Role of *Rad54*, *Rad54B* and *Snm1* in DNA

Damage Repair

De rol van *Rad54*, *Rad54B* en *Snm1* in herstel van schade aan DNA

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Contents

List of abbreviations

AID	activation induced cytidine deaminase
bp	base pair
DSB	double-strand break
DNA-PK$_{cs}$	DNA-dependent protein kinase catalytic subunit
dsDNA	double-stranded DNA
ES	embryonic stem
FACS	fluorescence activated cell sorting
FISH	fluorescent *in situ* hybridization
GC	gene conversion
HR	homologous recombination
HYM domain	hypermutation domain
ICL	DNA interstrand cross-link
IgλL	immunoglobulin lambda light chain
IR	ionizing radiation
MEFs	mouse embryonic fibroblasts
MMC	mitomycin C
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PCR	polymerase chain reaction
PNK	polynucleotide kinase
RS-SCID	radiosensitive severe combined immune deficiency
RSS	recombination signal sequences
SCID	severe combined immune deficiency
SCE	sister chromatid exchange
SHM	somatic hypermutation
SSA	single-strand annealing
ssDNA	single-stranded DNA
UV	ultraviolet light

Scope of the thesis

The aim of this thesis is to investigate the function of a number of genes involved in mammalian DNA damage repair, in particular in repair of DNA double-strand breaks (DSBs). Among a large number of different damages that can be introduced to DNA, DSBs are especially toxic. If left unrepaired, DSBs can trigger apoptosis or induce chromosomal rearrangements that can lead to carcinogenesis. Two main pathways are responsible for repair of DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). HR is generally an error-free mechanism that restores missing information on the basis of homologous sequence obtained from sister chromatid or homologous chromosome. By contrast, NHEJ is generally error-prone. During repair by NHEJ the DNA ends can be directly ligated or short stretches of homology at the ends can be used, leading to deletions or insertions at the site of the break.

A number of genes have been identified as players in DSB repair, both in prokaryotes and eukaryotes. Many of them are found in all the kingdoms of life and are similar in aspects of their sequence and function, although there are genes characteristic only for prokaryotes or eukaryotes.

Many subtle differences in function also emerge from studies on HR proteins in different species. These differences might have appeared because of diverse functions repair genes have to perform in more complexed organisms, or because the initial function(s) of a gene has been distributed over multiple paralogues.

Herein I concentrate on genes involved mainly in DSB repair via HR in mammalian systems. Two members of the group of HR genes are studied: *Rad54* and its paralogue *Rad54B*. Using mice and cells deficient in these genes we try to define the role of both *Rad54* and *Rad54B* in HR and their contribution to other cellular processes. Additionally, we investigate the link between *Rad54* and *Snm1*, a gene originally identified as being important for interstrand cross link (ICL) repair, with regard to ionizing radiation-induced DNA damage repair.

Chapter 1

DNA double-strand breaks: significance, generation, repair and link to cellular processes

DNA double-strand breaks: generation, significance, repair and link to cellular processes

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DNA double-strand breaks (DSBs) are introduced into DNA by a number of exogenous agents but are also generated during normal cellular processes such as DNA replication. Two major pathways repair this potentially harmful DNA damage: non-homologous end joining and homologous recombination. In this overview I discuss the significance of DSBs, describe the mechanisms of DSB repair processes and characteristics of the proteins involved. Subsequently, specialized processes that involve processing of physiological DSBs are described. The first is meiosis in which DSB processing leads to genetic diversity. The second and third are V(D)J recombination and somatic hypermutation. The latter two link the DSB repair systems to initiation of genetic diversity of immunoglobulins.

1. Generation of double-strand breaks, their significance and repair

In order to survive a cell needs to keep its genetic information, encoded in DNA, in an undamaged state. Sensing and repair of DNA damage is extremely important since cells are continuously exposed to a variety of DNA damaging agents. Depending on the particular agent, various lesions can be introduced in DNA, which forced cells to evolve a number of specialized DNA damage repair mechanisms. These different molecular machineries form a cross-talking network of DNA repair systems with functional overlap in the cell.

Double-strand breaks (DSBs), damage where both DNA strands of the double helix are affected, belong to the most genotoxic lesions. DSBs can be introduced by exogenous agents such as ionizing radiation (IR) or chemicals but also by free radicals generated during cellular metabolic processes or by collapsed forks during DNA replication (Cox *et al*, 2000). There are also specialized and programmed processes where DSBs are purposely introduced to initiate the generation of genetic diversity. In V(D)J recombination, immunoglobulin class switching, somatic hypermutation and
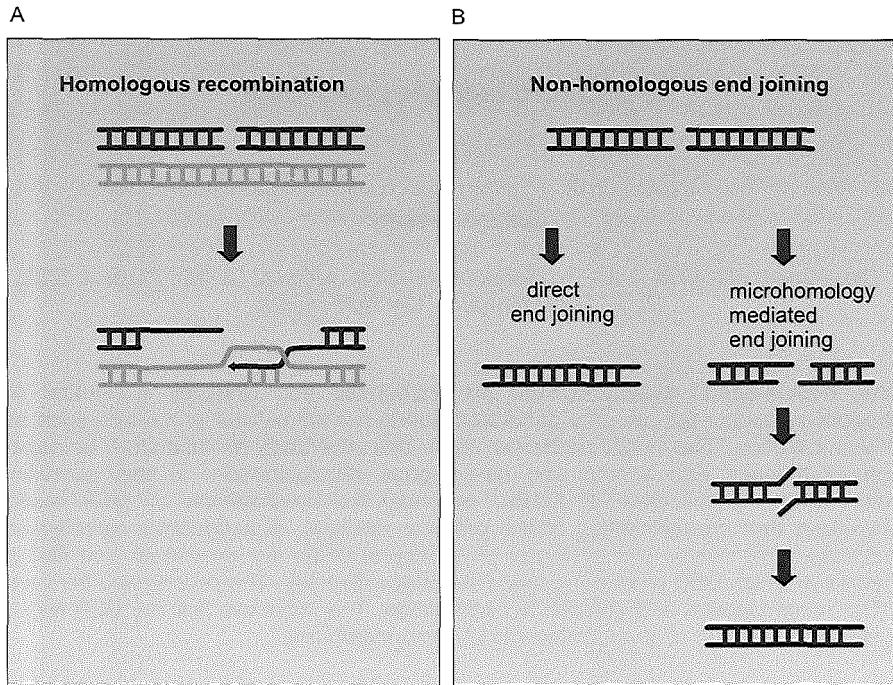
A

B

Figure 1. Schematic representation of DSB repair pathways. (A) Accurate repair by HR is a multi-step process leading to restoration of missing information using homologous sequences derived from sister chromatid or homologous chromosome (For details see Figure 2). (B) During precise NHEJ, indicated by "direct end-joining" in the figure, the ends are directly ligated and no loss or gain of nucleotides occurs. NHEJ can also make use of short stretches of homology (1-10 nucleotides). First, the ends are processed by nucleases to create 3' or 5' overhangs, then the overhangs are annealed and non-homologous single-stranded DNA tails are removed. Remaining gaps are filled and nicks are ligated.This process, indicated by "microhomology-mediated end-joining", can lead to deletions. In addition, nucleotides can be added to the break sites resulting in insertions.

meiosis DSBs are beneficial intermediates during genome rearrangements (Sekiguchi *et al.* 1999, Gellert, 2002, Paques *et al.* 1999).

