PCNA, Su(Var)2-10, Uba2 and Tosca (Figure 1A, lanes 3-6). Specific binding of [^{35} S]methionine

labelled Hus5 to GST-Rad22A and GST-Rad22B bound to glutathione-Sepharose beads is shown in Figure 1B, lanes 3 and 4. Interactions of DmRad51 with Ubc9 and among Uba2, Ubc9 and Su(Var)2-10 were also confirmed by GSTpull-down assays (results not shown).

To examine putative interactions of Hus5 with other members of the RAD52 group which are involved in HR in *S. pombe*, additional two-hybrid studies were performed. In addition to the interactions of Hus5 with Rad22A and Rad22B, we observed interaction of Hus5 with Ssb1, one of the three subunits of RPA. No interaction was detected with Rhp51, Rhp54, Rhp55, Rhp57 and Ssb2 (data not shown). To localize the regions that mediate association of Rad22A and Rad22B with Hus5, various deletion constructs were tested (gene fragments are indicated in Figure 2B).

		DNA binding fusions			
		DmRad51	Uba2	Ubc9	(no insert)
Activator fusions	Uba2	+	++	+	-
	Ubc9	+	+	+	-
	Su(Var)2-10	+	++	+	-
	(no insert)	-	-	-	-

Hus5							
Amino acid			Amino acid			Amino acid	
Rad22A-a	1 – 469	+/-	Rad22A-e	168 – 258	+/-	Rad22B-a	1 – 158
Rad22A-b	1 – 167	+	Rad22A-f	72 – 167	+	Rad22B-b	1 – 232
Rad22A-c	1 – 258	-	Rad22A-g	72 – 224	+	Rad22B-c	159 – 371
Rad22A-d	168 – 469	-	Rad22A-h	168 – 224	+	Rad22B-d	233 – 371

Figure 2: Protein-protein interactions detected by two-hybrid studies.

(A) Analysis of DNA binding domain fusions of *D. melanogaster* DmRad51, Uba2 and Ubc9 and activation domain fusions of Uba2, Ubc9 and Su(Var)2-10. Positive interactions were confirmed by β -galactosidase filter assays. ++, strong positive interaction; +, positive interaction; +/-, weak interaction; -, no interaction. **(B)** Two-hybrid analysis of Hus5 and fragments of *S. pombe* Rad22A and Rad22B. Amino acid regions of Rad22A and Rad22B used for two-hybrid analysis are indicated. In all experiments genes and gene fragments were tested both as DNA binding and activation domain fusions.

Two-hybrid experiments revealed that the region required for interaction of Rad22A with Hus5 is within gene fragment Rad22A-b coding for amino acids 1 to 258. A weak interaction with Hus5 was observed in case of Rad22A-a and Rad22A-e fragments. To localize the region of association more precisely, gene fragments Rad22A-f, Rad22A-g and Rad22A-h were tested. Two-hybrid experiments indicated that the region(s) required for interaction between Rad22A and Hus5 is located between amino acid 72 and 224. (Figure 2B). This clarifies that the region interacting with Hus5 is probably located in the

proximity of the (putative) SUMO modification site, the lysine residue at amino acid 135.

The small Rad22B-a N-terminal deletion fragment did not interact with Hus5, while all other fragments showed an interaction using the two-hybrid system with Hus5, indicating that the site(s) of interaction of Rad22B and Hus5 is located C-terminal of amino acid 158 (Figure 2B).

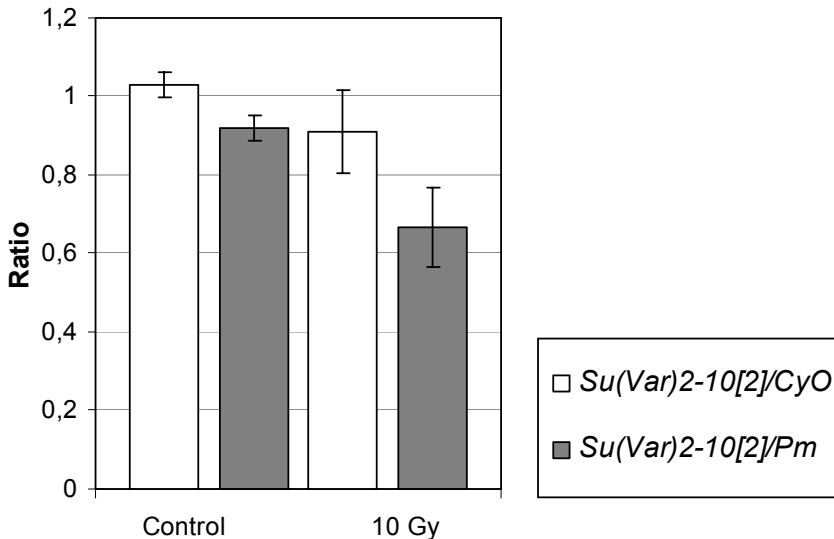


Figure 3: Survival of 0- to 24-hr *Drosophila* embryo's heterozygous for *Su(Var)2-10* after exposure to radiation. On the y-axis the ratio of *Su(Var)2-10 [2]/CyO* to *Pm/CyO* and of *Su(Var)2-10 [2]/Pm* to *Pm/CyO* flies from the same cross is indicated. If the defect in *Su(Var)2-10* leads to increased radiation sensitivity, these ratios will drop after exposure to X-rays. In agreement with Mendelian laws, the ratios in the untreated control are approximately 1.0. Standard errors of the mean are indicated for every experiment.

Su(Var)2-10 heterozygous flies are sensitive to X-rays.

To investigate the role of the *Su(Var)2-10* E3 ligase in the cellular responses to damage inflicted by ionizing radiation, we tested a *Drosophila melanogaster* strain mutated in *Su(Var)2-10* for increased X-ray sensitivity. Since homozygous *Su(Var)2-10* mutant flies are embryonic lethal, heterozygous flies were tested. *Su(Var)2-10[2]/CyO* females were crossed with *Pm/CyO* males and 0- to 24-hr embryos, 24- to 48-hr larvae and 48- to 72-hr larvae were exposed to a dose of 10 Gy of X-rays. In untreated offspring, the ratio of *Su(Var)2-10[2]/CyO*, *Su(Var)2-10[2]/Pm* and *Pm/CyO* flies and mutants was 1.0 : 0.9 : 1.0. The theoretically expected ratio for the three genotypes is 1:1:1 according to Mendelian laws (*CyO/CyO* flies are embryonic lethal). Increased radiation sensitivity will lead to a decrease in the *Su(Var)2-10[2]/Pm* to *Pm/CyO* and *Su(Var)2-10[2]/CyO* to *Pm/CyO* ratios. After irradiation of 0- to 24-hr embryos a slight increase in sensitivity was observed for the *Su(Var)2-10[2]/CyO* flies. For the *Su(Var)2-10[2]/Pm* flies the increase in radiation sensitivity was more

pronounced (Figure 3). Similar results were obtained for the reciprocal cross of *Su(Var)2-10[2]/CyO* males with *Pm/CyO* females (results not shown). Irradiation of 24- to 48-hr and 48- to 72-hr larvae did not result in enhanced radiation sensitivity (data not shown). These data indicate that *Su(Var)2-10* mediated SUMO modification is directly or indirectly involved in proper cellular responses to radiation-induced damage in *Drosophila*.

Mutational analysis of a putative SUMO modification site in Rad22A

Screening of the Rad22A amino acid sequence for putative SUMO modification sites (ψ KxE) revealed only one lysine residue at position 135 that may be a target for sumoylation. The putative acceptor lysine is flanked by a cysteine, a lysine and a glutamic acid ($C_{134}K_{135}K_{136}E_{137}$). To determine if sumoylation of K_{135} is required for proper repair through HR, the *rad22A*⁺ ORF was inserted in the pREP1 expression vector and mutations were introduced at position 135 and 136 or both (see materials and methods). Wildtype and mutated plasmid DNAs were introduced into *rad22A**rad22B* mutant *S. pombe* cells and tested for X-ray sensitivity.

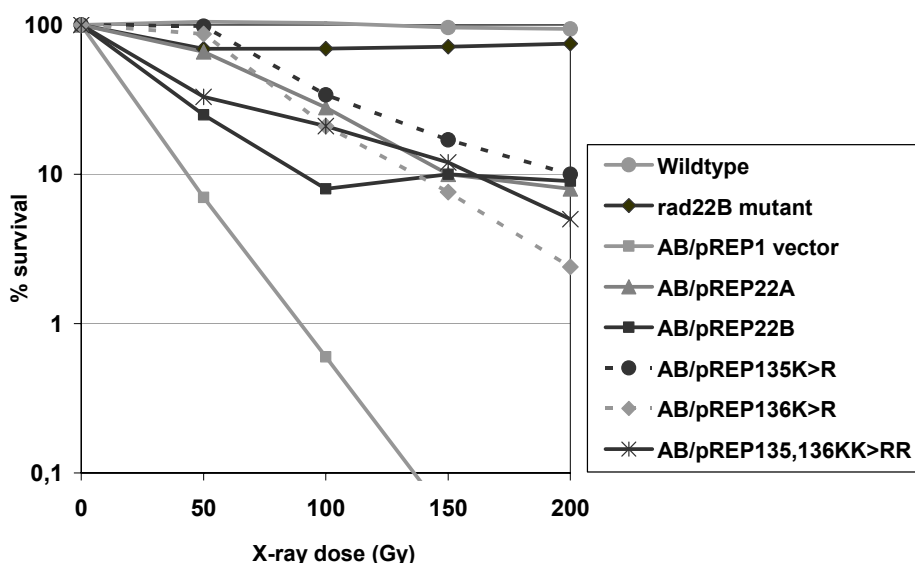


Figure 4: Survival of *S. pombe* strains after irradiation with X-rays. After irradiation of exponentially growing cells, appropriate dilutions were plated and colonies were counted after incubation of the plates for 3 days at 30°C. Each survival experiment was repeated at least twice. Strains used in this experiment: wildtype (Y4), *rad22B*^{-/-} (*rad22B* mutant), and double mutant *rad22A*^{-/-}*rad22B*^{-/-} strains containing expression vectors without insert (AB/pREP-), with Rad22A insert (AB/pREP22A), with Rad22B insert (AB/pREP22B) and with Rad22A inserts in which the putative SUMO acceptor site has been mutated (AB/pREP135 K>R, AB/pREP136 K>R, AB/pREP135,136 KK>RR). For details see Materials and methods. **See appendix for a colour version of this figure.**

As previously observed, *rad22Arad22B* mutant cells containing only vector DNA display a severely increased X-ray sensitivity (Figure 4) (van den Bosch et al., 2001). Introduction of pREP1 containing wildtype *rad22A*⁺ resulted in a considerable correction of the hypersensitivity to X-rays of the double mutant (see Figure 4). Overexpression of *rad22B*⁺ also resulted in significant complementation as was previously observed (van den Bosch et al., 2001). Constructs expressing mutated Rad22A in which the putative acceptor lysine residue was changed into an arginine (135 K>R) also corrected the radiation sensitivity of the *rad22Arad22B* mutant. Similar results were obtained using a 135 K>A allele (data not shown). Two hybrid experiments indicated that mutating Rad22A protein does not influence the interaction with Hus5. Conversion of the lysine flanking the putative lysine acceptor residue (136 K>R) or changing both lysines in the SUMO consensus motif into arginine (135, 136 KK>RR) did not influence the complementation of the X-ray hypersensitivity of the mutant drastically (see Figure 4).

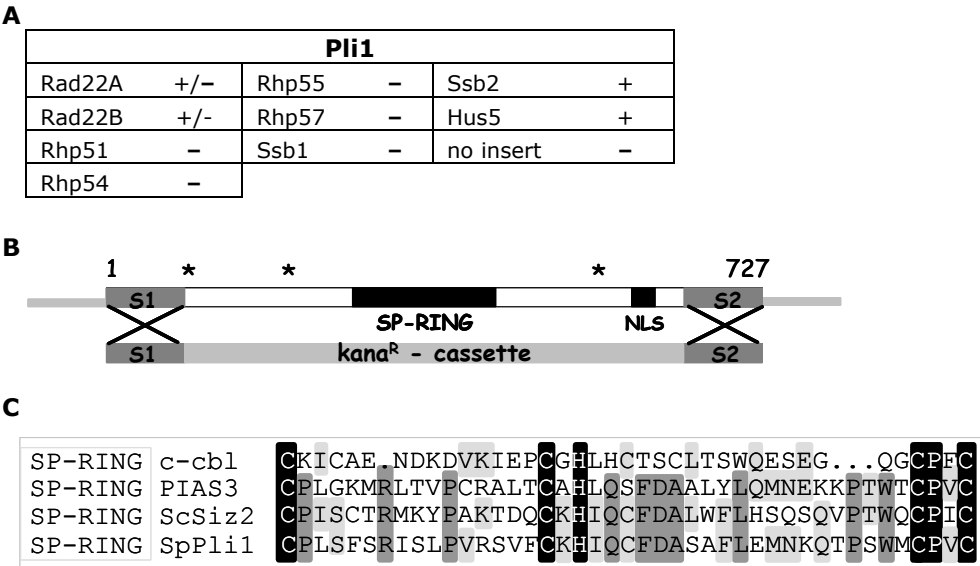


Figure 5
Analysis of *S. pombe* Pli1. **(A)** Interactions of Pli1 as determined by two-hybrid studies. All gene fragments were tested both as DNA binding and activator fusions. Positive interactions were confirmed by β -galactosidase filter assays. +, positive interaction; +/-, weak interaction; -, no interaction. **(B)** Schematic representation of the *pli1*⁺ gene (the 52 base pair intron is not shown). SP-RING domain and NLS coding sequences are indicated. Stars (*) represent the three putative SUMO modification motifs at position 41, 274 and 442 of the protein. The location of the arms (S1 and S2) used for disruption of the *pli1*⁺ gene by replacement with a kanamycin resistance cassette, is indicated. **(C)** Sequence alignment of SP-RING domains of the human ubiquitin ligase c-Cbl (GenPept accession number P22681), human PIAS3 (Q9Y6X2), *S. cerevisiae* Siz2 (NP_014799) and *S. pombe* Pli1 (AL031181). Identical amino acids are highlighted.

Isolation and disruption of the *pli1*⁺ gene

BLAST searches of the *S. pombe* database for sequences homologous to *S. cerevisiae* and human PIAS family members, led to the identification of a hypothetical open reading frame (ORF) on chromosome 1 with the reserved name of *pli1*⁺ (*S. pombe* geneDB). The predicted protein of 727 amino acid residues is homologous to Siz1 (Ull1) and Siz2 (Nfi1) from *S. cerevisiae* (21% and 25% identity, respectively). Like Siz1/2 and other members of the PIAS family, the Pli1 protein in *S. pombe* contains a SP-RING domain (Figure 5B), which is highly conserved (Figure 5C). In addition, a nuclear localization signal (NLS) and three putative sumoylation sites could be identified in Pli1 (Figure 5B). Two-hybrid studies were performed after cloning the *pli1*⁺ ORF in bait and prey vectors. The results showed weak interaction of Pli1 with Rad22A and Rad22B and a strong interaction with Hus5 and Ssb2. No interaction was observed with Rhp51, Rhp54, Rhp55 and Rhp55 (Figure 5A).

To examine the function of *pli1*⁺ and its role in DNA damage responses, a deletion mutant was generated (see materials and methods). When treated with hydroxyurea, *Pli1* mutant cells displayed the same sensitivity as wildtype cells (data not shown). The survival after X-ray irradiation is also very similar to the survival of the wildtype Y4 strain and the *rad22B* mutant. The data presented in Figure 6B, however, may indicate a slight but reproducible increase in radiation sensitivity of *pli1* (and *rad22B*) mutant cells.

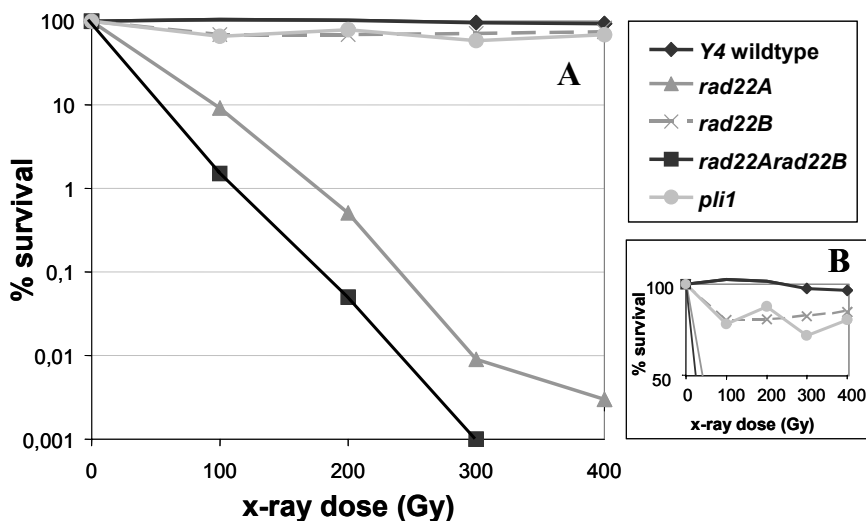


Figure 6

Survival following irradiation with X-rays of wildtype and mutant *S. pombe* strains after irradiation with X-rays **(A)** and a magnification of the survival curve of wildtype, *rad22B* mutant and *pli1* mutant cells **(B)**.

Discussion

To identify novel factors involved in the repair of DSBs in DNA through homologous recombination in *D. melanogaster* and *S. pombe*, yeast two-hybrid screens were performed. The use of *Drosophila* DmRad51 and Rad22A and Rad22B from *S. pombe* as baits, resulted in the identification of components of the sumoylation pathway. Interaction was detected between DmRad51 and SUMO activating, conjugating and ligating enzymes. Two-hybrid experiments and GST pull-down assays also showed interaction among the three components of the SUMO conjugation pathway itself (see Figure 2A). These data may suggest the formation of a sumoylation complex, in which SUMO is activated, conjugated and ligated to the substrate. The interactions between the various constituents may be highly dynamic and not all proteins have to be present in the complex at the same time during sumoylation.

The putative Su(Var)2-10 SUMO E3 ligase identified in our screen, was originally isolated as a suppressor of position-effect-variegation (PEV). Abnormal juxtaposition of genes near heterochromatic regions in the genome results in silencing of expression. Mutations in genes like *Su(Var)2-10* result in a mosaic or variegated expression of genes near centromeric or telomeric heterochromatin (for a review see Wallrath, 1998). At the chromosomal level abnormally condensed chromosomes and segregation defects were observed in *Su(var)2-10* homozygous mutants (Hari et al., 2001). In addition telomere-telomere and telomere-lamina associations are affected in this mutant. To investigate a role in the cellular responses to DNA damage, *Su(Var)2-10* mutant flies were assessed for increased sensitivity to ionizing radiation. As homozygous mutant flies die as late larvae and early pupae, heterozygous flies were tested. After a dose of 10 Gy, a decreased survival of 0- to 24-hr embryos was seen (see Figure 3). In contrast to most genes involved in DNA damage responses, increased radiation sensitivity was already manifest in heterozygous flies. The difference in hypersensitivity between *Su(Var)2-10[2]/Pm* and *Su(Var)2-10[2]/CyO* heterozygotes is most likely due to differences in the genetic background of *Pm* and *CyO* flies. At larval stages, no increased sensitivity was seen. Fly strains in which Rad54 or Ligase IV activity is abolished, also exhibit less severe radiation sensitivity at later stages of development (Gorski et al., 2003). The radiation-hypersensitivity seen in *Su(Var)2-10* heterozygous mutant flies indicates an important role for SUMO modification in signalling and/or repair of DNA damage. However, the role for SUMO in the repair of DSBs may be rather indirect. Alterations in chromosome architecture and organization in heterozygous *Su(Var)2-10* flies, as manifested by the PEV phenotype, may have serious effects on signalling and repair of damage and lead to reduced survival after irradiation.

Screening of two-hybrid libraries with Rad22A and Rad22B resulted in the identification of Hus5 as an interacting partner of both Rad52 homologs in *S. pombe*. Sumoylation of Rad22A *in vivo* has already been shown (Ho et al., 2001). Analysis of the Rad22A amino acid sequence revealed only one putative ψ KxE sumoylation consensus motif (C₁₃₄K₁₃₅K₁₃₆E₁₃₇). To test for sumoylation of

Rad22A at this position, various amino acid changes were introduced by PCR. However, overexpression of 135 K>R, 135 K>A, 136 K>R or 135,136 KK>RR alleles did not result in altered levels of complementation of *rad22rad22B* mutant cells as compared with the wildtype allele (see Figure 4). Apparently, SUMO conjugation of the lysine residue at position 135 is not strictly required for the role of Rad22A in HR. The possibility that Rad22A is sumoylated at other positions cannot be ruled out, neither can we exclude the possibility for a minor role of SUMO modification of Rad22A in HR, or a role in only certain types of recombination. The specific sumoylation of certain proteins in HR may be achieved by E3 ligases of the PIAS family. Two-hybrid studies demonstrated the interaction between proteins belonging to the RAD52 group and PIAS family homologs from various eukaryotes (Su(Var)2-10 with Rad51 in *Drosophila* (this study), PIASy with Mre11 in mice (C. Heyting and F. de Vries, unpublished results)). BLAST searches for PIAS family homologs in *S. pombe* revealed the Pli1 protein which is homologous to *S. cerevisiae* Siz1 (Ull1) and Siz2 (Nfi1). Recently, the *pli1*⁺ gene was also identified in a two-hybrid screen using the chromatin-associated switch-activating protein as a bait (Xhemalce et al., 2004). Disruption of the *pli1*⁺ gene did not result in increased ionizing radiation sensitivity (Figure 6). Possibly, Pli1 is not required for sumoylation of proteins involved in cellular responses to X-rays or its role is compensated by other SUMO ligases. Xhemalce and coworkers reported increased sensitivity of *pli1* mutant cells to the microtubule-destabilizing drug TBZ and enhanced loss of a minichromosome (Xhemalce et al., 2004).

Recently a second SUMO E3 ligase has been identified in *S. pombe*. Nse2, one of the components of the Smc5-6 complex, which is probably involved in recombinational repair mechanisms, contains an SP-RING motif and exhibits SUMO ligase activity (Andrews et al., 2005). Mutations in the SP-RING domain resulted in increased sensitivity to DNA damaging agents. Inactivation of the E3 ligase activity of Mms21, the *S. cerevisiae* homologue of Nse2, also leads to increased sensitivity to genotoxic agents as well as defects in nucleolar integrity and clustering of telomeres (Zhao and Blobel, 2005). Reduced survival after treatment with methyl methanesulfonate was observed in human cells after RNAi-mediated depletion of Mms21 (Potts and Yu, 2005). The phenotypical analysis of *Su(Var)2-10* and *mms21* mutants imply a crucial role for SUMO modification in maintaining proper chromosome architecture and nuclear organisation. Reduced levels of sumoylation lead to defects in the regulation of chromosome structure and functioning. Chromatin immunoprecipitation showed an enrichment of Hus5 in heterochromatin in *S. pombe* (Shin et al., 2005). In this study also the specific sumoylation of heterochromatin proteins was shown. Although the data presented here and those of others imply that SUMO modification of proteins involved in homologous recombination is probably important for the repair of DSBs (e.g. in the control of intracellular localization), sumoylation presumably also affects DSB-repair indirectly as a consequence of its essential role in maintaining proper chromosome structure and organization. Minor deviations in the spatial organization of chromosomes probably have

severe consequences for the efficacy of DNA repair mechanisms in preservation of the integrity of the genome.

Acknowledgements

The authors are grateful to Dr. Leon Mullenders for comments on the manuscript. This work was supported by the EU (RISC-RAD / FI6R-CT-2003-508842).

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Chapter 5

Mouse *Sycp1* functions in synaptonemal complex assembly, meiotic recombination, and XY body formation.

adapted from
Genes and Development, 2005, 19 (11):1376-1389

5 Mouse *Sycp1* functions in synaptonemal complex assembly, meiotic recombination and XY body formation

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Supplemental material is available at <http://www.genesdev.org>

Abstract

In meiotic prophase, synaptonemal complexes (SCs) closely appose homologous chromosomes (homologs) along their length. SCs are assembled from two axial elements (AEs), one along each homolog, which are connected by numerous transverse filaments (TFs). We disrupted the mouse gene encoding TF protein SYCP1 to analyze the role of TFs in meiotic chromosome behaviour and recombination. *Sycp1*^{-/-} mice are infertile, but otherwise healthy. *Sycp1*^{-/-} spermatocytes form normal AEs, which align homologously, but do not synapse. Most *Sycp1*^{-/-} spermatocytes arrest in pachynema, whereas a small proportion reaches diplonema, or, exceptionally, metaphase I. In leptotene *Sycp1*^{-/-} spermatocytes, γ H2AX (indicative of DNA damage, including double-strand breaks) appears normal. In pachynema, *Sycp1*^{-/-} spermatocytes display a number of discrete γ H2AX domains along each chromosome, whereas γ H2AX disappears from autosomes in wildtype spermatocytes. RAD51/DMC1, RPA and MSH4 foci (which mark early and intermediate steps in pairing/recombination) appear in similar numbers as in wildtype, but do not all disappear, and MLH1 and MLH3 foci (which mark late steps in crossing over) are not formed. Crossovers were rare in metaphase I of *Sycp1*^{-/-} mice. We propose that SYCP1 has a coordinating role, and ensures formation of crossovers. Unexpectedly, *Sycp1*^{-/-} spermatocytes did not form XY bodies.

Introduction

In meiosis, two rounds of chromosome segregation follow one round of replication. The first segregation, meiosis I, is reductional, as homologous chromosomes (homologs) move to opposite poles, whereas meiosis II is equational, because sister chromatids disjoin. The disjunction of homologs is prepared during the prophase of meiosis I, when homologs pair and non-sister chromatids of homologs recombine (reviewed by Zickler and Kleckner, 1999).

The resulting crossovers and cohesion between the sister chromatids connect the homologs and ensure their proper disjunction at meiosis I. In most analyzed eukaryotes, meiotic recombination is accompanied by the close apposition of homologs by a zipper like proteinaceous structure, the synaptonemal complex (SC). After premeiotic S-phase, the two sister chromatids of each chromosome develop a common axial structure, the axial element (AE), which consists of a linear array of protein complexes involved in sister chromatid cohesion (cohesin complexes), associated with various additional proteins (reviewed by Page and Hawley, 2004). Numerous transverse filaments (TFs) then connect the AEs of two homologs (synapsis) to form an SC. Within the SC, AEs are called lateral elements (LEs).

Genes encoding TF proteins have been identified in mammals (*Sycp1*), budding yeast (*ZIP1*), *Drosophila* (*c(3)G*) and *Caenorhabditis* (*Syp-1* and *Syp-2*). SYCP1, Zip1 and C(3)G are long coiled coil proteins with globular domains at both ends. Within SCs, they form parallel coiled-coil homodimers, which are embedded with their C-termini in the LEs, whereas the N-termini of TF protein molecules from opposite LEs overlap in the narrow region between the LEs of the two homologs. *Caenorhabditis* Syp-1 and Syp-2 are two short coiled coil proteins, which possibly take the place of a single longer coiled coil protein in other species (reviewed by Page and Hawley, 2004).

In the three species in which it has been analyzed, *Drosophila*, *Caenorhabditis* and yeast, TF-deficient mutants still initiate meiotic recombination, by induction of DNA double-strand breaks (DSBs) (Storlazzi et al., 1996; Colaiácovo et al., 2003; Jang et al., 2003), and align homologous chromosomes. However, they are deficient in crossover formation (reviewed by Zickler and Kleckner, 1999; and Page and Hawley, 2004). In *Caenorhabditis* and *Drosophila*, meiotic crossing over is abolished (Page and Hawley, 2001; MacQueen et al., 2002; Colaiácovo et al., 2003), whereas in yeast *zip1* null mutants, meiotic crossing over is reduced (Sym et al., 1993; Börner et al., 2004). Interestingly, Zip1 contributes to crossover formation even if no SC is assembled (Storlazzi et al., 1996), so not all functions of TF proteins in crossover formation require an intact SC structure; Storlazzi et al. (1996) proposed that Zip1 has a role in crossover designation before an SC is formed.

Börner et al. (2004) analyzed the crossover defect of yeast *zip1* null mutants in detail. In wildtype yeast meiosis, DSB ends are resected so that 3' single stranded tails arise (Sun et al., 1991). *zip1* mutants show wildtype levels and kinetics of DSB formation and resection, but coordinate defects in later steps, namely the formation of single end invasions (SEIs), double Holliday junctions (dHJs) and crossovers, which indicates that the progression from resected DSBs to SEIs is affected in *zip1* mutants. Because in wildtype yeast SEIs appear in late zygonema (Hunter and Kleckner, 2001), Zip1 fulfils its role in this step in crossover formation before an intact SC structure has been formed.

The relation between SC-formation and recombination implies more than a requirement of TF proteins for crossing over. Synapsis and recombination are interdependent in most species, and the interdependency differs between species. In yeast and mouse, but not *Drosophila*, disruption of genes involved in

meiotic DSB-formation, resection or strand invasion also affect synapsis, and most recombinational interactions in early meiosis probably serve to establish or stabilize homologue alignment and/or initiation of synapsis (reviewed by Hunter, 2003). Because of this interdependence it is important to analyze the localization of complexes of recombination related proteins relative to the SCs/AEs in TF-deficient mutants. Such complexes can be recognized immunocytochemically (Anderson et al., 1997; Moens et al., 2002). Upon immunofluorescent labelling they are visible as foci by light microscopy. The composition of foci changes as meiotic prophase proceeds, which most likely directly or indirectly reflects successive steps in homologue alignment and recombination (reviewed by Ashley and Plug, 1998).

The mouse provides excellent opportunities for studying the role of TFs in chromosome pairing and recombination by an immunocytological approach, because the cytology of mouse meiosis is very well developed, and successive stages of meiosis can be determined precisely (reviewed by Ashley, 2004). Furthermore, several SC components, including TF protein SYCP1 (Meuwissen et al., 1992; Sage et al., 1995) and many recombination-related proteins have been identified in the mouse, and the localization of these proteins in mouse meiosis has been studied in great detail (Heyting and Dietrich, 1991; Ashley and Plug, 1998; Moens et al., 2002). We disrupted the *Sycp1* gene, and analyzed the effect of the disruption on male meiosis, following an immunocytochemical approach. We focused on the state of the AEs and the formation of foci containing recombination-related proteins, in order to find out whether and how homologue alignment, meiotic recombination and SC assembly are affected in *Sycp1*^{-/-} mutants.

Materials and methods

Construction of the targeting vector

To inactivate the *Sycp1* gene, we designed a targeting construct to replace exons 2 to 8 by a neomycin gene, using pKO Scrambler V905 as a vector (Lexicon Genetics, Incorporated, The Woodlands, TX, USA). The neomycin phosphotransferase gene was isolated as an AscI fragment from pKO Select Neo (Lexicon Genetics) and inserted at the unique AscI site of pKO V905. The thymidine kinase gene was derived from pKO Select TK (Lexicon Genetics) by RsiII digestion and subcloned at the unique RsiII site of pKO V905. Genomic fragments were isolated after screening of a lambda FixII library derived from 129/Ola E14 cells (a gift of B. Vennström, Mouse Camp Transgene Facility, Karolinska Institute, Stockholm, Sweden). A 2.4 kb SalI (vector derived) – SacII fragment was used as a left arm. This fragment was first cloned in pGEM-T Easy (Promega), excised as a SalI-NotI fragment and cloned as a blunt fragment on the HpaI site of pKO V905. A 6 kb EcoRI fragment was used as the right arm and inserted in the EcoRI site of the targeting vector (Fig. 1). The final pKO plasmid mentioned above, containing all four elements, was linearized with SalI before electroporation.

Targeted inactivation of the Sycp1 gene

129/Ola derived IB10 ES cells (a subclone from E14 ES cells) were cultured on lethally irradiated mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Biocell Laboratories Inc., Rancho Dominguez, USA), 2 mM L-glutamate, 1 mM sodium pyruvate, non-essential amino acids, 0.1 mM β -mercaptoethanol, 10^3 U/ml leukemia inhibitory factor (LIF), penicillin (100 U/l) and streptomycin (100 μ g/l). 4×10^7 ES cells were resuspended in 300 μ l PBS, containing 65 μ g linearized targeting vector and electroporated at 800V and a capacitance of 3 μ F. Cells were seeded in five 9 cm dishes and after 24 and 72 h G418 (175 μ g/ml) and gancyclovir (1.3 μ g/ml) were added, respectively. Resistant colonies were isolated after 10 days of selection, expanded and genomic DNA was analyzed by PCR and blot-hybridization. The sequences of the upstream and downstream primers FW4 and neoFW3 are 5'-GGA TTG CAC GCA GGT TCT CC-3' and 5'-CAT ACA TGC CAC GGA GGA AG-3', respectively. Amplifications were performed using the Expand High Fidelity PCR system according to the manufacturer's instructions (Roche Applied Science, Basel, Switzerland). Primers were annealed at 60°C. Correct targeting results in a 3.8 kb PCR fragment, which was confirmed by blot-hybridization. We microinjected targeted ES cells into C57BL/6 blastocysts to generate chimeras, and chimeric males were mated with C57BL/6 females. To produce *Sycp1*^{-/-} mice, we intercrossed heterozygous offspring. We genotyped mice were genotyped by PCR on tail DNA using the primers scp40 (5'-CAT GCT CGA ACA GGT TAG TA-3'), scp41 (5'-GTG ACA ACT GCC AGA ATT AG-3'), neo7 (5'-CAT ACG CTT GAT CCG GCT C -3') and neo9 (5'-GAT GGC TGG CAA CTA GAA GG-3'). Scp40 and scp41 give a 382 bp fragment diagnostic of the wildtype *Sycp1* allele, while neo7 and neo9 give a 488 bp fragment diagnostic of the neomycin selectable marker. PCR conditions were 93°C for 1 min, 55°C for 1 min, 72°C for 2 min, for 35 cycles.

Western blot analysis

We prepared cell suspensions from testes of *Sycp1*^{-/-} and *Sycp1*^{+/-} mice (Heyting and Dietrich, 1991) and lysed the cells in Laemmli sample buffer. We loaded 5×10^5 lysed cells per 0.8-cm-wide slot of a 10% polyacrylamide gel, and separated the proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. After transfer of the proteins to nitrocellulose (Schleicher & Schuel) by electroblotting, we stained the resulting blots with Ponceau S and scanned them using an Agfa Snapscan 1212 flatbed scanner, before we probed them with antibodies. From each lane four strips were cut, which were each incubated in one of the anti-SYCP1 antisera, and then in secondary (antirabbit) antibodies conjugated to alkaline phosphatase (AP) (Promega), as described (Offenberg et al., 1998).

Histological analysis and TUNEL assay

Animals were killed by cervical dislocation. Testes, epididymides and seminal vesicles, or ovaries and uterus were examined and weighted. From each male, we fixed one testis and epididymis in Bouin's fixative for 24 h at room

temperature, and the other testis in phosphate-buffered formalin for 24 h at 4°C. Subsequently, organs were embedded in paraffin. Mounted sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. For TUNEL analyses, formalin-fixed sections were mounted on glass slides coated with a 2% solution of 3-aminopropyltriethoxysilane in acetone, deparaffinized and pre-treated with proteinase K (Sigma) and peroxidase (Gavrieli et al., 1992). Slides were subsequently washed in terminal deoxynucleotidyl transferase (TdT) buffer (100 mM cacodylate buffer, 1 mM CaCl₂, 0.1 mM dithiothreitol, pH 6.8) for five minutes (Gorczyca et al., 1993) and incubated for at least 30 minutes at 25°C in TdT buffer containing 0.01 mM Biotin-16-dUTP (Roche Diagnostics) and 0.4 U/μl TdT-enzyme (Promega, Wisconsin, MD, USA). The enzymatic reaction was stopped in TB-buffer (300 mM NaCl, 30 mM Na-citrate, pH 7.0), and the sections were washed (Gavrieli et al., 1992). Slides were then incubated with streptABComplex/horseradish peroxidase conjugate (Dako, Carpinteria, CA, USA) for 30 min and washed in PBS. dUTP-biotin labelled cells were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB)/metal concentrate (Pierce, Rockford, USA). Then we counterstained the sections with hematoxylin and counted the number of TUNEL-positive cells per cross-sectioned tubule. However, for *Sycp1*^{-/-} mice this was not possible, because numerous TUNEL-positive nuclei were clustered in single cross sectioned tubules. Therefore we counted the number of cross sectioned tubules with no, one to five, or more than five apoptotic nuclei. Tubules without germ cell development up to meiotic prophase were excluded from the analysis. We performed this analysis on two *Sycp1*^{+/-} and two *Sycp1*^{-/-} mice, classifying a minimum of 150 tubule sections for each genotype.

Cytology, immunocytochemistry and chromosome painting

The antibodies used in this study are listed in the Supplementary information. Paraffin and frozen sections of mouse testis (Meuwissen et al., 1992), and dry-down (Peters et al., 1997) or squash (Page et al., 1998) preparations of testis cell suspensions were prepared, incubated for immunocytochemistry and analyzed as described (Meuwissen et al., 1992; Eijpe et al., 2003). In some experiments, we exposed the cells to 1.25 μM okadaic acid for 5 h (Wiltshire et al., 1995) before spreading. For ultrastructural analysis we prepared uranyl-acetate-stained agar filtrates of lysed spermatocytes, and analyzed them as described (Heyting and Dietrich, 1991).