However, unrepaired DSBs can lead to permanent growth arrest or can activate programmed cell death via apoptosis. Persisting or incorrectly repaired DSBs can cause translocations, deletions, amplifications, inversions and fragmentations of chromosomes. These events can potentially trigger or contribute to carcinogenesis via activation of oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity (Ferguson *et al.* 2001, Pastink *et al.* 2001, Thompson *et al.* 2001).

To prevent illegitimate and mutagenic DNA rearrangements caused by unrepaired DSBs, the cell developed several mechanistically different repair

processes. Here I focus on two major DSB repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). HR is an error-free process during which the missing information is restored from an intact sister chromatid or homologous chromosome. By contrast, two broken ends can be also directly ligated at regions of little or no homology in an error-prone manner by NHEJ machinery. During this process, genetic information can be lost due to resection of the broken ends (see Figure 1).

The pathways can compete with each other in the repair of DSBs, but while NHEJ can recognize and repair only two-ended DSBs (with two overhangs created during nucleolytic processing), it has been proposed that HR has a wider specificity and can operate on both two-ended and single-ended DSBs that arise, for example, at collapsed replication forks (Cromie *et al.*, 2001, Dronkert *et al.*, 2001). Both HR and NHEJ play an important role in maintenance of genome stability although their relative contribution to DSB repair appears to be dependent on species, cell type, cell cycle phase and presence of regulatory proteins (see below).

2. Homologous recombination and non-homologous end joining - interplay and contribution to repair of DSBs

The single cell organism *Saccharomyces cerevisiae* uses HR as a dominant DSB repair pathway. Since most of the yeast DNA represents coding DNA, it is important that mutation-free genetic information is passed on to the progeny. In general, defects in NHEJ can be detected only when HR is impaired (Kramer *et al.*, 1994, Milne *et al.*, 1996, Siede *et al.*, 1996). The relative contribution of NHEJ depends on transcriptional regulation of a component of the NHEJ reaction (Astrom *et al.*, 1999, Lee *et al.*, 1999, Clikerman *et al.*, 2001, Valencia *et al.*, 2001).

One of the DSB repair pathways, HR, appears to be tightly bound to replication. The first data linking the two processes comes from prokaryotes. Some *Escherichia coli* mutants carrying mutations in genes involved in replication require DSB repair genes for viability (Michel *et al.*, 2001, McGlynn *et al.*, 2002). Mutations in prokaryotic DNA polymerases, ligases, helicases and topoisomerases lead to increased mitotic recombination (Klein, 1995, Rothstein *et al.*, 1995). Much less is known about the replication/recombination connection in eukaryotes. Experiments with haploid budding and fission yeast cells reveal the presence and increase of recombination intermediates (X - DNA structures) in S phase of the cell cycle. Inactivation of genes involved in HR leads to reduced levels of the intermediates in the mutant cells (Zou *et al.*, 1997, Seguardo *et al.*, 2002).

The absolute requirement of the mammalian DSB repair proteins, Rad51 and Mre11 (for details see section 3.1 and 3.5) for viability and S phase proliferation suggests an essential role of these DSB repair factors in replication (Sonoda et al., 1998, Yamaguschi-Iwai et al., 1999). Although direct evidence of the cooperation of the two processes in mammalian cells is still to be obtained, the data suggest that HR is important for progression of replication and/or rescue of arrested replication forks.

In order to repair IR-induced DNA damage, mammals preferably use NHEJ (Lieber, 1999). Large part of the mammalian genome is non-coding and non-regulating and the cells can afford small mutations in those parts of the genome. It is unlikely that such mutations will cause serious problems in somatic or differentiated cells. Another possible reason for why HR is not a dominant repair pathway for IR-induced DSBs is the presence of many repeats in the mammalian genome. HR could use ectopic DNA as the template, which could potentially lead to translocations, inversions and deletions.

During embryonic development, when fidelity of repair in rapidly dividing cells is very important, HR is the major player (Takata et al., 1998, Essers et al., 2000). The importance of HR during early stages of development is underscored by lethality of mice and/or IR sensitivity of embryonic stem (ES) cells lacking certain genes involved in HR (Thompson et al., 2001).

Although treated as two separate pathways, HR and NHEJ have functional overlap and interact with each other. They both contribute to IR resistance and maintenance of chromosomal integrity, but their relative contribution can vary depending on the species (Takata et al., 1998, Essers et al., 2000). There are indications of repair events combining features of HR and NHEJ, although specific proteins involved in this reaction are not known. The combined process is initiated by invasion of one DSB end onto homologous DNA on a different chromosome, subsequent DNA synthesis may be extended to regions of no homology and the junction of newly synthesized DNA-DSB has to be made by NHEJ (Moynahan et al., 1997, Johnson et al., 2000). Both pathways are involved in repair of different types of DSBs. For example V(D)J recombination, occurring in B and T cells, is completed by NHEJ (see section 6). Meiotic recombination, increasing genetic diversity in gametes and ensuring proper chromosome segregation, is performed by homologous recombination (see section 4) (Smith et al., 1998).
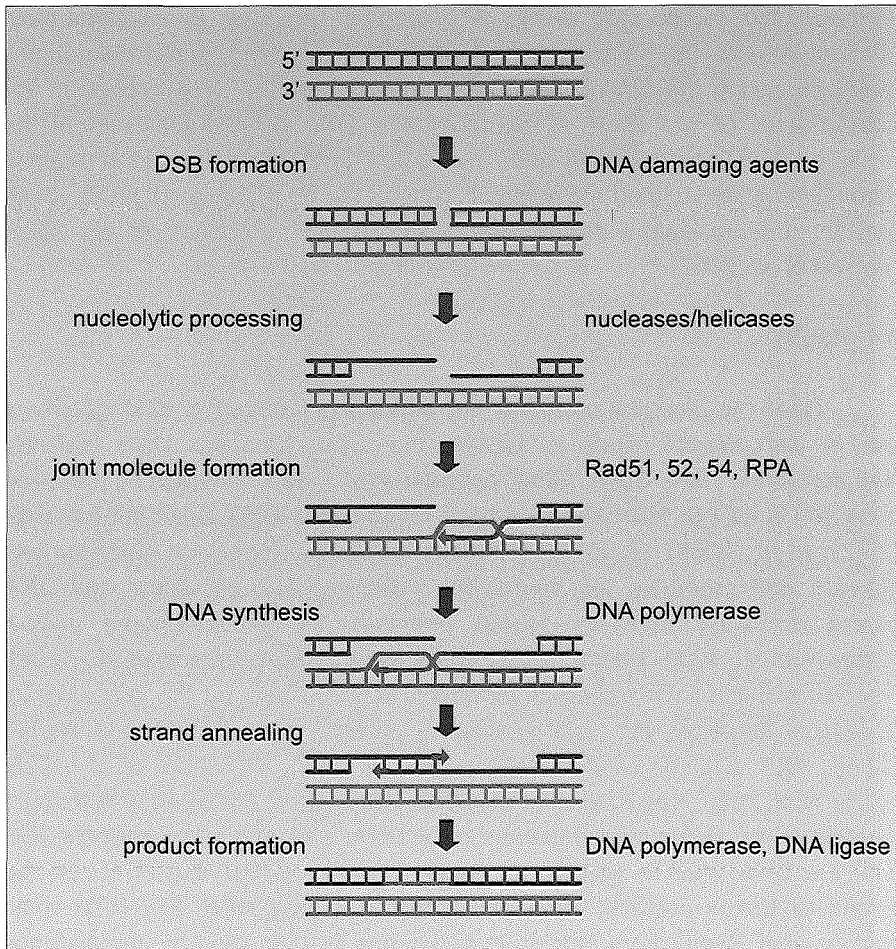
Figure 2. Biochemistry of homologous recombination. For description see section 3.

3. Biochemistry of homologous recombination

The sequence of events in the core reaction of HR is highly conserved throughout different species. During DSB repair via HR, missing DNA is restored using intact homologous sequence provided by either the sister chromatid or the homologous chromosome (Paques *et al.*, 1999, Johnson *et al.*, 2000). In the early stage of the reaction, referred to as presynapsis, the DNA ends are processed into 3' single-stranded (ss) overhangs, by yet unidentified endonucleases and/or helicases (see Figure 2). The ssDNA tails are coated with a strand exchange protein to form a nucleoprotein filament (see below) that can recognize a homologous DNA sequence. During

synapsis, the middle step of the recombination process, the nucleoprotein filament invades the homologous template DNA to form a joint heteroduplex molecule linking the broken end(s) and the undamaged DNA duplex. In the postsynaptic, or late stage of recombination, DNA polymerases restore the missing information and DNA ends are ligated. In this last step of the reaction resolution of recombined molecules into separate DNA duplexes by structure specific endonucleases occurs (Haber *et al.*, 2001, Modesti *et al.*, 2001, Nishino *et al.*, 2002).

3.1 Rad51

Rad51 is a central protein of HR and it is conserved in all kingdoms of life. *In vitro*, Rad51 binds to ssDNA and double-stranded DNA (dsDNA) (Benson *et al.*, 1994, Sung, 1994, Zaitseva *et al.*, 1999) although its preferred substrate is ssDNA tailed duplex, which resembles a DSB repair intermediate (Mazin *et al.*, 2000). Rad51 polymerizes on ssDNA forming a nucleoprotein filament that promotes the search for a homologous sequence and DNA strand exchange between dsDNA template and protein-coated ssDNA (Ogawa *et al.*, 1993, Benson *et al.*, 1994, Sung, 1994, Bianco *et al.*, 1998). Rad51-mediated joint molecule formation is stimulated by the presence of accessory proteins; the ssDNA binding protein RPA, Rad52 and Rad54 (Sung, 1997, Benson *et al.*, 1998, New *et al.*, 1998, Shinohara *et al.*, 1998, Mazin *et al.* 2000, Van Komen *et al.*, 2000, Krejci *et al.*, 2002). RPA is thought to remove the secondary structures on ssDNA, although when preincubated with the DNA prior to addition of Rad51, it has an inhibiting effect on the strand exchange reaction. Recently, it has been suggested that RPA could also prevent Rad51 from binding to secondary binding sites by sequestering ssDNA and enhancing the probability of formation of a stable joint molecule (Sung, 1994, Van Komen *et al.*, 2002). *In vivo* Rad51 filament is thought to perform homology search and invade the dsDNA template, thereby initiating the homologous recombination reaction.

Yeast cells lacking Rad51 are viable but display strongly reduced mitotic and meiotic recombination and are sensitive to IR (Shinohara *at al.*, 1992, Game, 1993). Depletion of Rad51 in chicken DT40 cells leads to accumulation of chromosomal abnormalities and cell death (Sonoda *et al.*, 1998). Targeted disruption of *Rad51* in mouse results in early embryonic lethality at day 8.5. *Rad51/p53* double mutant embryos progress to day 8.5 of development, but no full suppression by the *p53* mutation occurs (Lim *et al.*, 1996, Tsuzuki *et al.*, 1996). Together these observations suggest that Rad51 plays an important role in proliferation processes.

Rad51 interacts *in vitro* with a number of proteins involved in DSB repair: members of Rad52 epistasis group: Rad52, Rad54 and Rad51

paralogues, but also with DMC1, RPA, BRCA2. The latter protein was implicated in DSB repair recently and is thought to play a controlling role upstream of Rad51 (Shinohara et al., 1999, Davies et al., 2001, Moynahan et al., 2001, Orelli et al., 2001). Functional significance of Rad51 interaction with tumor suppressor p53 and SUMO-1 and UBL1/UBC9, two proteins involved in cellular protein traffic, has not yet been established (Shen et al., 1996, 1996, Sturzbecher et al., 1996).

During the S and G2 phase of the cell cycle mammalian Rad51 forms nuclear foci (local concentration of the protein). It is assumed that Rad51 foci are formed at sites of DNA damage, more specifically at sites of DSBs. It has been shown that the number of Rad51 foci increases after treatment of cells with DSB inducing agents and these foci accumulate at regions containing DNA damage and ssDNA (Raderschall et al., 1999). Rad51 foci are also present at stalled replication forks (Tashiro et al., 1996, 2000). Rad51 co-localizes with Bloom's syndrome protein (BLM), BRCA2, Rad54 and the phosphorylated form of histone variant (γH2AX) (Chen et al., 1998,1999, Tan et al., 1999, Paull et al., 2000, Wu et al., 2001). Rad51 foci are not formed in cells lacking BRCA1 and all the Rad 51 paralogues (see below). The functional significance of the foci has not been well understood.

Both in yeast and in vertebrates paralogues of Rad51 are present. Meiosis specific Rad51 homologue - DMC1 shares 54% amino acid sequence identity with Rad51. It promotes recombination between homologous chromosomes during meiosis, in contrast to sister chromatid recombination occurring during mitosis (Masson et al., 2001). Both Rad51 and DMC1 are required for proper HR during meiosis.

Paralogues arose probably by gene duplication and through evolution gained different function from the ancestor gene. In HR Rad51 paralogues are thought to assist Rad51 in the initiation of the process and/or processing of HR intermediates. There are two Rad51-like proteins in Saccharomyces cerevisiae - Rad55 and Rad57. They form a heterodimer interacting with Rad51 and stimulate Rad51-mediated strand exchange by removing RPA from ssDNA. The recombination defect and IR sensitivity of rad55 and rad57 mutants can be overcome by over-expression of Rad51 or Rad52 (Hays et al., 1995, Sung, 1997).