Results

Targeted inactivation of Sycp1

We disrupted the mouse *Sycp1* gene, using a targeting vector in which exon 2 to exon 8 of the gene had been replaced by a neomycin selection marker. The replaced sequence includes the splice donor sequence of intron 1, the ATG start codon in exon 2 and approximately 20% of the *Sycp1* ORF (Fig. 1A). The targeting vector was linearized and electroporated into embryonic stem (ES) cells. We tested approximately 600 neomycin and gancyclovir resistant ES cell

clones by PCR for correct targeting. About 2% of the clones tested contained the disrupted *Sycp1* allele. Correct targeting was confirmed by Southern blot analysis (Fig. 1B). We injected targeted ES clones into C57BL/6 blastocysts and obtained germ line transmitting chimeric animals. Intercrosses of *Sycp1*^{+/-} animals yielded *Sycp1*^{+/+}, *Sycp1*^{+/-} and *Sycp1*^{-/-} offspring in the expected Mendelian ratio. The *Sycp1*^{-/-} mice are viable and don't display obvious developmental defects. Antibodies against peptides covering the N-terminal, middle or C-terminal part of SCP1 (the rat protein homologous to SYCP1), or against nearly full-length SCP1 did not bind to any proteins in testis cell extracts from *Sycp1*^{-/-} mice (Fig. 1C), indicating that these mice do not express truncated SYCP1. Most likely the *Sycp1* disruption equals a null mutation.

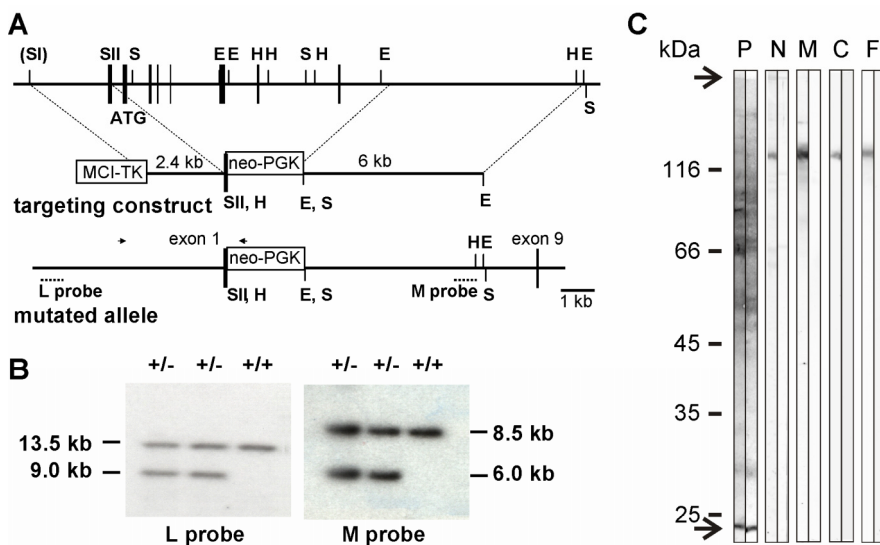


Figure 1: Targeted inactivation of mouse *Sycp1*.

(A) Structure of the targeted region of the wildtype *Sycp1* gene with exons 1-8 (top), targeting vector (center), and targeted allele (bottom). The ATG start codon is located in exon 2. Solid boxes indicate exons. Targeted integration results in a deletion including the 3'-end of exon 1 and exons 2-8. E, EcoRI; B, BglII; H, HindIII; S, SphI; SII, SacII. The SalI site indicated between brackets was derived from the lambda phage vector. Arrows indicate the primers used for screening for correctly targeted clones. **(B)** Blot-analysis of DNA from wildtype (+/+) and heterozygous (+/-) ES cells digested with HindIII (left) and SphI (right) and hybridized with the L probe and the M probe, respectively. The wildtype 13.5 kb HindIII fragment is replaced by a 9.0 kb fragment in the mutant and the 8.5 kb SphI fragment from the wildtype allele by a 6.0 kb fragment. **(C)** Western blot analysis of testis cell extracts from *Sycp1*^{-/-} mice. Strips carrying proteins from testis cell extracts from heterozygous mice (left in each pair of strips) or *Sycp1*^{-/-} mice (right strip) were probed with antibodies against the N-terminal (N), middle (M), or C-terminal part (C) of SCP1 (the rat protein homologous to SYCP1), or against nearly full length SCP1 (F). (P) Ponceau S-stained strips. Arrows indicate the the top of the gel and the electrophoresis front. (kDa) molecular mass in kilodaltons.

Sycp1^{-/-} mice are infertile

Whereas *Sycp1*^{+/-} mice were fully fertile, repeated breeding attempts of *Sycp1*^{-/-} males and females with wildtype animals did not yield any offspring. If the same wildtype animals were mated with heterozygous (*Sycp1*^{+/-}) males or females, pregnancy was readily achieved. *Sycp1*^{-/-} testes and ovaries were much smaller than those of *Sycp1*^{+/-} or wildtype mice (shown for testis in Fig. 2K), and *Sycp1*^{-/-} testes weighted on average 70% less than wildtype testes. Spermatozoa were lacking in epididymides of *Sycp1*^{-/-} knockout males (not shown). SYCP1 is thus required for correct development of the reproductive organs and for male and female fertility.

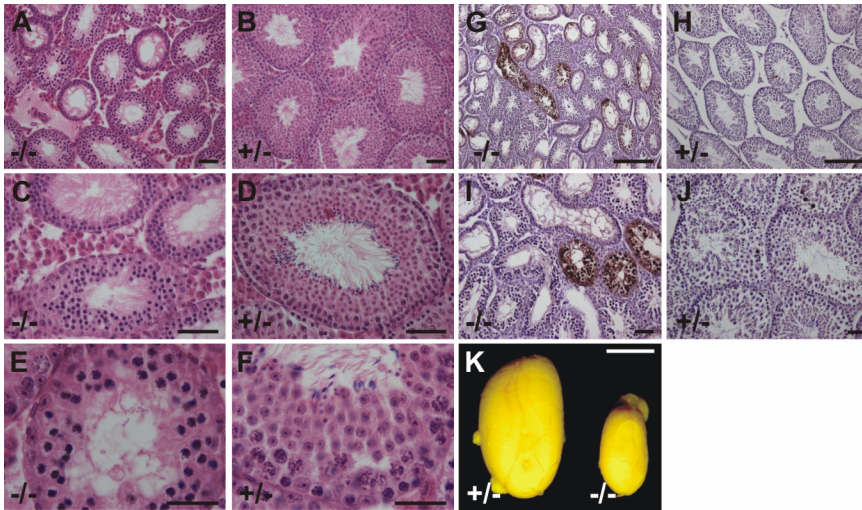


Figure 2: Morphology, histology and TUNEL analysis of testes from *Sycp1*^{-/-} mice.

The histological sections were stained with haematoxylin and eosin. **(A-F)** Testicular histology of adult *Sycp1*^{-/-} (-/-, **A,C,E**) and *Sycp1*^{+/-} (+/-, **B,D,F**) mice. Note the total absence of postmeiotic germ cells in *Sycp1*^{-/-} sections. Pachytene nuclei are abundant, but show aberrant nuclear morphology. **(G-J)** TUNEL analysis of testis sections of *Sycp1*^{-/-} (-/-, **G,I**) and *Sycp1*^{+/-} (+/-, **H,J**) mice. Tubule sections with numerous TUNEL-positive nuclei occur only in *Sycp1*^{-/-} mice. A few apoptotic nuclei are visible in tubule sections from *Sycp1*^{+/-} mice. **(K)** Testes from *Sycp1*^{+/-} (+/-) and *Sycp1*^{-/-} (-/-) mice. Bars: **(A-D,I,J)** 50 µm; **(E-F)** 25 µm; **(G-H)** 100 µm; **(K)** 2 mm. **See appendix for a colour version of this figure.**

Histological analysis of the gonads revealed various abnormalities. As is explained in detail in the Supplementary information, the mouse testis is organized in seminiferous tubules, in which cells differentiate coordinately. The tubules from *Sycp1*^{-/-} mice were much smaller than those from wildtype (Fig. 2A-F). They contained spermatogonia and spermatocytes, which appeared normal with respect to the presence of AE/LE proteins SYCP2 and SYCP3 (see below and Supplementary information), but the morphology of their nuclei was often abnormal. Furthermore, spermatocyte stages beyond diplotene were rare

in *Sycp1*^{-/-} testes, and post-meiotic spermatogenic cells (spermatids and spermatozoa) were completely lacking. Apparently, spermatogenic differentiation is interrupted predominantly at the pachytene stage of *Sycp1*^{-/-} spermatocytes, which most likely causes the sterility of *Sycp1*^{-/-} males. However, as has been found in other mouse meiotic mutants, the organization of the seminiferous tubules was not disrupted and the residual spermatocytes in *Sycp1*^{-/-} mice formed associations with similar cell types (except spermatids) as the corresponding spermatocytes in wildtype (Supplementary information). *Sycp1*^{-/-} ovaries weighted on average 35% less than *Sycp1*^{+/-} or *Sycp1*^{+/+} ovaries. Growing follicles and oocytes were lacking in sections of *Sycp1*^{-/-} ovaries, which suggests a disruption of oocyte development during meiosis, followed by apoptosis.

SYCP1-deficiency leads to increased apoptosis during pachynema

One possible explanation for the lack of spermatids in *Sycp1*^{-/-} testes is, that spermatogenic cells enter apoptosis during meiotic prophase (reviewed by de Rooij and de Boer, 2003). We tested this using TUNEL analysis of testis sections from approximately 8-week-old *Sycp1*^{+/-} and *Sycp1*^{-/-} mice. In *Sycp1*^{+/-} testes, we found on average 0.7 apoptotic nuclei per cross-sectioned tubule, which is slightly more than previously found in wildtype (Baarends et al., 2003). In wildtype, the TUNEL positive cells were most often in pachynema or metaphase/anaphase I (Fig. 2G-J). In testis sections of *Sycp1*^{-/-} but not of *Sycp1*^{+/-} animals, certain tubules contained many (10 or more) apoptotic nuclei. Accordingly, the percentage of tubule sections with 5 or more apoptotic nuclei had almost doubled in *Sycp1*^{-/-} compared to *Sycp1*^{+/-} testes (20% versus 11%). Because the percentage of tubules without apoptotic nuclei had not changed, we think that apoptosis occurs at similar developmental steps in *Sycp1*^{-/-} animals as in wildtype, but with a highly increased incidence, resulting in *Sycp1*^{-/-} tubules containing a whole layer of apoptotic nuclei. Thus, in *Sycp1*^{-/-} males, spermatogonia enter meiotic prophase, but most spermatocytes die of apoptosis at pachynema, and exceptionally some get to metaphase I.

AEs are formed in the absence of SYCP1 and align homologously

Sycp1^{-/-} spermatocytes assemble morphologically normal AEs (Fig. 3), which align homologously (Fig. 3 and Fig. S1), but are not connected by transverse filaments, and do not show a central element between them, i.e., they do not synapse (Fig. 3B). In *Sycp1*^{-/-} spermatocytes, AEs are only connected by a limited number of axial associations (AAs) (Fig. 3A,B), and are further apart (211±17 nm at AAs) than the LEs in pachytene spermatocytes of wildtype (in agar filtrates: 79±3 nm). This resembles the yeast *zip1* phenotype (Sym et al., 1993), including the size of the AAs, and is consistent with the idea that SYCP1 is a transverse filament component. Beyond the most centromere proximal and distal AAs, the AEs tend to be somewhat wider apart, so the AAs are the only or at least the shortest connections between the AEs. All analyzed components of wildtype mouse AEs/LEs were also present in *Sycp1*^{-/-} AEs (Fig. 3; for SYCP2 see Fig. 4).

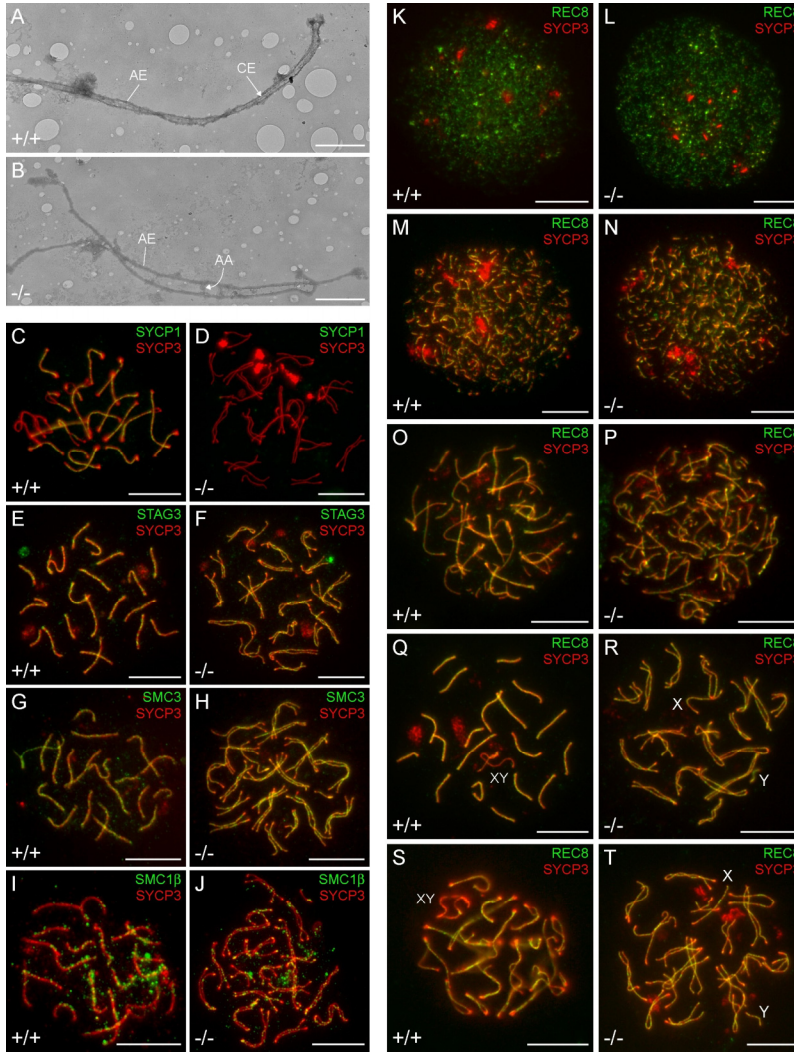


Figure 3: Assembly of AEs in *Sycp1*^{-/-} mice.

(A-B) Electron micrographs of AEs and SCs from wildtype (+/+) and *Sycp1*^{-/-} (-/-) male mice; **(A)** wildtype SC with closely apposed axial elements (AE) and a central element (CE); **(B)** homologically aligned axial elements (AE) from a *Sycp1*^{-/-} spermatocyte, connected by axial associations (AA). **(C-J)** Components of AEs and SCs in wildtype (+/+) and *Sycp1*^{-/-} (-/-) diplotene **(C-D)** or pachytene **(E-J)** spermatocytes; LE/AE protein SYCP3 and all analyzed cohesins are present in LEs/AEs of wildtype and mutant, whereas SYCP1 is not detectable in mutant spermatocytes. **(K-T)** formation of AEs/LEs, as shown by REC8/SYCP3 double labelling, in wildtype (+/+) and *Sycp1*^{-/-} (-/-) spermatocytes; **(K,L)** early leptotema; **(M,N)** late leptotema; **(O,P)** zygonema; **(Q,R)** pachynema; **(S,T)** diplonema; note the XY bivalent (XY) in wildtype cells **(Q,S)**, and separate X and Y chromosomes in the *Sycp1*^{-/-} cells **(R,T)**. Bars in **(A-B)** 1 µm; bars in **(C-T)** 10 µm. **See appendix for a colour version of this figure.**

In the Supplementary information we present evidence that the order of *Sycp1*^{-/-} and wildtype spermatocyte stages, as defined by AE morphology and extent of alignment/synapsis, is the same, and that corresponding *Sycp1*^{-/-} and wildtype stages have similar life spans, at least until the spermatocytes enter apoptosis. We use therefore AE morphology and alignment/synapsis as criteria for staging and comparing *Sycp1*^{-/-} and wildtype spermatocytes. The assembly and alignment of AEs, as detected by REC8/SYCP3 double labelling, proceeds similarly in *Sycp1*^{-/-} and wildtype (Fig. 3K-T). The pseudo-autosomal parts of the X and Y chromosome however, were not aligned in 28% of the *Sycp1*^{-/-} pachytene cells, whereas they were synapsed in 100% of the wildtype pachytene cells (examples shown in Figs. 3 and 4). Although most *Sycp1*^{-/-} spermatocytes are lost during pachynema (above), some reach diplonema: 0-3% (depending on the mouse) of the spermatocytes in spreads of *Sycp1*^{-/-} testis cell suspensions were in diplonema, compared to 15% of the spermatocytes in spreads of wildtype testis cell suspensions (late meiotic prophase stages are overrepresented in cell suspensions). Diplotene *Sycp1*^{-/-} AEs resemble wildtype LEs/AEs, including the thickened ends and the apparent repulsion of the LEs/AEs of homologous chromosomes (Fig. 3T).

Meiotic recombination is initiated in Sycp1^{-/-} spermatocytes, but repair is not completed

γ H2AX is a phosphorylated form of histone variant H2AX, which marks chromatin domains with DNA damage, including DSBs (Rogakou et al., 1999). γ H2AX appeared throughout *Sycp1*^{-/-} preleptotene and leptotene nuclei (Fig. 4F), as in wildtype (Fig. 4A) (Mahadevaiah et al., 2001). However, whereas γ H2AX becomes largely restricted to asynapsed portions of wildtype zygotene chromosomes (Fig. 4B) (Mahadevaiah et al., 2001), it occurs all along the AEs, including the aligned portions, of *Sycp1*^{-/-} zygotene chromosomes (Fig. 4G). The intensity of γ H2AX labelling along the *Sycp1*^{-/-} bivalents varied somewhat, but in most zygotene cells we could not distinguish separate γ H2AX positive domains (Fig. 4G and 5N). This pattern changed in *Sycp1*^{-/-} pachynema: some *Sycp1*^{-/-} pachytene cells displayed a mixture of long stretches of γ H2AX and narrow, intense γ H2AX positive domains, and other pachytene cells (presumably of a later stage) showed only narrow, γ H2AX positive domains along otherwise γ H2AX negative bivalents (Fig. 4H). Diplotene *Sycp1*^{-/-} spermatocytes displayed only the latter pattern (Fig. 4I). Late pachytene/early diplotene *Sycp1*^{-/-} spermatocytes, contained 110 ± 4.6 distinct, narrow γ H2AX positive domains per cell. In earlier spermatocyte stages the γ H2AX positive domains were too indistinct and heterogeneous to be counted. In wildtype, we found only distinct, narrow γ H2AX positive domains along synapsed stretches in late zygonema and early pachynema (Fig. 4B,C), and these domains were weakly labelled and disappeared during the course of pachynema (Fig 4 D) (Mahadevaiah et al., 2001). Taken together, the γ H2AX pattern suggests that meiotic DSBs are formed in *Sycp1*^{-/-} meiosis, but that least some DSBs are not repaired, or their repair gets stuck at some intermediate step that is still marked by γ H2AX. Another abnormality in the γ H2AX pattern was found on the sex chromosomes:

wildtype pachytene and diplotene spermatocytes have γ H2AX throughout the chromatin of the XY body (Fig. 4C-E) (Mahadevaiah et al., 2001) (a condensed chromatin structure containing the sex chromosomes formed during male meiotic prophase in mammals). In striking contrast, the sex chromosomes of *Sycp1*^{-/-} pachytene and diplotene spermatocytes displayed similar narrow γ H2AX positive domains as the autosomes (Fig. 4H,I).

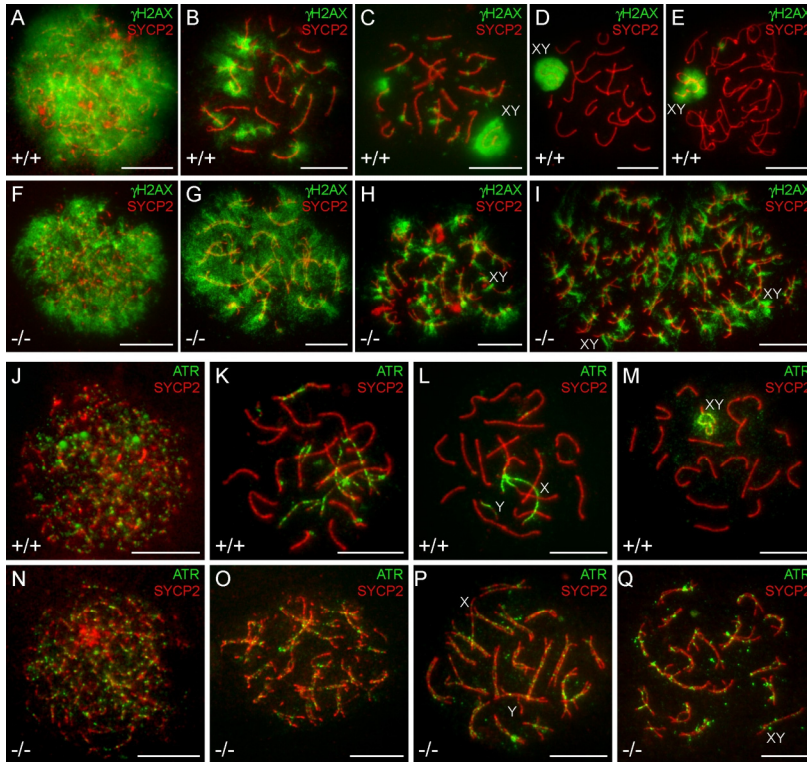


Figure 4: γ H2AX and ATR in wildtype (+/+) and *Sycp1*^{-/-} (-/-) spermatocytes. **(A-I)** γ H2AX ; **(A,F)** leptotema; **(B,G)** zygotema; **(C)** early pachynema; **(D,H)** mid-pachynema; **(E,I)** diplotema; the sex chromosomes (XY) form an XY-body in wildtype spermatocytes **(C-E)**, but not in *Sycp1*^{-/-} spermatocytes, even though the X and Y chromosomes are associated in the cells in **(H)** and **(I)**. **(J-Q)** ATR; **(J,N)** leptotema; **(K,O)** zygotema; **(L)** early pachynema and **(M)** and **(P)** mid-pachynema; **(Q)** diplotema; ATR is present throughout the chromatin of the XY bivalent in wildtype spermatocytes **(M)**, but forms foci and distinct domains along the X and Y chromosomes in *Sycp1*^{-/-} cells **(P-Q)**. Insets in **(J)** and **(N)** show the close association of ATR with the ends of AE fragments in wildtype (+/+) and *Sycp1*^{-/-} leptotema. Bars 10 μ m. **See appendix for a colour version of this figure.**

We also analyzed the putative H2AX phosphorylating kinase ATR (Turner et al., 2004). In leptotema of wildtype mouse ATR forms foci in association with AE segments (Fig. 4J), and in early zygotema, ATR foci occur along synapsed and

asynapsed portions of LEs/AEs (Fig. 4K). From mid-zygonema to early pachynema, ATR disappears from synapsed portions of SCs and accumulates along the non-autosomal parts of the XY bivalent and late pairing ("laggard") portions of autosomal LEs/AEs (Fig. 4L) (Turner et al., 2004 and references therein). In *Sycp1*^{-/-} leptonema and early zygonema, the ATR pattern was indistinguishable from wildtype (Fig. 4N). However, whereas the ATR signals disappeared from the synapsed portions of AEs in wildtype, they were present along the aligned AEs in *Sycp1*^{-/-} spermatocytes, usually in AE-associated foci, or incidentally in distinct domains that were reminiscent of the γ H2AX domains (Fig. 4P,Q). The dense ATR coating of laggard asynapsed portions of AEs as is found in wildtype (Fig. 4L) was not found in *Sycp1*^{-/-} spermatocytes. Strikingly, ATR shows the same aberrant pattern on the X and Y chromosome in *Sycp1*^{-/-} pachynema as γ H2AX: it forms few, discrete foci, or occasionally domains, on the AEs of the X and Y chromosome rather than covering all non-pseudo-autosomal parts of the AEs of the sex chromosomes (Fig. 4P,Q). In short, the ATR pattern in *Sycp1*^{-/-} spermatocytes differs in various respects from that in wildtype, but the similarity of the ATR and γ H2AX patterns found in wildtype (Turner et al., 2004), is also found in *Sycp1*^{-/-} spermatocytes (Fig. 4).

Sycp1^{-/-} spermatocytes barely form crossovers

To find out which step in meiotic recombination could be blocked in *Sycp1*^{-/-}, we analyzed proteins involved in later steps of meiotic recombination. Rad51 and Dmc1 are RecA homologs required for heteroduplex formation in meiosis, probably by assembling on 3' tails of resected DSB ends and initiating the strand invasion step (reviewed by Shinohara and Shinohara, 2004). In wildtype mouse, RAD51/DMC1 foci are formed along the AEs from leptonema on. In zygonema, they are located along synapsed and asynapsed portions of SCs, and in pachynema they gradually disappear (Fig. 5A,B) (Ashley and Plug, 1998). In *Sycp1*^{-/-} leptonema, RAD51/DMC1 foci appeared in similar numbers as in wildtype, but their number decreased more slowly (Fig. 5C,S; Fig. S4). Even late pachytene/early diplotene *Sycp1*^{-/-} spermatocytes displayed appreciable numbers of RAD51/DMC1 foci (Fig. 5D,S). 30-50% of the RAD51/DMC1 foci were between the aligned AEs in *Sycp1*^{-/-} late zygonema and late pachynema/early diplonema (Fig. 5D and Fig. S4). RAD51/DMC1 foci between aligned AEs occur also in wildtype mouse, maize and *Sordaria* (reviewed by Zickler and Kleckner, 1999; Tessé et al., 2003). Because homologous alignment requires DSBs (Tessé et al., 2003), the RAD51/DMC1 foci between aligned AEs might mark recombinational interactions between homologs. Part of the RAD51/DMC1 foci in *Sycp1*^{-/-} pachytene cells co-localize with γ H2AX domains (Fig. 5Q,R).

RPA binds to single-stranded DNA, and *in vitro* it enhances nucleoprotein formation by RAD51 if added to the reaction mixture after RAD51 (Pâques and Haber, 1999). In wildtype spermatocytes, RPA foci appear and disappear on average later than RAD51/DMC1 foci (Fig. 5E,F,S; Fig. S4) (Moens et al., 2002).

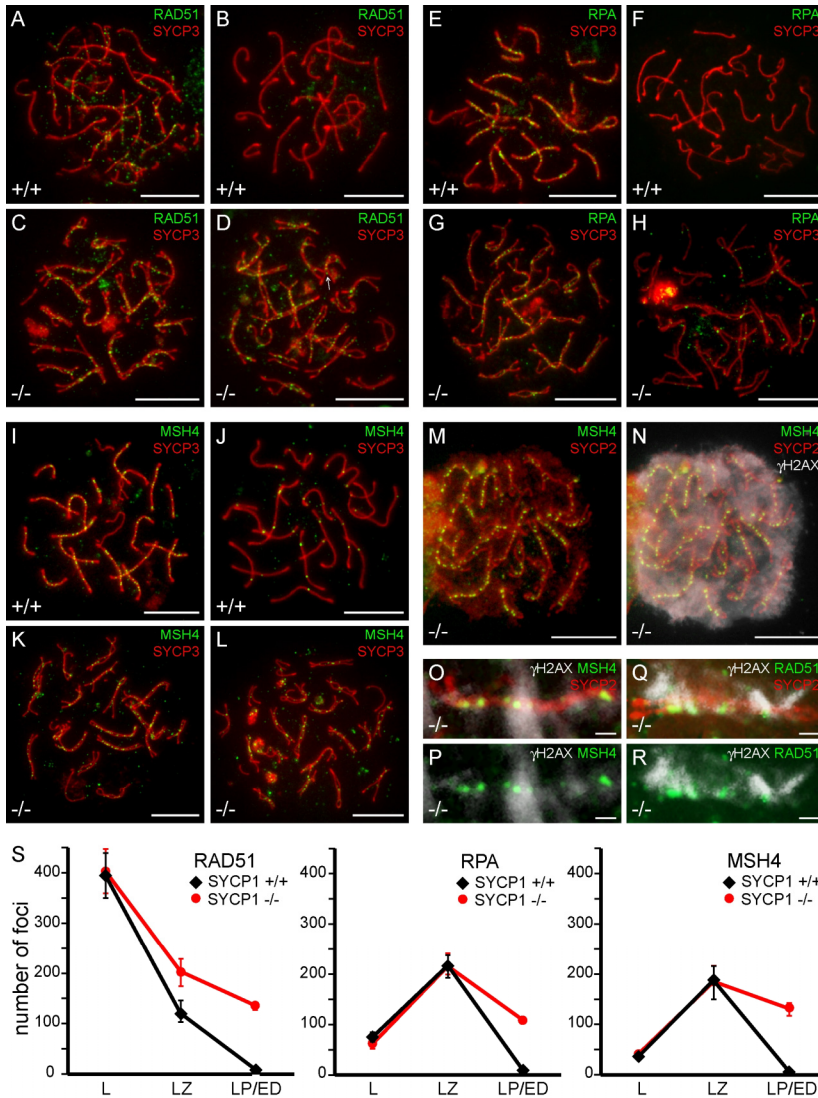


Figure 5: Recombination-related proteins along AEs and SCs in wildtype (+/+) and *Sycp1*^{-/-} (-/-) spermatocytes.

(A-D) RAD51/DMC1; **(A,C)** late zygonema; **(B,D)** late pachynema. **(E-H)** RPA; **(E,G)** late zygonema; **(F,H)** diplonema. **(I-L)** MSH4; **(I,K)** late zygonema; **(J)** mid-pachynema; **(L)**, diplonema. **(M-N)** MSH4/SYCP2/ γ H2AX triple labelling of a zygotene *Sycp1*^{-/-} spermatocyte; the number and localization of MSH4 foci appears normal, but the persistence of γ H2AX throughout the chromatin is abnormal. **(O-P)** MSH4/ SYCP3/ γ H2AX triple labelling of a late pachytene *Sycp1*^{-/-} bivalent, to show that part of the γ H2AX domains co-localize with an MSH4 focus. **(Q-R)** RAD51/SYCP2/ γ H2AX triple labelling of a late pachytene *Sycp1*^{-/-} bivalent, to show that part of the γ H2AX domains co-localize with a RAD51 focus. **(S)** Counts of RAD51, RPA and MSH4 foci in successive stages of meiotic

prophase; the vertical axes represent the number of AE or SC associated foci per cell; the vertical bars represent the observed range of the number of foci per cell in a given spermatocyte stage. For more details of the counts, see Supplementary Information, Fig. S4. Bars in (A-N) 10 μm ; bars in (O-R) 1 μm . **See appendix for a colour version of this figure.**

In *Sycp1*^{-/-} leptotene and late zygotene spermatocytes, the number of RPA foci and their time of appearance in relation to alignment/synapsis were similar as in wildtype zygonema (Fig. 5G,S; Fig. S4). However, *Sycp1*^{-/-} diplotene spermatocytes still have appreciable numbers of RPA foci (Fig. 5H,S; Fig. S4). About 80% of the RPA foci were located between two aligned AEs of *Sycp1*^{-/-} spermatocytes (Fig. 5G; Fig. S4). RPA foci between aligned AEs occur also in wildtype zygonema (Fig. 5G), and between homologously aligned but not synapsed LE/AE-segments between translocation breakpoints in *Sycp1*^{+/+} pachynema (Plug et al., 1997).

Msh4 is a MutS homologue, which forms a heterodimeric complex with another MutS homologue, Msh5. The Msh4/Msh5 heterodimer probably recognizes and stabilizes meiotic recombination intermediates (Ross-Macdonald and Roeder, 1994; Snowden et al., 2004). Yeast Msh4 localizes to sites of synapsis initiation. *msh4* mutants show partial and delayed synapsis and 30-50% of the wildtype level of crossing over, and *msh4* mutations affect the same subset of crossovers as *zip1* mutations (Novak et al., 2001). In mouse, MSH4 foci colocalize extensively with RPA foci, but appear and disappear slightly later (Moens et al., 2002). Otherwise than in yeast, the number of MSH4 foci in mouse far exceeds the number of chiasmata that will be formed (Fig. 5I,S; Fig. S4). Neyton et al. (2004) proposed that in mouse meiosis, MSH4 cooperates first in zygonema with RAD51/DMC1 in homologue alignment, synapsis initiation and/or in resolution of early DNA-DNA interactions, and subsequently, in pachynema, with MLH1 and MLH3 in crossover formation. In *Caenorhabditis*, MSH-4 and MSH-5 appear to fulfill only this second role: *msh-4* or *msh-5* mutants align homologs and assemble SC, but RAD-51 foci persist and crossovers are not formed, which suggests a role for MSH-4/5 downstream RAD-51 in crossover formation in *Caenorhabditis* (Colaiácovo et al., 2003). In mouse, *Sycp1*^{-/-} leptotene and zygotene spermatocytes display similar numbers of MSH4 foci as wildtype spermatocytes (Fig. 5K,M,S; Fig. S4). Most *Sycp1*^{-/-} MSH4 foci are between aligned AEs, indicating that they mark DNA interactions between homologs. However, in *Sycp1*^{-/-} late pachynema/early diplonema, the number of MSH4 foci is still 70% of that in late zygonema (Fig. 5L,S; Fig. S4). This might suggest that the DNA-DNA interactions to which MSH4 binds are formed normally in *Sycp1*^{-/-}, but that most of these cannot be processed.

MLH1 is essential for crossover formation, both in mammals and yeast (Baker et al., 1996; Hunter and Borts, 1997). In mouse, MLH1 foci appear in mid-pachynema, and their position and number closely correlate with those of chiasmata (Froenicke et al., 2002). MLH3 foci largely co-localize with MLH1 foci in the mouse (Svetlanov and Cohen, 2004), and MLH3 most probably cooperates with MLH1 in crossover formation (Wang et al., 1999; Lipkin et al., 2002).

Sycp1^{-/-} spermatocytes do not form MLH1 and MLH3 foci (Fig. 6), which indicates that SYCP1 is required for crossover formation. Accordingly, we observed only univalents in the two natural metaphases I that we found among spread spermatocytes of *Sycp1*^{-/-} mice (Fig. 6E). If we forced pachytene or diplotene *Sycp1*^{-/-} spermatocytes to condense their chromosomes, using okadaic acid (OA), most chromosomes formed univalents (Fig. 6F). Chromosomal fragments were rare in natural metaphase I or OA-induced metaphase I-like configurations of *Sycp1*^{-/-} (Fig. 6E,F).

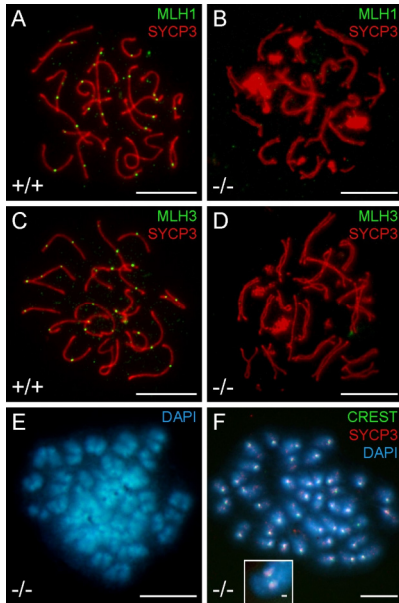


Figure 6: Formation of crossovers and chiasmata.

(A,B) MLH1 labelling and **(C,D)** MLH3 labelling of wildtype (+/+) or *Sycp1*^{-/-} (-/-) pachytene spermatocytes. The *Sycp1*^{-/-} spermatocytes do not assemble MLH1 or MLH3 foci. **(E,F)** A natural **(E)** and an okadaic acid-induced **(F)** metaphase I spermatocyte of *Sycp1*^{-/-}. In the cells shown here, only univalents can be identified; the inset in **(F)** shows a bivalent found in another OA-induced *Sycp1*^{-/-} metaphase I. Bars in **(A-F)** 10 μ m; bar in the inset in **(F)** 1 μ m. **See appendix for a colour version of this figure.**

Taken together, the immunofluorescence labelling of foci suggests that *Sycp1*^{-/-} spermatocytes can initiate meiotic recombination at wildtype level and establish stable homologous alignment of autosomes. However, many repair/recombination intermediates are not repaired and crossovers are not formed.

Sycp1^{-/-} spermatocytes do not form XY bodies

In 28 % of the *Sycp1*^{-/-} pachytene spermatocytes, the X and Y chromosome were associated, but this did not ensure formation of an XY body. In *Sycp1*^{-/-} pachynema, γ H2AX and ATR occurred in a similar discrete pattern along the AEs

of the XY bivalent as along autosomal AEs (Fig. 4I,P,R) (rather than covering the non-autosomal parts of the sex chromosomes), the characteristic DAPI-intense domain of the XY body was not formed, and the AEs of the X and Y chromosomes were not curled or bent, as is usually seen in wildtype XY bodies (compare Fig. 4D,E with Fig. 4H,I and Fig. 4Q). We will analyze the XY bivalent in *Sycp1*^{-/-} spermatocytes in more detail in a separate study.

Discussion

In this study we disrupted the mouse *SYCP1* gene and analyzed the effect on meiotic recombination and chromosome behavior, by an immunocytochemical approach. We will assume that immunofluorescence signals represent functional protein complexes and that orthologous proteins fulfill similar roles in mouse and yeast meiosis, unless there are indications that this is not so. In addition, we will have to make assumptions when and how the proteins act that we detect by immunofluorescence, to link the cytological observations in *Sycp1*^{-/-} mice to studies at the DNA and cytological level in other organisms.