In addition to Dmc1, in mammalian cells five Rad51 paralogues have been identified: XRCC2, XRCC3, Rad51B, Rad51C, Rad51D. They have limited sequence homology to Rad51, mainly limited to Walker A and B domain potentially involved in ATP hydrolysis. Each of the paralogues interacts with Rad51 and with each other but not with itself (Thacker, 1999, Schild et al., 2000). Disruption of the paralogues in vertebrate cells leads to chromosomal instability, moderately increased IR sensitivity and significantly increased sensitivity to the cross-linking agent mitomycin C (MMC) (Cui et al.,

1999, Takata *et al.*, 2001). It has been reported that XRCC2 promotes DSB repair via HR whereas XRCC3 most likely takes part in the later stage of HR (Griffin *et al.*, 2000, Brenneman *et al.*, 2002). Similarly to *Rad51*-deficient mice, targeted disruption of *Rad51B*, *Rad51D* and *Xrcc2* results in embryonic lethality (Pittman *et al.*, 1998, Shu *et al.*, 1999, Deans *et al.*, 2000). Although data strongly suggest the involvement of the paralogues in HR, none of them can substitute for Rad51 hinting towards functional diversity and supporting the key role of Rad51 in HR.

3.2 Rad52

Rad52 is a central HR protein in yeast. *rad52* mutants display the most severe recombination phenotype of all *RAD52* epistasis group mutants. *rad52* mutants are extremely sensitive to DNA damaging agents and are almost completely deficient in all pathways of HR (Game *et al.*, 1974, Paques *et al.*, 1999). In vertebrates *Rad52* mutants have only two-fold decreased HR level in comparison to wild type cells (Rijkers *et al.*, 1998, Yamaguchi-Iwai *et al.*, 1998). It is likely that the function of Rad52 in mammals is taken over by Rad51 paralogues (Fujimori *et al.*, 2001, Modesti *et al.*, 2001). *In vitro* Rad52 binds to ssDNA and protects the ends from nucleolytic degradation and forms rings interacting with DNA (Shinohara *et al.*, 1998, Van Dijk *et al.*, 1999, Parsons *et al.*, 2000, Stasiak *et al.*, 2000, Ranatunga *et al.*, 2001, Kagawa *et al.*, 2002, Loyd *et al.*, 2002, Singleton *et al.*, 2002). It stimulates Rad51-mediated strand exchange by overcoming the inhibitory role of RPA. It interacts also with Rad51 and RPA and forms foci colocalizing with both proteins. (Sung *et al.*, 1997, Benson *et al.*, 1998, Gasior *et al.*, 1998, New *et al.*, 1998, Shionara *et al.*, 1998, Liu *et al.* 1999, 2000).

In yeast, Rad59, a sequence homologue of Rad52 is present. Similarly to Rad52, it stimulates annealing of complementary ssDNA molecules, but in contrast to Rad52 in an RPA independent manner. Rad59 is required for the completion of Rad51-dependent recombination (Bai *et al.*, 1996, Pethukova *et al.*, 1999, Davis *et al.*, 2001).

3.3 Rad54

Rad54 belongs to the SWI2/SNF2 family of proteins involved in many biological processes such as transcriptional activation and repression, destabilization of nucleosomes, DNA repair and chromosome segregation (Pazin *et al.*, 1997). Although Rad54 contains seven conserved helicase motifs, characteristic for the SWI2/SNF2 family, no helicase activity has been detected for Rad54. It is a dsDNA-dependent ATPase, and forms damage-induced foci colocalizing with Rad51 foci (Petukova *et al.*, 1998,

Swagemakers *et al.*, 1998, Tan *et al.*, 1999). Rad54 interacts with Rad51 in yeast two-hybrid system and stimulates Rad51-mediated strand exchange *in vitro* by binding to assembled Rad51-ssDNA nucleoprotein filament (Golub *et al.*, 1997, Petukhova *et al.*, 1998, Mazin *et al.*, 2000). It also stimulates the Rad51-mediated extension of heteroduplex DNA in estabilished joint molecules and disassembly of Rad51 from the dsDNA in the postsynaptic stage of recombination (Solinger *et al.*, 2001, 2002). Rad54 induces topological changes in DNA. Rad54 introduces supercoiled regions into nicked plasmid in an ATP-dependent manner and the reaction is stimulated by addition of Rad51 nucleoprotein filament (Van Komen *et al.*, 2000, Mazin *et al.*, 2000, Ristic *et al.* 2001). *In vivo* Rad54 could translocate along DNA resulting in a change of the local helix that facilitates the invasion of Rad51 nucleoprotein filament. In this way, partial unwinding of the DNA could make it more accessible for other proteins, allowing recombination to proceed.

Yeast, mouse and chicken cells deficient in *Rad54* are viable although the decreased levels of recombination and increased sensitivity to IR and cross linking agents points to defective HR (Bezzubova *et al.*, 1997, Shinohara *et al.*, 1997, Essers *et al.*, 1997, Dronkert *et al.*, 2000).

3.4 Rad54B

In yeast a Rad54 homologue, called RDH54/TID1, has been identified (Dresser *et al.*, 1997, Klein, 1997). Both proteins share a homology limited to the ATPase Walker A and B domains and seven helicase motifs characteristic for the SWI2/SNF2 protein family. TID1 is a DNA binding protein, similarly to Rad54 - it is a dsDNA dependent ATPase and in yeast cooperates with Rad51 in D-loop (joint molecule) formation (Petukhova *et al.*, 2000, Tanaka *et al.*, 2002). Just as Rad54, TID1 can introduce positive and negative supercoils to a plasmid in the presence of ATP. In two hybrid studies TID1 was shown to interact with Rad51 and DMC1. It promotes colocalization of both proteins in meiosis, likely by directing DMC1 to sites of Rad51 (Shinohara *et al.*, 2000). In yeast there is a clear difference in roles of Rad54 and TID1. While Rad54 is important in mitosis using sister chromatid as a template for recombination, TID1 is important in meiosis and directs recombination towards using the homologous chromosome. Yet the functions of the two proteins overlap, since Rad54 can partially substitute for TID1 (Shinohara *et al.*, 1997, Klein, 1997, Arbel *et al.*, 1999). In haploid cells TID1 is required for adaptation from G2/M arrest after induction of DSB. It is suggested that it could interact with RPA and other ssDNA-binding proteins in assessment of ssDNA generated after processing of DSBs (Lee *et al.*, 2001). This is a first indication that TID1 could have a function independent of other recombination proteins, including Rad54.

Recently human *Rad54B* has been cloned (Tanaka *et al.*, 2000). Rad54B binds to both ssDNA and dsDNA and is a dsDNA-dependent ATPase. Its ATPase activity is low as compared to human Rad54 (Tanaka *et al.*, 2002). Human Rad54B is a nuclear protein. Following IR treatment human Rad54B forms foci that colocalize with Rad51, Rad54 and Brca1 foci (Tanaka *et al.*, 2000.

In Chapter 4 of this thesis we describe cloning of murine *Rad54B*. Described is generation of *Rad54B* deficient mice and cells and their phenotypes.