Early meiosis in Sycp1^{-/-} spermatocytes

We used AE morphology as detected by SYCP2 or SYCP3 labelling and alignment/synapsis as a basis for staging (Fig. 3; Fig. S3). *Sycp1*^{-/-} and wildtype spermatocyte stages that correspond by these criteria also show similar patterns of cohesins (shown for REC8 in Fig. 3K-T), but display largely different patterns of recombination related proteins other than cohesins. Leptonema is the only analyzed stage in which *Sycp1*^{-/-} and wildtype spermatocytes show similar immunofluorescence patterns of all analyzed proteins: the γ H2AX and ATR patterns are similar, and RAD51/DMC1, RPA and MSH4 foci occur in similar numbers in wildtype and *Sycp1*^{-/-} leptonema, indicating that DSBs are induced and that some post-DSB step(s), presumably at least resection of DSB ends (Xu et al., 1997) take place at wildtype levels in *Sycp1*^{-/-} leptonema. However, differences (other than synapsis) between *Sycp1*^{-/-} and wildtype become apparent between leptonema and zygonema, in particular with respect to γ H2AX and ATR (Fig. 4K). Furthermore, the number of RAD51/DMC1 foci has decreased between leptonema and zygonema, but less so in *Sycp1*^{-/-} than in wildtype (Fig. 5S; Fig. S4). The numbers of RPA and MSH4 foci on the other hand are similar in late zygonema in mutant and wildtype (Fig. 5S; Fig. S4). In wildtype, RPA and MSH4 foci most likely arise from RAD51/DMC1 foci, and then lose RAD51/DMC1 (Moens et al., 2002). Although the RAD51/DMC1 foci occur in similar numbers per cell as MSH4 and RPA foci in *Sycp1*^{-/-} late zygonema (Fig. 5S), they cannot completely overlap with these foci, because more than 80% of the MSH4 and RPA foci are between the aligned AEs, but only 46% of the RAD51/DMC1 foci (Fig. S4). Possibly MSH4 foci are normal in *Sycp1*^{-/-} late zygonema, but some repair pathway is affected that is marked by RAD51/DMC1 but not by MSH4 foci. However, as the turnover of MSH4 and RAD51/DMC1 foci is not known, other explanations are conceivable.

Important questions about the immunofluorescence signals in zygonema are: what does the γ H2AX signal throughout the *Sycp1*^{-/-} nuclei indicate? And what do the MSH4 foci represent? In yeast, the number of Msh4 foci per cell roughly equals the number of Zip1-dependent crossovers per cell (Novak et al., 2001), and it seems likely that most or all MSH4 foci in yeast mark sites of future crossovers. In mammals and *Arabidopsis*, the number of MSH4 foci exceeds the number of crossovers by far, so in these organisms most MSH4 foci will not become crossovers, but mark possibly recombinational interactions that serve homologue alignment (Higgins et al., 2004; Neyton et al., 2004); the MSH4 images of late zygotene cells (Fig. 5K,M and Fig. S4) suggest that such interactions occur normally in *Sycp1*^{-/-}. An ensuing question is whether there are any crossover-designated MSH4 foci at all among the MSH4 foci in mouse zygonema. In wildtype mouse this might be the case, because some MSH4 foci co-localize with MLH1 in pachynema (Santucci et al., 2000), but the question remains whether these co-localizing MSH4 foci were already present in zygonema. Based on work in yeast (Börner et al., 2004) we would expect so. If so, then they would comprise less than 10% of all late zygotene MSH4 foci in wildtype. It would probably have escaped us if *Sycp1*^{-/-} would lack this type of MSH4 foci (if any) in zygonema. So we do not know whether crossover-designated MSH4 foci are missing from *Sycp1*^{-/-} zygotene spermatocytes (either because crossovers are not designated, or because crossover-designated intermediates fail to form MSH4 foci), or whether crossover-designated MSH4 foci are assembled in *Sycp1*^{-/-} zygonema, but fail to become crossovers in a later stage.

Similar questions arise with respect to γ H2AX. γ H2AX is not restricted to AEs, but occurs throughout chromatin loops. Possibly, a single meiotic DSB causes H2AX phosphorylation on megabases of DNA (which corresponds to tens of loops), like DSBs in somatic cells (Rogakou et al., 1999). Therefore γ H2AX positive domains might contain only one or a few DSBs (or other γ H2AX marked recombination intermediates) associated with the AEs (Fig. 5O-R), but do not necessarily also have DSBs in the loops. However, even if we assume this, it is not clear which lesions γ H2AX might mark in *Sycp1*^{-/-} zygonema. Late zygotene spermatocytes of wildtype and *Sycp1*^{-/-} have similar numbers of RPA and MSH4 foci (Fig. 5S and Fig. S4), yet γ H2AX is restricted to asynapsed AEs and some weak domains in synapsed SC segments in wildtype (Fig. 4B), but covers all the chromatin in *Sycp1*^{-/-} (Figs. 4 C and 5 M). Possible explanations for this difference are: (i) MSH4, RPA and/or RAD51/DMC1 foci in wildtype and *Sycp1*^{-/-} look similar but contain different recombination intermediates; only those present in *Sycp1*^{-/-} are marked by γ H2AX. (ii) The 70% "extra" RAD51/DMC1 foci in *Sycp1*^{-/-} late zygonema (Fig. 5S) bring about the overall γ H2AX labelling. We doubt whether this relatively small number of RAD51/DMC1 marked DSBs or recombination intermediates (60-70 per cell) could cause this. (iii) Late zygotene *Sycp1*^{-/-} spermatocytes contain besides the RAD51/DMC1, RPA and MSH4 marked DNA structures other DNA lesions that are not marked by any of these proteins, but are marked by γ H2AX, e.g. unresected DSBs. This seems unlikely: if yeast *zip1* mutants are similar to *Sycp1*^{-/-} in this respect, it would predict an elevated level

of that type of DNA-lesions in *zip1*; there are no indications for this (Börner et al., 2004). (iv) The γ H2AX labelling in late zygotene *Sycp1*^{-/-} spermatocytes reflects some disorganization in the *Sycp1*^{-/-} cell that is not related to the presence of DSBs. (v) Loss of DSBs or recombination intermediates (due to repair) is uncoupled from loss of γ H2AX staining in *Sycp1*^{-/-}. This may result in the persistence of γ H2AX labelling at sites where there are no breaks (anymore). Although there are no conclusive arguments against the other possible explanations, we prefer the last one, because it accounts for the close correlation between asynapsis and the presence of γ H2AX in wildtype. A similar correlation exists between asynapsis and the presence of ATR (Turner et al., 2004) and RAD50 and MRE11 (Eijpe et al., 2000). This correlation could either mean that synapsis can only occur in chromosomal regions where these proteins have been lost, or that these proteins are lost from chromatin loops upon synapsis (or some local SYCP1-dependent event preceding synapsis). The presence of γ H2AX all over the chromatin in *Sycp1*^{-/-} zygotene nuclei argues for the second interpretation: perhaps, synapsis/SYCP1 causes first the loss of ATR, which is then followed by loss of γ H2AX. In *Sycp1*^{-/-} mid to late pachynema, most γ H2AX eventually disappears from the chromatin, except from a number of distinct domains (Fig. 4H). Since most of these domains have RAD51/DMC1 (Fig. 5Q,R) or MSH4 (Fig. 5O,P) foci at their bases, they probably represent loops in which repair has not been completed.

Role of SYCP1 in later steps of meiotic recombination

In wildtype late pachynema/early diplonema, most or all RAD51/DMC1, RPA and MSH4 foci have disappeared, whereas *Sycp1*^{-/-} late pachytene/early diplotene cells still have 50-70% of the number of foci found in zygonema (Fig. 5S; Fig. 54). Similar observations have been made in TF mutants of *Caenorhabditis* (Colaiácovo et al., 2003; Alpi et al., 2003) and yeast (Novak et al., 2001). Apparently meiotic recombination is blocked or impeded at a step where these proteins act, possibly single end invasion, because yeast Rad51 and Dmc1 are required for strand invasion (Hunter and Kleckner, 2001). 30-50% of the RAD51/DMC1, RPA and MSH4 foci disappear between zygonema and late pachynema/diplonema of *Sycp1*^{-/-}. Whether these foci represent a specific subpopulation or a random sample of the foci in late zygonema is not clear.

Mouse *Sycp1*^{-/-} mutants have in common with TF mutants in other species that crossover formation is affected. More than 90% of the crossovers in the mouse depend on synapsis/SYCP1 (Fig. 6 E,F). However, the number of RAD51/DMC1, RPA and MSH4 foci and γ H2AX signals that are still present in *Sycp1*^{-/-} diplonema exceeds the number of crossovers in wildtype about fivefold: we counted 117±17 MSH4 foci per diplotene *Sycp1*^{-/-} spermatocyte, whereas there are on average 21-25 exchanges per cell in male mouse meiosis (Koehler et al., 2002). SYCP1 is therefore not only required for crossover formation, but also for repair of DSBs that will not become crossovers, at least if persisting MSH4 foci in *Sycp1*^{-/-} still mark DNA lesions. Upon exposure to OA, *Sycp1*^{-/-} spermatocytes repair the recombination intermediates (if any) that underlie the RAD51/DMC1, RPA and MSH4 foci, because chromatid breaks are rare in OA-induced

metaphases I (Fig. 6F). Possibly, OA opens up a DNA repair pathway that is not normally used in wildtype, for instance by releasing the sister chromatid as template for repair. However, exposure to OA reveals little or no crossing over in *Sycp1*^{-/-}, whereas it reveals crossover formation in wildtype. Therefore, SYCP1 must have a role in crossover formation besides its proposed role in the repair of breaks that will not become crossovers.

To summarize the role of SYCP1 in recombination: a substantial fraction of meiotic DSBs does not require SYCP1 for repair (Fig. 5S); it is not known whether these breaks are a random sample or a specific subset of breaks. 100-200 breaks per cell (as estimated from the number of foci and γ H2AX signals in late pachynema) require SYCP1 and/or synapsis for repair. And the formation of more than 90% of the crossovers depends on SYCP1 and/or synapsis. This resembles the yeast *zip1* phenotype. The role of SYCP1 in crossover formation is a conserved TF function in all species analyzed thus far. Possibly SYCP1/the SC serve as support for the assembly of MLH1 foci and/or enhance crossing over by providing a close apposition of homologs. Alternatively, or in addition, TF proteins/synapsis might ensure certain overall structural alterations in the bivalents that lead to crossover formation (Börner et al., 2004).

Role of SYCP1 in XY body formation

This study revealed an unexpected role of SYCP1 in the formation of the XY body. Turner et al. (2004) presented recently evidence that coating of asynapsed portions of AEs with BRCA1 and ATR was correlated with H2AX phosphorylation and transcriptional inactivation. In the XY bivalent this would ultimately result in the formation of an XY body. The aberrant distribution of ATR and γ H2AX in *Sycp1*^{-/-} pachytene spermatocytes provides therefore an obvious explanation for the failure to form XY bodies. However, the question remains why ATR is distributed aberrantly. Perhaps ATR relocates to asynapsed portions of AEs after it has disappeared from synapsed portions of AEs; this might explain the dense coating (rather than discrete foci) of ATR along the last asynapsed portions of AEs, including those of the sex chromosomes. Possibly ATR does not relocate in *Sycp1*^{-/-}, because it is sequestered at unrepaired DNA breaks, and/or because SYCP1/synapsis is directly or indirectly required for relocation of ATR.

Comparison with other meiotic recombination-deficient mouse mutants

Besides *Sycp1*, other mouse genes homologous to yeast genes involved in the Zip1-dependent pathway of crossover formation have been knocked out, namely *Msh4* (Kneitz et al., 2000) *Msh5* (Edelmann et al., 1999; de Vries et al., 1999) and *Dmc1* (Pittman et al., 1998; Yoshida et al., 1998). Contrary to *Sycp1*^{-/-} mice, these knockouts display partial and nonhomologous alignment/synapsis rather than full-length homologous alignment. Presumably, MSH4, MSH5 and DMC1 are indispensable for establishment of stable recombinational interactions between homologs in the mouse, whereas SYCP1 contributes only to a minor extent to the stability of such interactions, at least in leptotema till pachynema.

Msh4, *Msh5* and perhaps *Dmc1* knockouts enter apoptosis when the spermatogenic epithelium is in developmental stage IV and the spermatocytes should be in early/mid-pachynema (de Vries et al., 1999; de Rooij and de Boer, 2003). At least a small proportion of *Sycp1*^{-/-} spermatocytes progresses further and reaches diplotene or exceptionally metaphase I. This could be related to the ability of *Sycp1*^{-/-} spermatocytes to establish reasonably stable homologous alignment. Among other mutant mice with a less defined but on average later arrest in meiosis than early/mid pachytene (stage IV), there are several that can align or synapse chromosomes homologously, including *Mlh1*, *Mlh3* and *Brca1* mutants (reviewed by de Rooij and de Boer, 2003).

Acknowledgements

We thank C. Beerends, P. de Boer, P. Cohen, M.A. Handel, Ch. Her, R. Jessberger, R. Kanaar, T. de Lange, P. Moens, J.-M. Peters and M. Volker for antibodies, and B. Vennström for the lambda FixII library. We are indebted to the Central Mouse Facility at the LUMC for blastocyst injections and general mouse facilities. We thank Jaap Jansen (LUMC) for his advice in culturing ES cells, and M. Eijpe, H.H. Offenberg and A.J.J. Dietrich for help and practical advices, and two anonymous reviewers for several useful suggestions. The Netherlands Society for Scientific Research (NWO) (grant 901-01-097) and the EU (contract QLK3-2000-00365) financially supported this work.

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Summary

Summary

All organisms are composed of cells, the basic units of structure capable of performing the activities of life. Every cell's nucleus contains DNA, the heritable material that directs the many functions of the cell. In eukaryotic cells, the DNA is organized within the nucleus along with certain proteins into structures called chromosomes. In humans, each somatic cell (any cell other than reproductive cells) has 46 chromosomes. The chromosomes are found in pairs and we inherit one chromosome of each pair from each parent. The 46 chromosomes in our somatic cells are therefore actually two sets of 23 homologous chromosomes, a set from our mother and a set from our father. The chromosome is divided in hereditary units called genes. Genes are segments of DNA that encode a specific hereditary trait. DNA is a double-stranded helical polymer of four different kinds of monomers called nucleotides. Each nucleotide consists of three components: a nitrogen-containing base, a sugar (deoxyribose), and a phosphate group. The base can be adenine (A), thymine (T), guanine (G) or cytosine (C). A–T and C–G bases are paired in the interior of the double helix. Inherited information is passed on in the form of each gene's specific sequence of nucleotides. Cells are programmed to transcribe and translate genes and doing so cells synthesize specific enzymes and other proteins. It is the cumulative action of these proteins that produces an organism's inherited traits.

The transmission of hereditary traits has its molecular basis in the precise replication of DNA, which produces copies of genes that can be passed along from parent to offspring. When a cell divides normally, during a process called mitosis, two daughter cells are formed that are genetically identical to the parent cell. The reproductive cells, or gametes, that transmit genes from one generation to the next are sperm and ova (unfertilized eggs). Sperm and ova are made by meiosis. Meiosis, like mitosis, is preceded by the replication of chromosomes. However, this single replication is followed by two consecutive cell divisions, called meiosis I and meiosis II. These divisions result in four daughter cells (rather than the two daughter cells of mitosis), each with only half as many chromosomes as the parent cell. After meiosis, each of the gametes has a single set of 23 chromosomes. After a sperm cell unites with an ovum, chromosomes from both parents are present in the nucleus of the fertilized egg.

The induction of DNA damage is a threat to all organisms. First, the correct functioning of the cell is assured by gene expression by transcription and translation. DNA damage possibly leads to blockage of transcription and delay of gene expression. Second, during nuclear division (mitotic and meiotic division) the DNA of the mother cell is first replicated before it is equally distributed over two new nuclei. In this way damaged DNA can be passed on to daughter cells. Accumulation of damaged DNA in cells can lead to mutations and chromosome aberrations. Proteins encoded by mutated DNA can be altered leading to lack of

or a modified function, the genetic basis of hereditary diseases. In addition, mutations and chromosome aberrations can lead to unlimited cell growth (carcinogenesis). Signalling of DNA damage and subsequent repair is of substantial importance to all organisms. This importance is underlined by the existence of various damage signalling and DNA repair pathways in all prokaryotic and eukaryotic cells.

Chapter 1 of this thesis introduces one of the most genotoxic lesions: the DNA double-strand break (DSB), which affects both DNA strands of the double helix. DSBs can be induced by ionizing radiation and other exogenous DNA damaging agents or free radicals generated during cellular metabolic processes or collapsed forks during normal replication. DSBs also arise as intermediates in several endogenous cellular processes such as the initiation of recombination and assurance of proper chromosome segregation during meiosis, mating-type switching in yeast and the assembly of immunoglobulins and T cell receptors from variable (V), diversity (D), and joining (J) gene segments (V(D)J rearrangement). Persisting or incorrectly repaired DSBs in DNA can result in chromosomal rearrangements (including chromosomal fragmentation, translocations and deletions). Genetic instability resulting from unrepaired DSBs can lead to programmed cell death via apoptosis. In multicellular organisms chromosomal rearrangements can lead to or contribute to carcinogenesis by activation of oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity. The importance of DSB repair is underlined by the evolution of several DSB repair pathways. The two major pathways are non-homologous endjoining (NHEJ) and homologous recombination (HR). By non-homologous endjoining, broken ends of DNA are sealed together, irrespective of their sequence in a not necessarily error-free way. Due to resection of the broken ends, genetic information can be lost resulting in inaccurate DNA repair. HR, in which damaged chromosomes are restored using intact sister chromatids or homologous DNA molecules as a template, accurately repairs DSBs.

Genes involved in HR are introduced in **Chapter 1.3**. These genes belong to the RAD52 epistasis group and are well characterized in *S. cerevisiae*. Key proteins in the RAD52 epistasis group are Rad51, Rad52 and Rad54. In HR, Rad51, stimulated by the activities of Rad52 and Rad54, mediates the pairing of homologous DNA molecules and strand exchange. In this thesis we mainly focus on the Rad52 protein. Rad52 is a key protein in recombinational repair in *S. cerevisiae*. In the fission yeast *S. pombe*, two RAD52 homologs, *rad22A*⁺ and *rad22B*⁺, are present. *S. pombe rad22A* mutants resemble *S. cerevisiae rad52* mutants in radiosensitivity, while inactivation of *rad22B*⁺ does not lead to such obvious repair defects. This demonstrates that Rad22A is crucial in recombination and repair of vegetative *S. pombe* cells, while Rad22B has an auxiliary role in DSB repair. Meiosis in *S. pombe* is dependent on the presence of either Rad22A or Rad22B. Interestingly, the level of Rad22B protein is strongly enhanced in *S. pombe* cells from 5 to 7 hours after induction of meiosis in comparison to vegetatively dividing cells and cells in early and late meiosis. We

wondered whether differences in phenotypes of *rad22A* and *rad22B* mutants can be clarified by differences in biochemical activities. In mammals, Rad52 deficiency has no obvious effect on cells and the mild phenotype of *rad52* mutants suggests the existence of additional mammalian proteins functionally redundant with Rad52. Recently, it was demonstrated that the biochemical function of Rad52 can probably be compensated for by the breast cancer-associated factor BRCA2.

In **Chapter 1.4** we stress the importance of recombination between homologous chromosomes during meiosis. This recombinational behaviour of homologous chromosomes during meiosis is responsible for genetic variation and gives rise to the process of evolution. During prophase of the first meiotic division, the duplicated chromosomes pair with their homologues, a process called synapsis. During part of prophase I, a protein "zipper", the synaptonemal complex (SC), holds the homologous chromosomes tightly together all along their lengths. When the SC disappears in late prophase, chiasmata can be seen, the physical manifestations of recombination between homologous chromosomes also called crossing overs. SCs are assembled from two axial elements (AEs), one along each homolog, which are connected by numerous transverse filaments (TFs). Meiotic HR, in contrast to recombination in mitotically dividing cells, is actively induced in high frequencies (100 to 1000 fold higher than in mitotic cells). During meiosis, the homologous chromosome is the preferred template, whereas in mitotically dividing cells the sister chromatide is used predominantly as a template molecule. In most organisms meiotic recombination is initiated by the formation of a double-strand break by the Spo11 protein. In addition to Spo11, the formation and resection of breaks during meiosis requires factors belonging to the RAD52 group and a number of meiosis-specific recombination proteins.

In **Chapter 1.5** we describe post-translational modification as a way to alter the function of a protein. We discuss peptide modifications, post-translational modifications of target proteins by attaching other peptide groups to it. Ubiquitination is the most common mechanism to regulate the properties of a protein by marking target proteins for degradation. Also covalent attachment of the small ubiquitin-like modifier, SUMO, to proteins plays a major role in regulating cellular functions through changes in cellular localization, biochemical activation, or through protection from ubiquitin-dependent degradation. SUMO modification of a specific target occurs in three enzymatic steps: first E1 enzymes, known as activating enzymes, modify SUMO so that it is in a reactive state, then E2 enzymes, known as conjugating enzymes, catalyze the attachment of SUMO to the substrate protein. E3 enzymes, or ligases, play a role in recognizing the substrate protein.

In **Chapter 2** we analyse the biochemical properties of the two Rad52 homologs in *S. pombe*, Rad22A and Rad22B. *S. cerevisiae* Rad52 is a key protein in the repair of DNA double-strand breaks by homologous recombination. *In vitro*, Rad52 displays DNA binding and strand annealing activities and promotes

Rad51-mediated strand exchange. To obtain insight in the functions of the Rad22A and Rad22B proteins, both proteins are overexpressed in *E. coli* as his-tagged proteins and purified to near homogeneity. Rad22A and Rad22B are tested for DNA binding by gel retardation and filter binding assays and we demonstrate that both proteins are capable of binding to short single-stranded DNAs. Rad22A and Rad22B do not bind to duplex oligonucleotides or linearized plasmids containing blunt ends or 5' single-strand overhangs. In contrast to Rad22A, Rad22B promptly binds to DNA fragments containing 3' overhangs. As expected, Rad22A as well as Rad22B efficiently promote strand annealing of complementary oligonucleotides. In a reaction containing Rad22A, almost 90% of the complementary DNAs is annealed to duplex molecules, whereas in reactions containing Rad22B, the maximum level of annealing is 60%. We demonstrate that the presence of duplex DNA inhibits the annealing activity of Rad22B. The presence of Rad22A can overcome this inhibitory effect. Rad22A and Rad22B are self-associating proteins and exist as multimeric structures as was shown by electron-microscopy.

In **Chapter 3** we illustrate that combined mutations in *RAD52* and *RAD54* homologs (*rad22A*⁺, *rad22B*⁺ and *rhp54*⁺) in *S. pombe* results in identical sensitivities to X-rays, cis-diammine-dichloroplatinum and hydroxyurea. In this respect, these fission yeast homologs resemble the epistatic interaction (*i.e.* single, double and triple mutant strains are equally sensitive to DNA damaging agents) of their counterparts in *S. cerevisiae*. To verify whether inactivation of *RAD52* affects the DNA damage sensitivities of Rad54 deficient mice, we have studied double mutant mice and bone marrow cells derived from these animals. Haemopoietic depression in bone marrow and the formation of micronuclei after *in vivo* exposure to mitomycin C (MMC), is not increased in either single or double mutants in comparison to wildtype mice. The induction of sister chromatid exchanges in splenocytes is slightly reduced in *rad54* single and double mutants. We show that the MMC survival of *rad54*^{-/-} mutant mice and the survival of bone marrow cells after exposure to X-ray is aggravated by a deficiency of Rad52. These findings demonstrate that double mutants have additive defects in HR. Possibly, the single-strand annealing activity of Rad52 may be more prominent in the absence of Rad54.

In **Chapter 4** we present the identification of novel proteins involved in DSB repair by homologous recombination. Using *Drosophila* DmRad51 as bait protein in yeast two-hybrid studies, we have identified Uba2, Ubc9 and Su(Var)2-10, the SUMO E1 activating, E2 conjugating and E3 ligating enzymes, respectively, as associating factors. Su(Var)2-10, which is originally identified as a dominant suppressor of position-effect-variegation, is essential for viability and is involved in the regulation of chromosome structure. We observe an increased X-ray sensitivity in heterozygous *Su(Var)2-10* flies, which implies that sumoylation has a role in DNA damage responses to X-rays. Both Rad22A and Rad22B display interaction with Hus5, the E2 component of the SUMO conjugation pathway in *S. pombe*. To investigate the importance of sumoylation of Rad22A, we have

changed the single SUMO consensus motif of Rad22A. This alteration does not influence DSB repair by Rad22A after X-ray. We have screened the *S. pombe* database for proteins belonging to the PIAS family of SUMO ligases and identified the SP-RING domain protein Pli1. Although, the protein-protein interactions suggest a connection between SUMO modification and DSB repair by HR, *pli1* fission yeast mutants are not hypersensitive to X-rays and hydroxyurea. Defects in chromosome structure in *su(var)2-10* mutants imply an essential role for SUMO in maintaining proper nuclear organization, suggesting that the role for SUMO in DNA repair may be indirect.

In **Chapter 5** we introduce *sycp1*^{-/-} mice. Sycp1 is a component of the transverse filament of the mouse synaptonemal complex, which tightly connects homologous chromosomes during meiotic prophase and assures meiotic recombination. *Sycp1*^{-/-} mice are infertile, but otherwise healthy. The axial elements of the SC are normally formed in *sycp1*^{-/-} spermatocytes, but synapsis does not occur. Most *sycp1*^{-/-} spermatocytes arrest in pachynema, whereas a small proportion reaches diplotema, or, exceptionally, metaphase I. γ H2AX foci (indicative of double-strand breaks) appear during leptotene in both *sycp1*^{-/-} and wildtype spermatocytes. Significant numbers of discrete γ H2AX foci are found along each chromosome in pachynema *sycp1*^{-/-} spermatocytes, whereas γ H2AX disappears from autosomes in wildtype spermatocytes. Rad51/Dmc1, RPA and Msh4 foci (which mark early and intermediate steps in pairing/recombination) appear in similar numbers as in wildtype, but do not all disappear in *sycp1*^{-/-} mutants. Mlh1 and Mlh3 foci (which mark late steps in crossing over) are not formed. Unexpectedly, *sycp1*^{-/-} spermatocytes do not form XY bodies. Crossovers were rare in metaphase I of *sycp1*^{-/-} mice. We propose that Sycp1 has a coordinating role and ensures formation of crossovers.

Samenvatting

Samenvatting

DNA, in kranten vaak de blauwdruk van het leven genoemd, wordt continu van buitenaf bedreigd. Gelukkig beschikken cellen over een uitgebreid pakket aan noodmaatregelen om het genetische materiaal intact te houden. Gaat het in enkele gevallen toch fout, dan kan dat leiden tot kanker. Dit proefschrift gaat over één van die onmisbare maatregelen, waarmee de cel haar DNA beschermt. Om deze samenvatting te kunnen begrijpen, zal ik eerst een globale uitleg moeten geven over wat die allerbelangrijkste bouwsteen van levende wezens: het DNA, is.

Elke vorm van leven op aarde is opgebouwd uit één of meer cellen. De kleinste organismen bestaan uit slechts één cel, terwijl grotere organismen uit enkele tot honderd biljoen (een 1 met 14 nullen) cellen zijn opgebouwd. In alle cellen van levende wezens zit DNA, dat niet los in de celvloeistof ligt, maar stevig verpakt is binnen de membranen van de celkern. Niet al te lang geleden, in 1944, bewees Oswald T. Avery dat in het DNA, in codevorm, de erfelijke aanleg van organismen wordt beschreven.

De celkern ligt vol met DNA. Ieder soort organisme heeft een verschillend aantal DNA-moleculen per celkern. Die afzonderlijke DNA-moleculen worden chromosomen genoemd. Zo hebben mensen in elke celkern 46 chromosomen, die samen alle erfelijke eigenschappen van een mens dragen. Die chromosomen zijn veelal verschillend van grootte, maar toch steeds twee aan twee gelijk. De gelijke chromosomen vormen een paar. Menselijke cellen hebben dus 23 paar chromosomen in elke celkern. De informatie op de chromosomen is in elke cel gelijk. Om te zorgen dat deze informatie steeds goed wordt doorgegeven als een cel zich deelt, worden alle chromosomen voor de deling exact gekopieerd. Bij de normale celdeling (mitose) worden de verdubbelde chromosomen vervolgens simpelweg verdeeld over twee nieuwe cellen. Iedere nieuwe cel krijgt op deze manier de volledige set erfelijke eigenschappen mee. Bij de bijzondere celdeling die voorafgaat aan de vorming van voortplantingscellen (meiose), wordt het DNA ook eerst verdubbeld maar daarna in twee stappen (meiose I en II) verdeeld over uiteindelijk vier voortplantingscellen. Elke op deze manier gevormde geslachtscel bevat zo de helft van het aantal chromosomen, slechts één chromosoom van elk paar. Wanneer nu bij de bevruchting twee geslachtscellen versmelten, komen de chromosomen in het nieuw gevormde leven weer in paren voor: één chromosoom van elk paar is afkomstig van vader, één van moeder.

Kleinere stukjes van een chromosoom, de genen, bepalen telkens één van de erfelijke eigenschappen. In 2001 is aangetoond dat een mens 20.000 tot 25.000 verschillende genen heeft. Uiterlijke kenmerken van organismen worden bijvoorbeeld voor een belangrijk deel door erfelijke eigenschappen bepaald; hierbij kun je denken aan de oogkleur van mensen. Soms wordt een eigenschap

door één enkel gen bepaald, maar veel vaker zijn er bij eigenschappen meer genen betrokken. Elk gen geeft de instructie voor de bouw van één bepaald eiwit met een eigen specifieke functie. Eiwitten kunnen betrokken zijn bij de opbouw van cellen, maar ze kunnen ook een veel belangrijkere taak hebben, namelijk het uitvoeren van alle levensfuncties. Deze laatste groep eiwitten, de werklieden van de cel die alle reacties in goede banen leiden, heten enzymen.

Als we chromosomen wat verder uitvergroten, dan zien we zeer lange ketens DNA. In 1953 waren de chemische componenten van het DNA bekend. James Watson en Francis Crick deden toen een voorstel voor de structuur van het DNA, zonder dat zij DNA ooit hadden kunnen zien. Later bleek het door Watson en Crick beschreven model juist te zijn. DNA lijkt op een wenteltrap en bestaat uit twee in elkaar gedraaide moleculen waarvan de ruggengraat gevormd wordt door een keten van afwisselend suiker en fosfaat groepen. De verbinding tussen de twee spiraalvormige ketens wordt gevormd door de vier verschillende basen van het DNA: A (adenine), C (cytosine), G (guanine) en T (thymine). C hecht altijd aan G in de tegenoverliggende keten en A hecht aan T in de tegenoverliggende keten. Zo vinden we op het DNA een lange reeks basenparen G-C, A-T, C-G en T-A, in een wisselende volgorde die uniek is voor ieder individu. De code in het DNA, geschreven in een vier-letterig alfabet, wordt gebruikt als recept om eiwitten op te bouwen en biedt de mogelijkheid om de erfelijke eigenschappen van een individu vast te leggen.

Er kunnen spontaan veranderingen (mutaties) in het erfelijk materiaal ontstaan. We spreken van mutaties op het moment dat één of meer letters in het molecuul anders is/zijn (bijvoorbeeld een A wordt een C), of als letters zijn weggevallen (een TCAG wordt een TAG) of toegevoegd. Mutaties zijn vaak handig, ze zorgen voor variatie in erfelijke eigenschappen en maken evolutie mogelijk. Soms heeft een mutatie ernstige gevolgen: als de DNA-code van een gen beschadigd wordt, kan een bepaald eiwit of enzym niet meer of nog maar gedeeltelijk functioneren. De specifieke taak van het eiwit bepaalt de ernst van de gevolgen.

Mutaties hebben veel verschillende oorzaken. Allereerst kan DNA beschadigd worden door normale scheikundige processen in de cel. Daarnaast wordt het DNA van buitenaf bedreigd, door bijvoorbeeld kosmische straling of chemische stoffen die met DNA reageren en genetische veranderingen veroorzaken. Schade veroorzaakt door straling kan bijvoorbeeld het gevolg zijn van een dagje strand (ultraviolette straling) of een bezoekje aan de tandarts (röntgenstraling). De chemische stoffen die DNA-schade veroorzaken, genotoxische agentia, kun je binnenkrijgen door het eten van gefrituurde kroketten of het inademen van tabaksrook. De hoeveelheid DNA-schades die we oplopen is enorm en wordt geschat op 10.000 gebeurtenissen per cel per dag. Om organismen te behoeden voor de mogelijk ernstige gevolgen van DNA-schade beschikt de cel over een heel scala aan noodgrepen. Er bestaan honderden eiwitten die betrokken zijn bij het opsporen, verwijderen en herstellen van DNA-schade. Gelukkig is reparatie in veruit de meeste gevallen succesvol. Wanneer de verdediging tegen DNA-

schade dan toch faalt, kunnen beschadigde cellen zichzelf opofferen en afsterven (apoptosis). Soms echter ontwikkelen cellen zich tot kankercellen, die ongeremd groeien en tumoren veroorzaken. Het hele proces van schadeherstel wordt extra gecompliceerd, als je bedenkt dat de instructie voor het opbouwen van de DNA-schadehersteleiwitten ook gegeven wordt door genen in het DNA. Iedereen kan zich voorstellen dat als de genen beschadigd worden die coderen voor één van die hersteleiwitten, de gevolgen dramatisch zijn en leiden tot zeer complexe ziekteverschijnselen.

De integriteit van het DNA wordt het meest bedreigd, wanneer beide ketens van een DNA- molecuul doorbroken worden, een zogenaamde dubbel-strengs breuk (DSB). De veroorzakers van speciaal dit type DNA-schade, de DSB, zijn ioniserende straling (b.v. röntgenstraling en straling afkomstig uit radioactieve stoffen) en specifieke chemische stoffen. DSBs ontstaan ook spontaan tijdens de mitose en meiose. Bij zo'n DSB kunnen de twee gebroken einden van een chromosoom uit elkaar drijven en de gevolgen daarvan kunnen natuurlijk rampzalig zijn. Herstel van deze DSBs is dan ook onontbeerlijk. De cel kan dubbel-strengs DNA-breuken op twee manieren repareren: 1. door de losse uiteinden simpelweg weer aan elkaar te plakken (DNA-eindverbinding ofwel non-homologous endjoining (NHEJ)). Dit proces is vanzelfsprekend niet altijd foutloos. 2. door de juiste informatie van een homolog chromosoom (bijvoorbeeld het tweede chromosoom van een paar of een zuster-chromatide) te kopiëren (homologe recombinatie ofwel homologous recombination (HR)). Het homologe molecuul dient dan als matrijs voor foutloos DSB-herstel.

Dit proefschrift gaat vooral over de tweede manier van dubbel-strengs breukherstel via homologe recombinatie. Met het beschreven onderzoek hebben we geprobeerd te begrijpen wat de biologische gevolgen van bijvoorbeeld blootstelling aan straling zijn, onder andere omdat röntgenstraling veelvuldig wordt gebruikt als anti-kanker therapie. Daarnaast probeerden we met dit onderzoek inzicht te krijgen in hoe het herstel van DSBs moleculair in zijn werk gaat. Uiteindelijk probeerden we te begrijpen hoe homologe recombinatie bijdraagt aan het voorkomen van kanker. Voor ons onderzoek maakten we o.a. gebruik van modelorganismen als *Schizosaccharomyces pombe* (spleijtgist) en muizen met een genetisch defect in één van hun DNA-reparatiemechanismen. Het gebruik van gisten als model voor menselijke cellen is niet zo ver gezocht als het lijkt. Ook al staan gisten en de mens evolutionair mijlenver uit elkaar, de genetische informatie is nog enigszins identiek. Dit blijkt uit het feit dat gistcellen die zich niet langer kunnen delen, gered kunnen worden door stukken menselijk DNA in de cel te spuiten.

In **hoofdstuk 1**, de inleiding, wordt uitgebreid verteld hoe dubbel-strengs breuken ontstaan, hoe we denken dat het herstel van DSBs via homologe recombinatie in zijn werk gaat en welke eiwitten daarbij betrokken zijn. Er wordt een onderscheid gemaakt tussen het DSB-herstel tijdens de mitose en tijdens de meiose.

Met behulp van röntgegevoelige bakkersgistcellen zijn veel eiwitten geïdentificeerd, die betrokken zijn bij het herstel van dubbel-strengs breuken in mitotisch delende cellen. Deze eiwitten behoren allemaal tot een familie van eiwitten betrokken bij stralingsgevoeligheid (radiation sensitivity), de RAD52-groep. Er zijn verschillende genen die coderen voor deze groep van eiwitten, onder andere het Rad51 gen, het Rad52 gen en het Rad54 gen. De functies van die eiwitten worden beschreven in **paragraaf 1.3**. In dit proefschrift houden wij ons vooral bezig met de functie van het Rad52 enzym. Rad52 is van vitaal belang voor bakkersgistcellen, maar zoogdiercellen waarin Rad52 ontbreekt, zijn normaal levensvatbaar. In splijtgist *S. pombe* zijn twee eiwitten gevonden die homoloog zijn aan bakkersgist Rad52, namelijk Rad22A en Rad22B. Een interessante vraag is of deze twee eiwitten, Rad22A en Rad22B, ook functioneel verschillend zijn. De resultaten van zo'n onderzoek helpen om de functie van zoogdier Rad52 te begrijpen en kunnen ons op het idee brengen welk eiwit mogelijk de functie van Rad52 in zoogdieren heeft overgenomen. Recent is ontdekt dat de functies van bakkersgist Rad52 in zoogdieren waarschijnlijk worden uitgevoerd door het BRCA2 enzym. BRCA2 (breast cancer 2) is het eiwit dat ontbreekt of gemuteerd is bij mensen met een erfelijke vorm van borstkanker.