3.5 Rad50/ Mre11/ NBS1 complex

The presence of Rad50/Mre11/NBS1 complex is required for proper functioning of DSB repair, although its role is still elusive (Paques *et al.*, 1999). The complex consists of two proteins, Rad50 and Mre11, conserved from yeast to human, while the third subunit, NBS1 in mammals and XRS2 in yeast, is less conserved at the amino acid level (Johzuka *et al.*, 1995, Dolganov *et al.*, 1996, Carney *et al.*, 1998). In yeast the complex is involved in NHEJ, sister chromatid repair by HR, telomere maintenance and in formation and processing of DSBs in meiosis (Ivanov *et al.*, 1992, Boulton *et al.*, 1998, Bressan *et al.*, 1999). Mre11 is a core protein of this complex. Mammalian Mre11 forms homodimers and multimers and interacts with Rad50 and NBS1. Biochemical analysis of Mre11 revealed its strand dissociation, strand annealing and 3'-5' exo/endo dsDNA nuclease activity properties (Paull *et al.*, 1998, Trujillo *et al.*, 1998, De Jager *et al.*, 2001). Mre11, Rad50 and NBS1 localize in damage-induced foci (MRN foci). NBS1 is required for phosphorylation and translocation of the complex to the nuclear foci after induction of DNA damage (Carney *et al.*, 1998). MRN foci do not colocalize with Rad51 foci (Maser *et al.*, 1997). The complex also forms DNA-replication foci observed throughout S phase of the cell cycle. These foci colocalize with replication protein PCNA and are associated with DNA replication forks (Maser *et al.*, 2001, Cleaver *et al.*, 2002).

Conditional inactivation of *Mre11* in chicken cells causes accumulation of chromosomal breaks, increased radiosensitivity and reduced targeted integration frequencies, a measure of HR efficiency (Yamagushi-Iwai *et al.*, 1999). *NBS1*-deficient chicken cells display similar defects as the *Mre11* knockout cells, with additional reduction of gene conversion levels and lower rates of sister chromatid exchanges (Tauchi *et al.*, 2002). *Mre11, Rad50* and *NBS1* null mutations in mice leads to embryonic lethality indicating the importance of this complex for the function of the cell (Xiao *et al.*, 1997, Luo *et al.*, 1999, Zhu *et al.*, 2001). Mutations in *Mre11* gene have been found in patients with ataxia telangiectasia-like disorder, mutations in *NBS1* cause the

Nijmegen breakage syndrome (Carney et al., 1998, Steward et al., 1999). Cells derived from patients with both disorders are sensitive to DSB inducing agents and display radioresistant DNA synthesis (Kraakman-van der Zwet et al., 1999).

The exact role of the MRN complex in DSB repair is unclear. The MRN complex mutant cells perform mitotic HR with efficiency comparable to wild type cells, with an exception of sister chromatid exchanges - specific type of HR and defective gene-targeting capability in NBS1- and Mre11- deficient chicken cells, respectively (Yamagushi-Iwai et al., 1999, Tauchi et al., 2002). The direct involvement of Mre11 complex in NHEJ has only been well documented in Saccharomyces cerevisiae (Ivanov et al, 1994, Schiestl et al., 1994, Boulton et al., 1998, Moreau et al., 1999). NHEJ appears to work in the absence of Mre11 in chicken cells (Yamagushi-Iwai et al., 1999). In NBS1 patient cell lines the V(D)J recombination is unaffected (Harfst et al., 2000). The complex was proposed to be responsible for the nucleolytic processing of DSBs, but the in vitro 3'-5' nuclease activity of Mre11 does not match the 5'-3' nuclease activity required in vivo. The architecture of Mre11/ Rad50 complex hints towards ends-bridging activity of the complex. Holding the two DNA ends together might stimulate the search for short or extended homology regions, used by NHEJ and HR, respectively (de Jager et al., 2001, Hopfner et al., 2002).

There are indications that the Mre11 complex takes part in the cell cycle checkpoint cascade. Mammalian Mre11 and NBS1 are phosphorylated in response to IR by ATM. Ultraviolet light (UV), hydroxyurea or methylmethane sulfonate (MMS) treatment also leads to phosphorylation of both proteins but most likely by ATR (Gatei et al., 2000, Lim et al., 2000). ATM and ATR are crucial components of the damage signaling pathway, phosphorylating downstream proteins responsible for cell cycle arrest, DNA repair, induction of apoptosis and halting of replication (Jackson, 2002). The presence of the Mre11 complex is required for proper activation of checkpoints throughout all cell cycle phases (D'Amours et al., 2002). The complex could serve as a signal modifier, it could be responsible for nucleolytic modification of the lesions to make them "visible" for the checkpoint machinery.

3.6 Brca1 and Brca2

Mutations in BRCA1 and BRCA2 – breast cancer susceptibility genes, predispose to breast, ovarian, prostate and pancreatic cancers (Rahman et al., 1998, Zheng et al., 2000). The gene products are two nuclear proteins that colocalize in somatic cells and on early meiotic chromosomes and relocate to replication sites after UV and hydroxyurea treatment (Chen et al., 1998).

Mouse Brca1- and Brca2- deficient and human mutant cell lines display chromosome instability and sensitivity to DNA damaging agents (Scully *et al.*, 2000). Both proteins are required for homology-directed repair and gene targeting events. In comparison to wild type cells gene targeting is 20-fold and 2-fold decreased in Brca1 and Brca2 mutant cells, respectively (Moynahan *et al.*, 1999, 2001). Similarly to the Rad51 phenotype, targeted disruption of *Brca1* and *Brca2* in mice leads to embryonic lethality associated with a proliferation defect partially suppressed by a p53 mutation (Gowen *et al.*, 1996, Hakem *et al.*, 1996, Ludwig *et al.*, 1997, Suzuki *et al.*, 1997). Both Brca1 and Brca2 colocalize in foci with Rad51 during S phase, after induction of DNA damage and at early synaptonemal complexes during meiosis (Scully *et al.*, 1997, Chen *et al.*, 1998). Although both proteins are involved in DSB repair they seem to have a different contribution to the process. Brca1 interacts with Mre11-Rad50-Nbs1 complex and can be found in damage-induced Mre11 foci (Scully *et al.*, 1997, Zhong *et al.*, 1999). It is required not only for homology directed repair but also facilitates microhomology-mediated end joining (Moynahan *et al.*, 1999, Zhong *et al.*, 2002). Brca2 interacts with Rad51 and is needed for Rad51 foci formation (Wong *et al.*, 1997, Marmorstein *et al.*, 1998, Yuan *et al.*, 1999). Crystallographic data, characterizing the conserved BRC repeats and C-terminal ssDNA binding folds of Brca2 suggest that Brca2 most likely recruits Rad51 to a DSB and regulates spatial distribution of Rad51 (Pellegrini *et al.*, 2002, Yang *et al.*, 2002).

The Brca proteins could link DSB repair and cell cycle control processes potentially transducting DNA damage signals to effector proteins responsible for cell cycle arrest, induction of apoptosis and other processes required to be halted. Brca1 is phosphorylated by ATM and ATR. Additionally *Brca1* mouse knockout cells display abnormalities in G2/M checkpoint enforcement whereas Brca2 could be involved in metaphase progression (Futamura *et al.*, 2000, Ventiktaranen, 2001, Jackson, 2002). Although there is evidence connecting Brca proteins to the cell cycle their exact role in the chain of events in this process remains to be established.