Om geslachtscellen te kunnen vormen, moet een bijzondere kerndeling, de meiose, ervoor zorgen dat er van elk paar chromosomen, slechts één enkel chromosoom in elke voortplantingscel komt. Hiervoor is het nodig dat de homologe chromosomen van één paar met elkaar verbonden worden in een ritssluitingachtige structuur, het synaptonemale complex (SC; zie figuur 3 van hoofdstuk 1). Deze structuur houdt de chromosomen van één paar bijeen tijdens de eerste fase van de meiose, maar belangrijker is, dat er nu binnen de hekwerken van het SC ook homologe recombinatie kan plaatsvinden tussen de gepaarde chromosomen. Deze homologe recombinatie tijdens de meiose zorgt voor nieuwe combinaties van genen, omdat genen worden uitgewisseld met genen op het homologe tweede chromosoom van een paar (een zogenaamde cross-over). Deze cross-overs zorgen voor herrangschikking van de eigenschappen, zodat er extra variatie onder nakomelingen ontstaat en liggen aan de basis van de evolutie.

Om genen uit te kunnen wisselen tussen homologe chromosomen worden er tijdens de meiose in de context van de vorming van het SC actief dubbel-strengs breuken gecreëerd. Deze dubbel-strengs breuken worden vervolgens foutloos hersteld door homologe recombinatie. Er zijn verschillen tussen homologe recombinatie in de mitose en in de meiose. Ten eerste worden de DSBs in de meiose actief geïnduceerd, ten tweede komt homologe recombinatie tijdens de meiose 100 tot 1000 keer vaker voor dan in mitotisch delende cellen en ten derde wordt tijdens de meiose bij voorkeur het homologe chromosoom gebruikt voor herstel, terwijl dit in normaal delende cellen ook een tweede kopie (chromatide) van het eigen chromosoom kan zijn. De eiwitten betrokken bij homologe recombinatie in mitose en meiose zijn veelal gelijk. In **paragraaf 1.4**

worden specifieke eiwitten betrokken bij homologe recombinatie tijdens de meiose verder besproken.

Tot slot wordt in hoofdstuk 1 aandacht besteed aan het reguleren van de functies van de verschillende eiwitten betrokken bij DSB-herstel. Om eiwitten te kunnen afbreken nadat zij hun functie hebben uitgevoerd of om eiwitten op de juiste plek in de cel te krijgen, worden eiwitten vaak gemarkeerd met een ander eiwit. Vooral het markeren met ubiquitine is bekend. De wetenschappers die ontdekten dat eiwitten die met ubiquitine gemarkeerd zijn, afgevoerd worden naar de afvalverwerking van de cel, noemden dit de kiss of death. Zij ontvingen een Nobelprijs voor hun werk. Behalve ubiquitine zijn er meer eiwitten die eiwitten kunnen markeren, zoals SUMO (small ubiquitin-like modifier). Hoe SUMO aan eiwitten wordt gekoppeld en wat er dan mogelijk met die eiwitten gebeurt, staat beschreven in **paragraaf 1.5**.

In **hoofdstuk 2** concentreren we ons op de verschillen in functie tussen Rad22A en Rad22B in *S. pombe*. Om dit onderzoek uit te kunnen voeren hebben we eerst via gentechnologie bacteriën gemaakt, die we splijtgist Rad22A en Rad22B lieten produceren. Uit de bacteriecelextracten hebben we Rad22A en Rad22B gezuiverd. Met de gezuiverde Rad22A en Rad22B eiwitten hebben we proeven gedaan. We hebben ontdekt dat zowel Rad22A als Rad22B in staat zijn aan DNA te binden, maar dat Rad22A alleen bindt aan enkel-strengs DNA, terwijl Rad22B ook bindt aan DNA dat gedeeltelijk enkel- en gedeeltelijk dubbel-strengs is. Zowel Rad22A als Rad22B kunnen twee enkel-strengs DNA ketens aan elkaar plakken tot dubbel-strengs DNA (een proces dat annealing heet), maar Rad22A doet dat veel sneller en efficiënter. De annealings-reactie van Rad22B wordt geremd wanneer teveel enkel-strengs DNA is omgezet en er dus teveel dubbel-strengs DNA is ontstaan. Als vervolgens Rad22A wordt toegevoegd aan de reactie, heft Rad22A de remming op en zorgt ervoor dat het enkel-strengs DNA dat nog in de reactie aanwezig is, wordt omgezet in dubbel-strengs DNA. Van de gezuiverde Rad22A en Rad22B eiwitten hebben we ook opnamen gemaakt met een elektronenmicroscop (zie de omslag van dit proefschrift). Uit deze opnamen blijkt dat zowel Rad22A als Rad22B hun functies niet uitvoeren als losse eiwitten, maar dat ze allebei grotere complexen vormen, waarbij een aantal (waarschijnlijk zeven) Rad22A of Rad22B eiwitten aan elkaar vastzitten.

In **hoofdstuk 3** wordt duidelijk hoe Rad52 samenwerkt met Rad54, een ander eiwit van de Rad52 groep. We hebben deze samenwerking onderzocht, door splijtgistcellen te maken waarin we Rad22A, Rad22B en Rhp54 (Rad54 homoloog pombe) in verschillende combinaties uitschakelden. We onderzochten de enkel, dubbel of trippel mutante splijtgistcellen op röntgegevoeligheid en gevoeligheid voor de chemische stoffen cis-platinum en hydroxyurea. Het blijkt dat de functies van Rad22A, Rad22B en Rhp54 bij het herstel van DSB zo verweven zijn, dat wanneer één van deze eiwitten uitgeschakeld is, het dubbel-strengs breukherstel via homologe recombinatie niet meer goed kan verlopen.

We hebben de onderlinge betrokkenheid van Rad52 en Rad54 ook onderzocht in muizen. In muizen waarin we zowel Rad52 als Rad54 uitschakelden, blijkt dat meestal de defecten veroorzaakt door de afwezigheid van Rad54 niet worden versterkt door afwezigheid van Rad52. In twee specifieke situaties leidde de aangebrachte schade echter wel tot versterkte defecten, namelijk in het geval dat we röntgenshade aanbrachten aan beenmergcellen van de muis en in het geval dat we hele muizen inspotten met de chemische stof MMC. We concludeerden hieruit dat wanneer Rad52 en Rad54 afwezig zijn, behalve de homologe recombinatie ook bijvoorbeeld de annealing van enkel-strengs DNA (een rol van Rad52) niet meer goed verloopt.

In **hoofdstuk 4** beschrijven we dat eiwitten betrokken bij dubbel-strengs breukherstel gemarkeerd worden door SUMO. We ontdekten dat Rad22A en Rad22B in splijtgist *S. pombe* waarschijnlijk gemodificeerd worden door SUMO, doordat beide eiwitten interacteren met Hus5, een enzym dat SUMO koppelt aan haar doelwit. We vonden dezelfde interacties bij homologe eiwitten in *Drosophila melanogaster*, de fruitvlieg. We onderzochten het effect van afwezigheid van Su(Var)2-10, een enzym dat SUMO bij een specifiek doelwit in de buurt brengt, op de levensvatbaarheid van fruitvliegen na bestraling. We vonden dat als er onvoldoende Su(Var)2-10 aanwezig is, fruitvliegen een verlaagde overlevingskans hebben na blootstelling aan röntgenstraling. We zochten ook naar een homoloog van Su(Var)2-10 in *S. pombe* en vonden een stuk *S. pombe* DNA dat informatie bevat voor een eiwit dat homoloog is aan Su(Var)2-10 en soortgelijke enzymen in andere organismen die SUMO aan een specifiek doelwit koppelen. We noemden dit vermoedelijke enzym Pli1 en hebben het gen dat codeert voor Pli1 uitgeschakeld in splijtgistcellen. Afwezigheid van Pli1 leidde niet tot verhoogde röntgegevoeligheid in splijtgist. We vermoeden dat, ondanks dat er duidelijke aanwijzingen zijn dat eiwitten betrokken bij homologe recombinatie gemodificeerd worden met SUMO, deze modificatie slechts indirect betrokken is bij DSB-herstel. We denken dat zonder SUMO modificatie de structuur van de celkern niet langer intact kan worden gehouden, met bijbehorende effecten op bijvoorbeeld DNA herstelprocessen.

In **hoofdstuk 5** geven we het belang aan van een goed functionerend synaptonemaal complex (SC) tijdens de meiose. De ritssluitingstructuur van het SC bestaat uit twee axiale ketens die parallel aan de homologe chromosomen liggen en transversale elementen die de homologe chromosomen met elkaar verbinden. Wanneer we in muizen het gen uitschakelen dat codeert voor één van de transversale eiwitten, Sycp1, wordt het SC niet goed opgebouwd. De muizen zijn dan onvruchtbaar. Uit verder onderzoek van de meiose in deze muizen blijkt, dat de eerste stappen van de meiose, waarin de paring van de homologe chromosomen en de homologe recombinatie plaatsvinden, wel ingezet worden, maar dat de meiose niet wordt afgemaakt. Daadwerkelijke uitwisselingen van genen tussen de homologe chromosomen (cross-overs) vinden niet plaats. We denken daarom dat Sycp1 een coördinerende rol speelt tijdens de meiose en cross-overs mogelijk maakt.

Abbreviations

AE	axial element
ATP	adenosine triphosphate
BIR	break-induced replication
bp	base pair(s)
BRCA	breast cancer-associated factor
CDK1	cyclin dependent kinase 1
DNA	deoxyribonucleic acid
DNA-PKcs	DNA protein kinase catalytic subunit
DSB	double-strand break
DSBR	double-strand break repair
dsDNA	double-strand DNA
ES	embryonic stem
HR	homologous recombination
IR	ionizing radiation
LE	lateral element
MMC	mitomycin C
MMR	mismatch repair
MMS	methylmethane sulfonate
NBS	nijmegen breakage syndrome
NHEJ	non-homologous endjoining
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PNK	polynucleotide kinase
RAD	radiation-sensitive
RPA	replication protein A
RHP	rad homologue of <i>S. pombe</i>
SC	synaptonemal complex
SCE	sister chromatid exchange
SDSA	synthesis-dependent strand annealing
SSA	single-strand annealing
ssDNA	single-strand DNA
SUMO	small ubiquitin-like modifier
TF	transverse filament
UBL	ubiquitin-like
UV	ultraviolet light

Curriculum vitae

De auteur van dit proefschrift werd geboren op 24 oktober 1977 te Nijmegen. Na het behalen van het Gymnasium bèta diploma aan het Gymnasium Ceeleum te Zwolle, begon zij in 1996 met de studie biologie aan de Universiteit van Wageningen. De doctoraalfase omvatte twee afstudeerprojecten en een stage. Het eerste afstudeerproject werd gedaan in het Laboratorium voor Erfelijkheidsleer van de Universiteit van Wageningen onder begeleiding van Prof. Dr. C. Heyting, waar een moleculair genetisch onderzoek werd uitgevoerd. Het tweede afstudeerproject vond plaats aan de vakgroep Antropogenetica van het Academisch Medisch Centrum van de Universiteit van Amsterdam, waar genetisch-diagnostisch onderzoek werd verricht. De eindstage was in het laboratorium van Prof. Dr. L. Sherman van het Department of Immunology van het Scripps Institute in La Jolla, California (VS), waar kankeronderzoek werd gedaan. In september 2000 studeerde zij af en startte een promotie onderzoek op de afdeling Toxicogenetica van het Leids Universitair Medisch Centrum onder leiding van Dr. A. Pastink en Prof. Dr. Ir. A.A. van Zeeland. De resultaten van het aldaar uitgevoerde onderzoek zijn in dit proefschrift weergegeven. In december 2002 werd daarnaast begonnen aan een parttime baan als docent biologie aan het Adelbert College in Wassenaar. Na de afronding van haar promotie onderzoek zal zij blijven doceren aan het Adelbert College.



Appendix

Selected coloured figures



Chapter 3 Figure 2

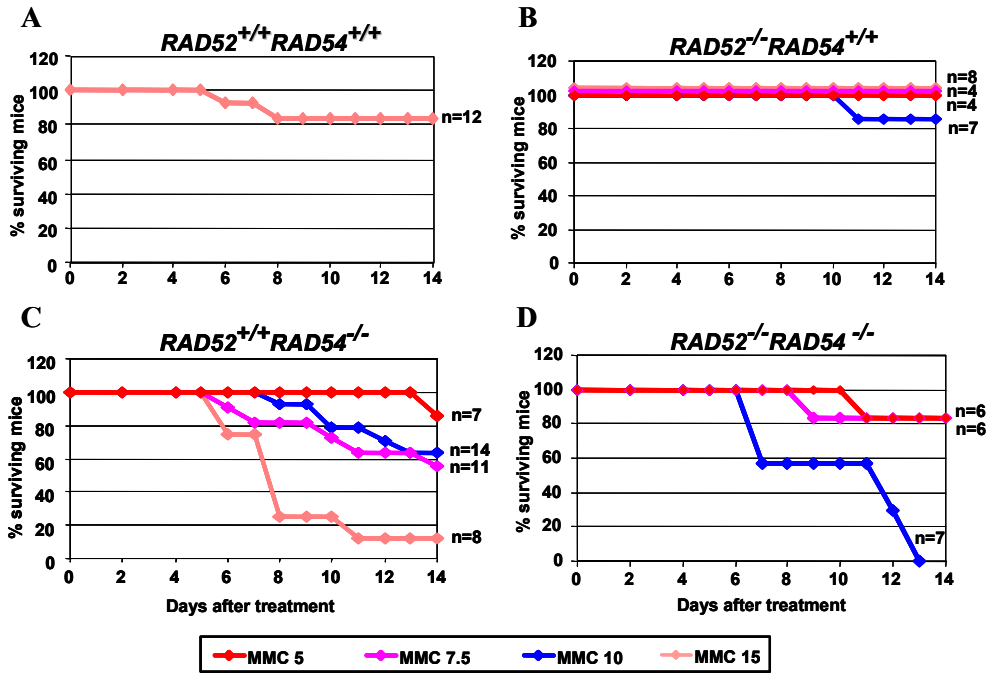


Figure 2: Survival of mice following mitomycin C treatment. Animals were injected intraperitoneally with 5, 7.5, 10 or 15 mg/kg bodyweight MMC. Total numbers of individually treated mice are indicated per dose. Control (A) $RAD52^{-/-}$ (B), $RAD54^{-/-}$ (C) and $RAD52^{-/-} / RAD54^{-/-}$ (D) mice.

Chapter 3 Figure 4

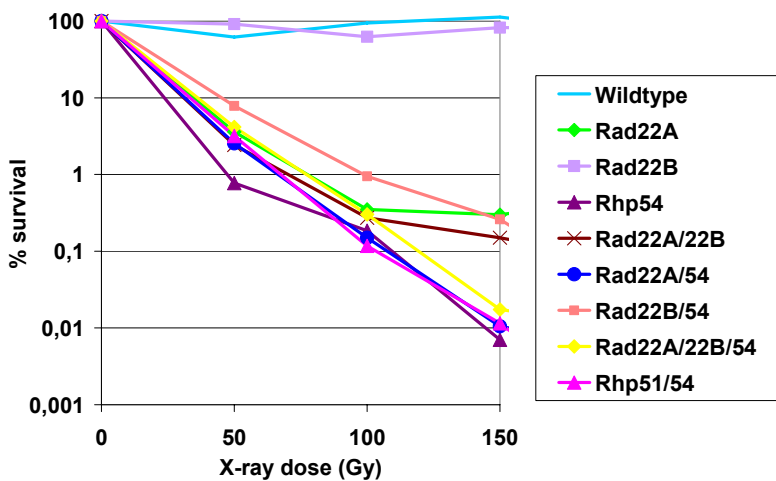


Figure 4: X-ray survival of wildtype and single, double and triple mutant *S. pombe* strains. Exponentially growing cells were harvested, irradiated and appropriate dilutions were plated in triplicate on YES media. After 3 days of incubation at 30°C the colonies were counted. Each survival experiment was repeated at least twice.

Chapter 4 Figure 4

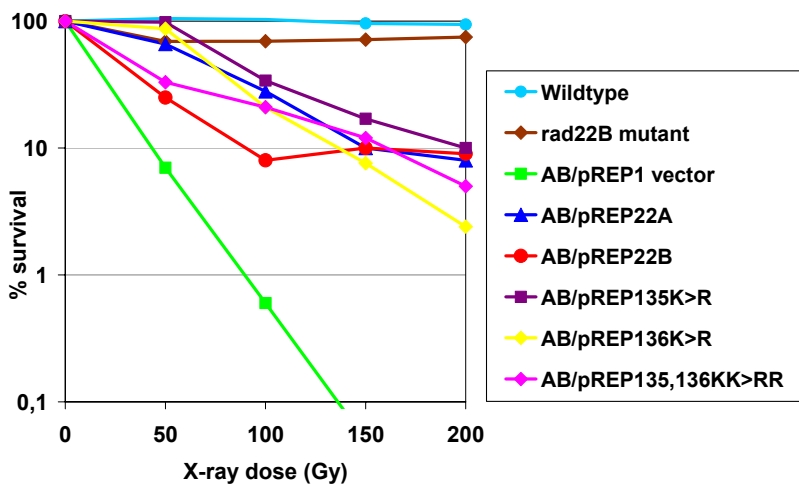


Figure 4: Survival of *S. pombe* strains after irradiation with X-rays. After irradiation of exponentially growing cells, appropriate dilutions were plated and colonies were counted after incubation of the plates for 3 days at 30°C. Each survival experiment was repeated at least twice. Strains used in this experiment: wildtype (Y4), *rad22B*^{-/-} (*rad22B* mutant), and double mutant *rad22A*^{-/-}*rad22B*^{-/-} strains containing expression vectors without insert (AB/pREP-), with *Rad22A* insert (AB/pREP22A), with *Rad22B* insert (AB/pREP22B) and with *Rad22A* inserts in which the putative SUMO acceptor site has been mutated (AB/pREP135 K>R, AB/pREP136 K>R, AB/pREP135,136 KK>RR). For details see Materials and methods.