3.7 Other proteins involved in homologous recombination

HR is a complex process and not all proteins involved in this reaction have been identified. The number of proteins known to participate in or to be connected to HR has significantly increased over the last years. The variety of substrates HR acts on could explain the diversity of proteins required for successful completion of HR.

Human homologues of *E.coli* RecQ helicase: Bloom, Werner and Rothmund-Thomson proteins (BLM, WRN and Recql 4, respectively) are

thought to resolve abnormal replication structures after the replication forks stall or collapse, preventing occurrence of DSBs (Frei *et al.*, 2000). These proteins could also promote joint molecule formation and take part in the resolution of joint molecules. The three proteins are ATP dependent 3'-5' helicases and can unwind forked DNA structures and synthetic Holliday junctions *in vitro* (Chakraverty *et al.*, 1999, Karow *et al.*, 2000). Mutations in BLM, WRN or SGS1 the yeast *RecQ* homologue: SGS1, lead to chromosomal instability and increased risk of tumor formation in patients (German *et al.*, 1974, Fukuchi *et al.*, 1989, Vennos *et al.*, 1995). Patient derived cell lines accumulate abnormal replication intermediates (Chakraverty *et al.*, 1999). BLM mutant cells are characterized by hyperrecombination and increased numbers of sister chromatid exchanges. WRN cells have increased levels of translocations and deletions (Chakraverty *et al.*, 1999). The Blm protein interacts with Rad51 and RPA, WRN protein with DNA-PK (see section 5) and RPA (Brosh *et al.*, 2000, Constantinou *et al.*, 2000, Cooper *et al.*, 2000, Wu *et al.*, 2000, 2001). After induction of DSBs the Wrn and Blm proteins form foci partially colocalizing with Rad51 (Bischof *et al.*, 2001, Sakamoto *et al.*, 2001).

Mismatch repair proteins, like Msh2, Msh3, Msh6, Mlh1 and Pms1 inhibit ectopic recombination between sequences with heterologous regions, preventing translocations (Chen *et al.*, 1998, 1999). They are also required for correction of heteroduplex DNA formed during HR in meiosis (Weng *et al.*, 1998, Elliot *et al.*, 2001).

4. DSB in meiosis

The purpose of meiosis, a process highly conserved in eukaryotes, is to generate haploid gametes from diploid cells. Meiosis differs from somatic cell division - mitosis, since in this process two rounds of cell division, meiosis I and meiosis II, follow a single round of chromosome replication. During the first division, reductional chromosome segregation occurs. After replication, in the prophase of first meiotic division, replicated maternal and paternal chromosomes pair (see Figure 3). Each replicated chromosome pairs with its homologue and forms a bivalent containing four chromatids. At this step meiotic recombination, exchange of genetic information, occurs between non-sister chromatids. These recombination events, also termed meiotic crossovers, play an important role in creation of genetic diversity among individuals.

In mammals meiotic prophase I can be divided in four distinct stages: leptotene, zygotene, pachytene and diplotene. All the events of prophase I occur within a proteinaceous structure called synaptonemal complex (SC), that serves as a framework tethering the homologous chromosomes. The SC
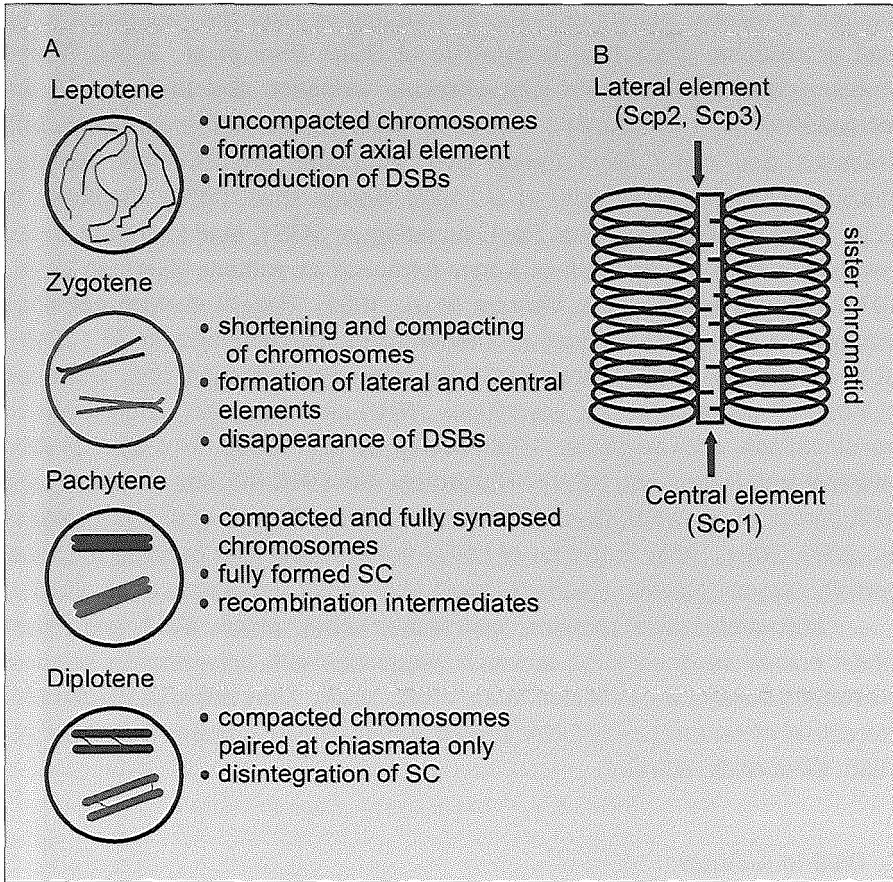
Figure 3. Meiosis in mammals. (A). Meiotic prophase I. After DNA replication, in leptoptene single chromosomes comprised of sister chromatids are visible. As the cell progresses through prophase I, chromosomes become dense, pair and recombine. (B). Simplified schematic representation of mature synaptonemal complex (SC). Chromatin loops are anchored to the lateral elements of the SC, comprised of Scp3 and Scp2, the central elements consists of Scp1. Adapted from Roeder, 1997.

contains two axial elements that are connected by a central element, which functions as a zipper during synapsis. The central element is composed mainly of a meiotic specific protein Scp1 (synaptonemal complex protein 1). The axial/lateral elements contain the meiosis specific proteins Scp2 and Scp3 (Meuwissen et al. 1992, Schalk et al., 1998) (see Figure 3B).

In leptotene long, uncompacted single homologues are visible, unsynapsed axial elements composed of Scp3 start to form, meiotic recombination is initiated and DSBs appear. Shorter and compacted chromosomes that start to synapse characterize zygotene. Mature SC starts to form; Scp1 accumulates

at the central elements. Scp3-containing axial elements become closely connected to each other and from that moment are referred to as lateral elements. At this phase DSBs disappear. In pachytene all the chromosomes are compacted, fully synapsed, except in male meiosis and the presence of HR intermediates (Holliday junctions) can be detected. In diplotene SC disassembles, still condensed chromosomes are paired only at the sites of recombination, represented by so-called chiasmata (For details see Scp3 distribution during meiotic prophase I, Chapter 4, Figure 7A).

In later steps of the reductional first meiotic division, in metaphase the bivalents become attached to the meiotic spindle and line up at the metaphase plate, in anaphase the two replicated chromosomes are separated and pulled to the opposite poles. The interphase is followed by the second meiotic division: metaphase II, anaphase II and telophase II and leads to formation of haploid gametes.

Both in *S. cerevisiae* and in mammals Spo11 is important early in meiotic recombination because it induces a DSB that is required for subsequent HR between homologous chromosomes. Expression of this gene is restricted almost entirely to gonadal tissues, both, in human and mice (Romanienko *et al.*, 1999). Mice lacking Spo11, both females and males, are infertile. Oocytes and spermatocytes progress through the early prophase, but failure in synapsis of homologous chromosomes, triggers programmed cell death (apoptosis) (Baudat *et al.*, 2000, Romanienko *et al.*, 2000).

In mice, proteins involved in processing of recombination intermediates are localized to, so-called, recombination nodules that first outnumber the recombination sites and eventually, in pachytene, reflect the true sites of HR. Rad51 is a component of early recombination nodule (Barlow *et al.*, 1997). Already in Rad51 leptotene starts to localize to meiotic chromosomes in the form of foci. The foci persist till pairing of homologous chromosomes occurs and disappear in pachytene (see Chapter 4, Figure 7A).

Localization and expression pattern of Dmc1, a meiosis specific Rad51 paralogue, mirrors that of Rad51 (Yoshida *et al.*, 1998). Both proteins colocalize on SC in zygotene and pachytene in mouse spermatocytes (Tarsounas *et al.*, 1999). Although mice deficient in Dmc1 are viable, disruption of *Dmc1* causes sterility. In *Dmc1* knockout meiotic cells the chromosomes fail to condense and pair and as a result undergo apoptosis (Pittman *et al.*, 1998, Yoshida *et al.*, 1998). The role of Dmc1 in meiosis has not been clarified in detail. However, it appears that Dmc1 is important for pairing between homologous chromosomes in a manner analogous to Rad51's function in pairing sister chromatids during mitotic recombination (Bishop *et al.*, 1992, 1994, Arbel *et al.*, 1999).

5. Non-homologous end joining

In the NHEJ reaction the two ends of the DSB are ligated directly or with use of microhomology – a few homologous bases near the ends. The presence of template DNA is not required for this process. In the first step of the repair reaction the Ku70/80 heterodimer binds to the DNA ends, probably to protect the break from nucleolytic processing (see Figure 4). Binding of the
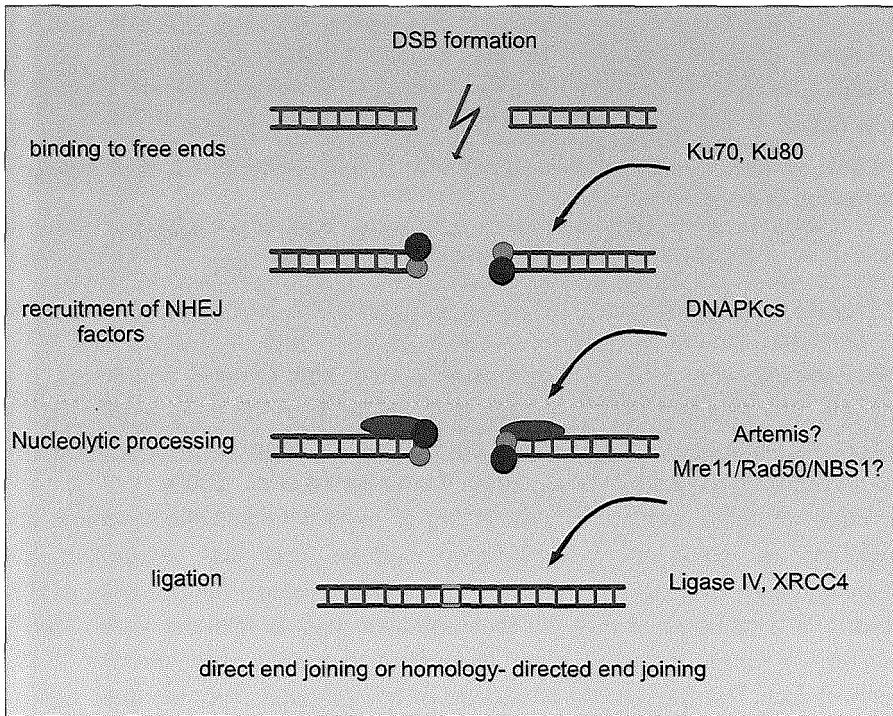

Figure 4. Proteins involved in non-homologous end joining. Different factors and their possible function in the repair by NHEJ are depicted. For details see section 5.

Ku heterodimer attracts the third component of the DNA-PK complex: DNA-PK catalytic subunit (DNA-PKcs). The complex could be responsible for bringing the ends together and for phosphorylation and recruitment of other NHEJ factors. Ligase IV and XRCC4 perform the final step of the reaction – ligation of the broken ends. A subset of DSBs needs nucleolytic processing prior to ligation. The Mre11 complex is most likely the nuclease candidate in yeast cells, although flap-specific nuclease Rad27 could also be involved. In mammalian cells FEN1 (Rad27 in yeast) and recently identified Artemis (see below) might perform the end processing (Kanaar *et al.*, 1998, Hoeijmakers *et*

al., 2001, Pastink *et al.* 2001, Jackson, 2002). Human polynucleotide kinase could also be a reasonable candidate to execute the processing, but only within the context of NHEJ (Chappel *et al.*, 2002).

6. DNA rearrangements in the immune system

In order to recognize a wide range of antigens, the immune system needs to produce a large diversity of immunoglobulins and T cell receptors interacting with the antigens. Various vertebrates developed a number of processes leading to gene-specific modifications ensuring a production of a large variety of immunoglobulins and T cell receptors: V(D)J recombination, gene conversion, somatic hypermutation and class switch recombination V(D)J recombination occurs during development of B and T cells. It is a site-specific recombination/repair process between the V (variety), D (diversity) and J (joining) segments, which produce the mature T-cell receptor and immunoglobulin genes.
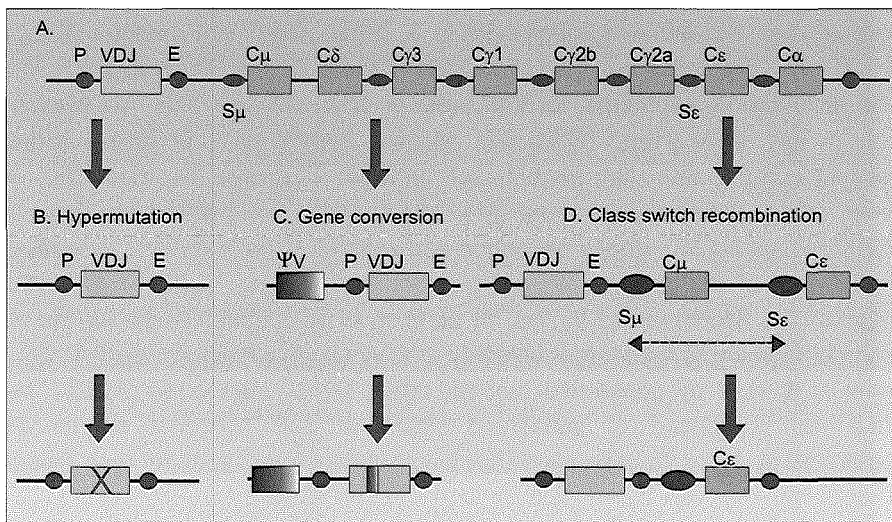