Chapter 5 Figure 2

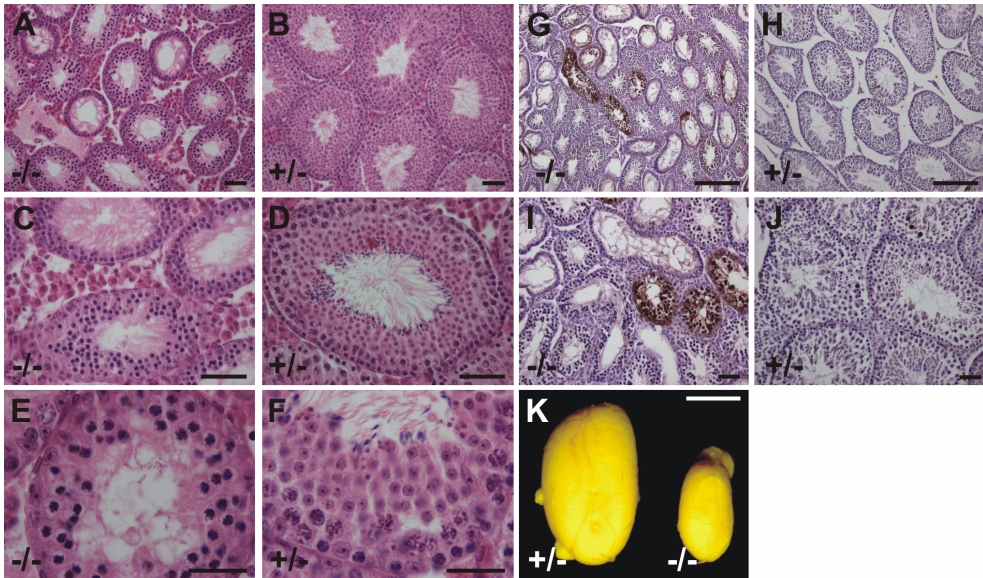
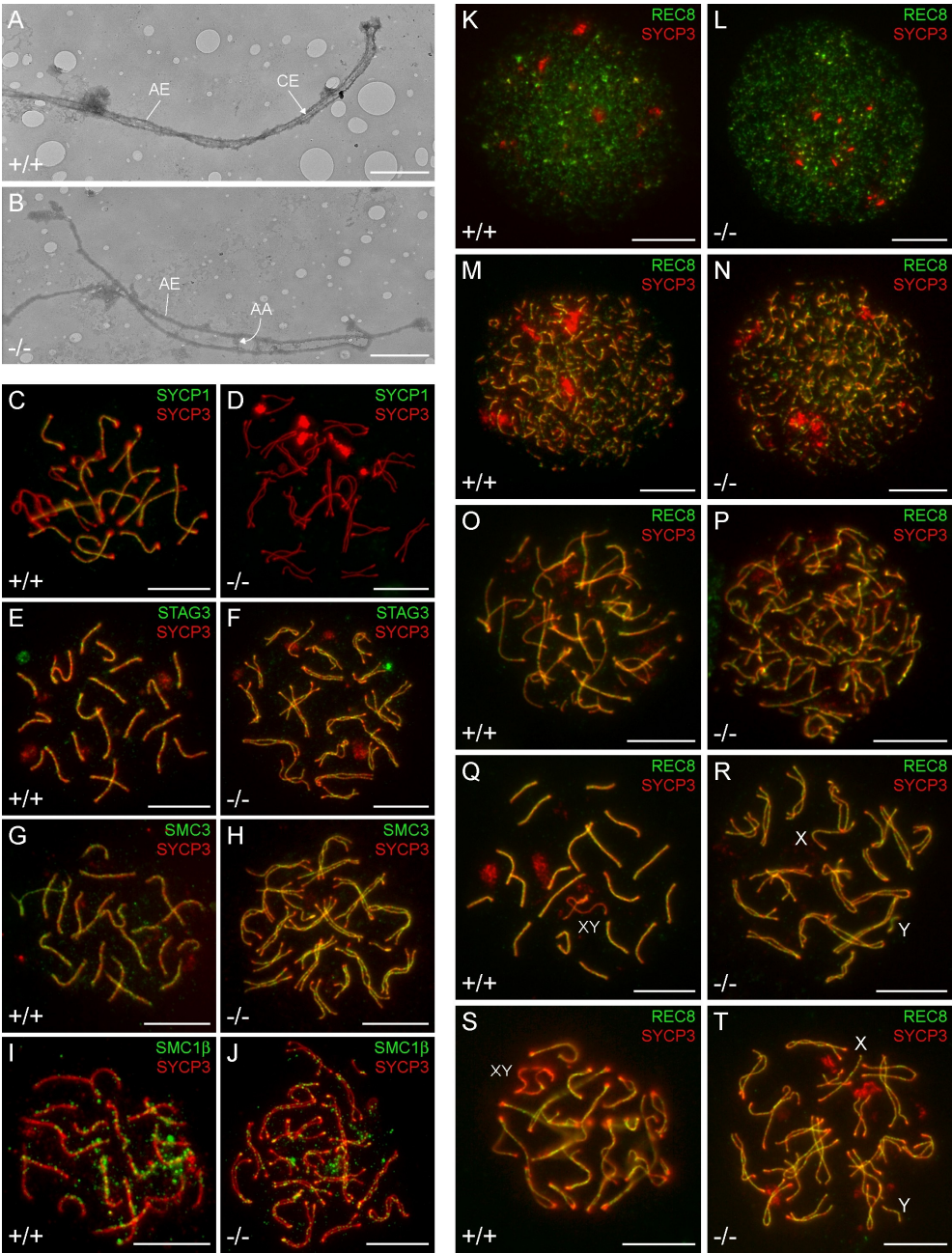


Figure 2: Morphology, histology and TUNEL analysis of testes from *Sycp1*^{-/-} mice. The histological sections were stained with haematoxylin and eosin. **(A-F)** Testicular histology of adult *Sycp1*^{-/-} (-/-, **A,C,E**) and *Sycp1*^{+/-} (+/-, **B,D,F**) mice. Note the total absence of postmeiotic germ cells in *Sycp1*^{-/-} sections. Pachytene nuclei are abundant, but show aberrant nuclear morphology. **(G-J)** TUNEL analysis of testis sections of *Sycp1*^{-/-} (-/-, **G,I**) and *Sycp1*^{+/-} (+/-, **H,J**) mice. Tubule sections with numerous TUNEL-positive nuclei occur only in *Sycp1*^{-/-} mice. A few apoptotic nuclei are visible in tubule sections from *Sycp1*^{+/-} mice. **(K)** Testes from *Sycp1*^{+/-} (+/-) and *Sycp1*^{-/-} (-/-) mice. Bars: **(A-D,I,J)** 50 µm; **(E-F)** 25 µm; **(G-H)** 100 µm; **(K)** 2 mm.

Figure 3: Assembly of AEs in *Sycp1*^{-/-} mice.

(A-B) Electron micrographs of AEs and SCs from wildtype (+/+) and *Sycp1*^{-/-} (-/-) male mice; **(A)** wildtype SC with closely apposed axial elements (AE) and a central element (CE); **(B)** homologously aligned axial elements (AE) from a *Sycp1*^{-/-} spermatocyte, connected by axial associations (AA). **(C-J)** Components of AEs and SCs in wildtype (+/+) and *Sycp1*^{-/-} (-/-) diplotene (**C-D**) or pachytene (**E-J**) spermatocytes; LE/AE protein SYCP3 and all analyzed cohesins are present in LEs/AEs of wildtype and mutant, whereas SYCP1 is not detectable in mutant spermatocytes. **(K-T)** formation of AEs/LEs, as shown by REC8/SYCP3 double labelling, in wildtype (+/+) and *Sycp1*^{-/-} (-/-) spermatocytes; **(K,L)** early leptotema; **(M,N)** late leptotema; **(O,P)** zygonema; **(Q,R)** pachynema; **(S,T)** diplonema; note the XY bivalent (XY) in wildtype cells (**Q,S**), and separate X and Y chromosomes in the *Sycp1*^{-/-} cells (**R,T**). Bars in **(A-B)** 1 µm; bars in **(C-T)** 10 µm.

Chapter 5 Figure 3



Legends figure 3: see page 175

Chapter 5 Figure 4

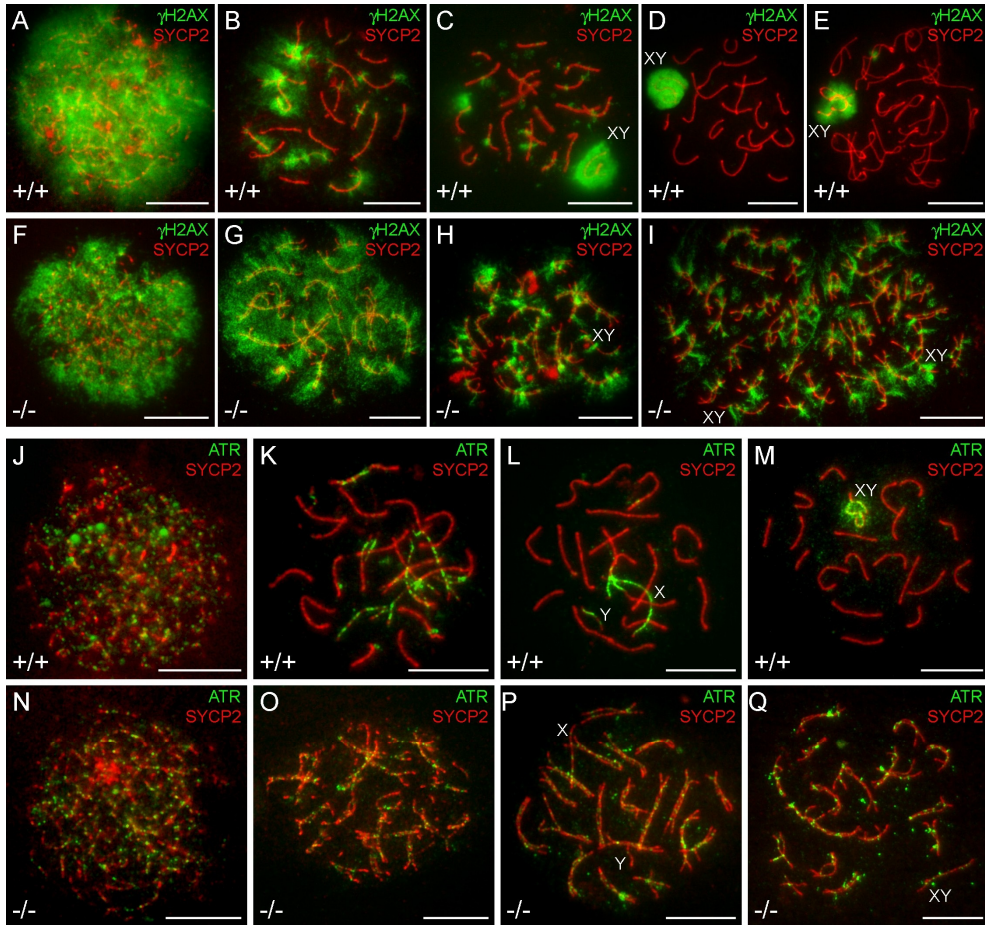
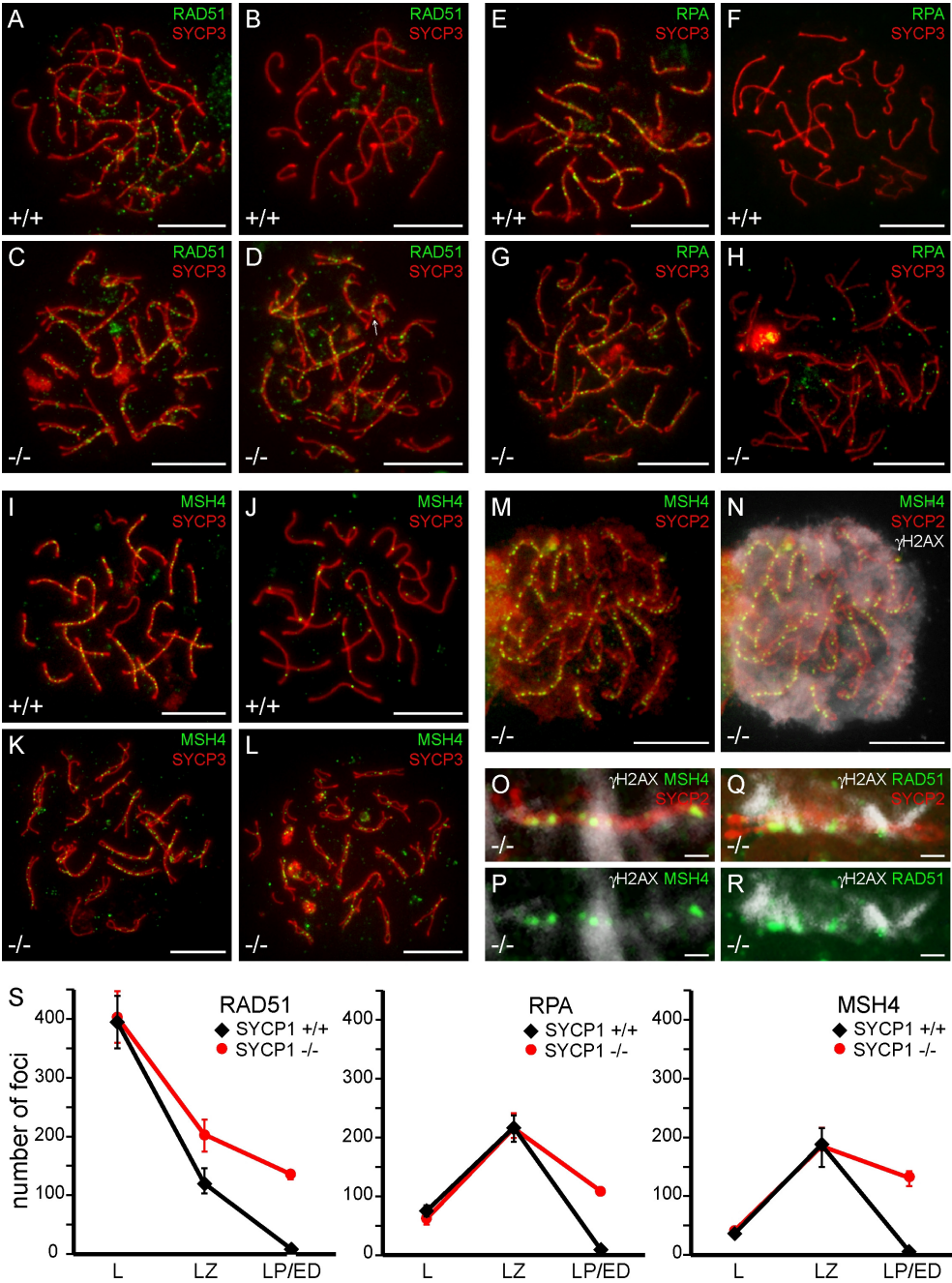


Figure 4: γ H2AX and ATR in wildtype (+/+) and *Sycp1*^{-/-} (-/-) spermatocytes.

(A-I) γ H2AX ; (A,F) leptotema; (B,G) zygotema; (C) early pachynema; (D,H) mid-pachynema; (E,I) diplonema; the sex chromosomes (XY) form an XY-body in wildtype spermatocytes (C-E), but not in *Sycp1*^{-/-} spermatocytes, even though the X and Y chromosomes are associated in the cells in (H) and (I). (J-Q) ATR; (J,N) leptotema; (K,O) zygotema; (L) early pachynema and (M) and (P) mid-pachynema; (Q) diplonema; ATR is present throughout the chromatin of the XY bivalent in wildtype spermatocytes (M), but forms foci and distinct domains along the X and Y chromosomes in *Sycp1*^{-/-} cells (P-Q). Insets in (J) and (N) show the close association of ATR with the ends of AE fragments in wildtype (+/+) and *Sycp1*^{-/-} leptotema. Bars 10 μ m.

Chapter 5 Figure 5



Legends figure 5: see page 179

Figure 5: Recombination-related proteins along AEs and SCs in wildtype (+/+) and *Sycp1*^{-/-} (-/-) spermatocytes.

(A-D) RAD51/DMC1; **(A,C)** late zygonema; **(B,D)** late pachynema. **(E-H)** RPA; **(E,G)** late zygonema; **(F,H)** diplonema. **(I-L)** MSH4; **(I,K)** late zygonema; **(J)** mid-pachynema; **(L)**, diplonema. **(M-N)** MSH4/SYCP2/ γ H2AX triple labelling of a zygotene *Sycp1*^{-/-} spermatocyte; the number and localization of MSH4 foci appears normal, but the persistence of γ H2AX throughout the chromatin is abnormal. **(O-P)** MSH4/ SYCP3/ γ H2AX triple labelling of a late pachytene *Sycp1*^{-/-} bivalent, to show that part of the γ H2AX domains co-localize with an MSH4 focus. **(Q-R)** RAD51/SYCP2/ γ H2AX triple labelling of a late pachytene *Sycp1*^{-/-} bivalent, to show that part of the γ H2AX domains co-localize with a RAD51 focus. **(S)** Counts of RAD51, RPA and MSH4 foci in successive stages of meiotic prophase; the vertical axes represent the number of AE or SC associated foci per cell; the vertical bars represent the observed range of the number of foci per cell in a given spermatocyte stage. For more details of the counts, see Supplementary Information, Fig. S4. Bars in **(A-N)** 10 μ m; bars in **(O-R)** 1 μ m.

Chapter 5 Figure 6

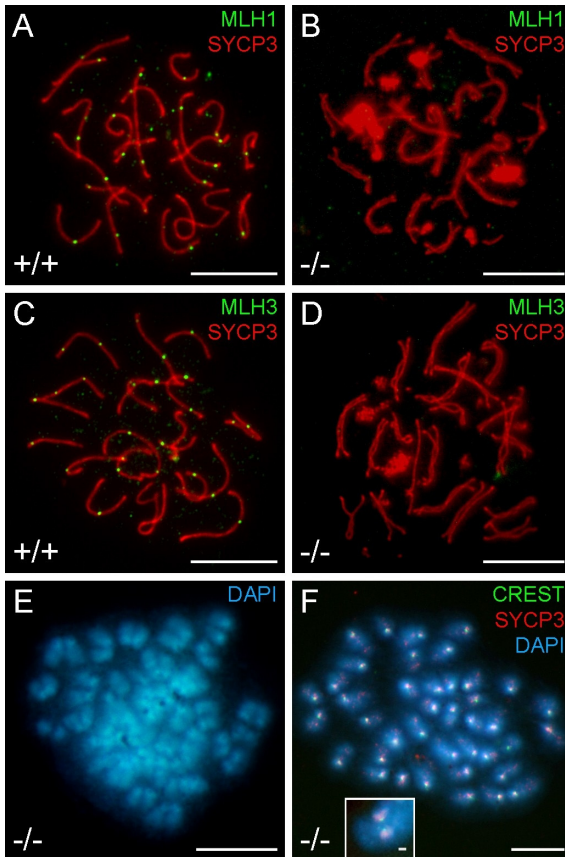


Figure 6: Formation of crossovers and chiasmata.

(A,B) MLH1 labelling and **(C,D)** MLH3 labelling of wildtype (+/+) or *Sycp1*^{-/-} (-/-) pachytene spermatocytes. The *Sycp1*^{-/-} spermatocytes do not assemble MLH1 or MLH3 foci. **(E,F)** A natural **(E)** and an okadaic acid-induced **(F)** metaphase I spermatocyte of *Sycp1*^{-/-}. In the cells shown here, only univalents can be identified; the inset in **(F)** shows a bivalent found in another OA-induced *Sycp1*^{-/-} metaphase I. Bars in **(A-F)** 10 μ m; bar in the inset in **(F)** 1 μ m.