Figure 5. Processes leading to diversity of Ig genes. (A) Schematic representation of the Ig heavy chain locus: V, D, J and constant (C) regions are depicted as boxes. P and E indicate promoter and enhancer, respectively. The constant region (C_H) consists of an array of C_H genes. The $C\mu$ gene is adjacent to rearranged V_H, D_H, J_H segments. Each C_H gene except for C_δ is flanked by a repetitive switch (S) region. (B) During somatic hypermutation point mutations are introduced in the "hot spots" of VDJ exon. Point mutations are schematically depicted by the cross in the VDJ exon box. (C) During gene conversion sequence information is transferred from a pseudo V gene (ψV) into the functional V segment. (D) CSR occurs between two S regions and results in deletion of intervening region in the form of circular DNA (not depicted in the figure). Adapted from Papavasiliou *et al.*, 2002.

Gene conversion (GC), somatic hypermutation (SHM) and class switch recombination (CSR) are adaptive processes and occur in B cells after exposure to an antigen, in most cases (see Figure 5). After antigen recognition B cells migrate to germinal centers in the spleen and the peripheral lymph nodes to undergo clonal expansion and mutations. These processes lead to selection of B cells carrying the immunoglobulins with the highest affinity to the antigen. GC introduces templated change using sequences of pseudogenes. During SHM the random mutations are introduced in the VDJ exon. As a result, both V(D)J recombination and SHM changes the antigen specificity of the immunoglobulin. CSR affects the constant region of immunoglobulin heavy chain and alters its effector functions. The three processes are used to create the antibody repertoire to a different extent in different species (Papavasiliou *et al.*, 2002).

6.1 V(D)J recombination

During V(D)J recombination, immunoglobulin and T cell receptor genes are assembled from existing gene segments. Mammals have seven antigen receptor loci: the immunoglobulin (Ig) H, κ, λ loci and T cell receptor β, α, γ and δ loci. Each locus consists of variable (V) and joining (J) gene segments. The diversity (D) segments, located between V and J segments, are present only in IgH, TCR β and TCRδ loci. Multiple copies of V, D, and J exons present in the mammalian genome are arranged in arrays. The possibility of using one of many copies of the gene segments is one mechanism (next to V(D)J recombination, gene conversion and somatic hypermutation) increasing the variety of the antigen receptors repertoire. Joining of the V, D and J segments is accompanied by loss or addition of nucleotides at the junctions, also contributing to creation of diversity of the antigen receptors. Each V, D, J gene is flanked by conserved recombination signal sequences (RSS). The RSS are composed of conserved heptamer and nonamer sequences separated by 12 or 23 base pairs (bp) long nonconserved spacers (see Figure 6).

The first step of the V(D)J recombination is recognition of RSS sites and introduction of the DSB at the border of the RSS. The intervening DNA is excised and the gene segments are joined. The process results in generation of coding and signal joints. Signal joints are formed by fusion between two heptamer sequences, which is rarely associated with loss or gain of nucleotides. The coding joints are variable. The loss of several nucleotides from one or both ends occurs very often, but the factors involved in this process have not been identified. The non-templated insertions of one or a few base pairs (up to 15) - are introduced by deoxynucleotidyl transferase (TdT), with preference for G residues resulting in GC - rich N (from non-
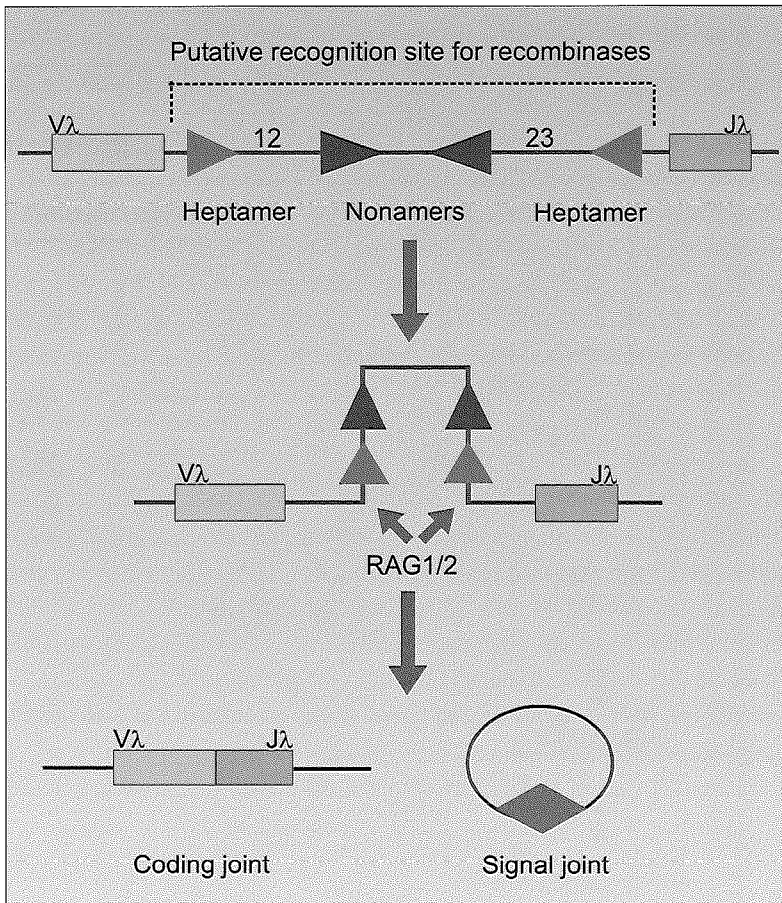
Figure 6. Schematic representation of V(D)J recombination. The following stages of the reaction are depicted: recognition of the RSS and cleavage of the DNA by the RAG1and RAG2 proteins and formation of the products: coding and signal joints. Coding joint consists of the Vλ and Jλ gene segments; signal joint contains RSS and is discarded.

templated) regions. Templated types of additions originate from off-center nicking of hairpin intermediates formed by RAG proteins (see below and Figure 7B) (Bassing *et al.*, 2002, Gellert, 2002).

6.2 Biochemistry of V(D)J recombination

V(D)J recombination is a process combining actions of lymphoid specific RAG proteins and several factors involved in NHEJ-mediated DSB repair. RAG1 and RAG2 are indispensable for initiation and progression of the V(D)J recombination (Mombaerts *et al.*, 1992, Shinkai *et al.*, 1992). In the first
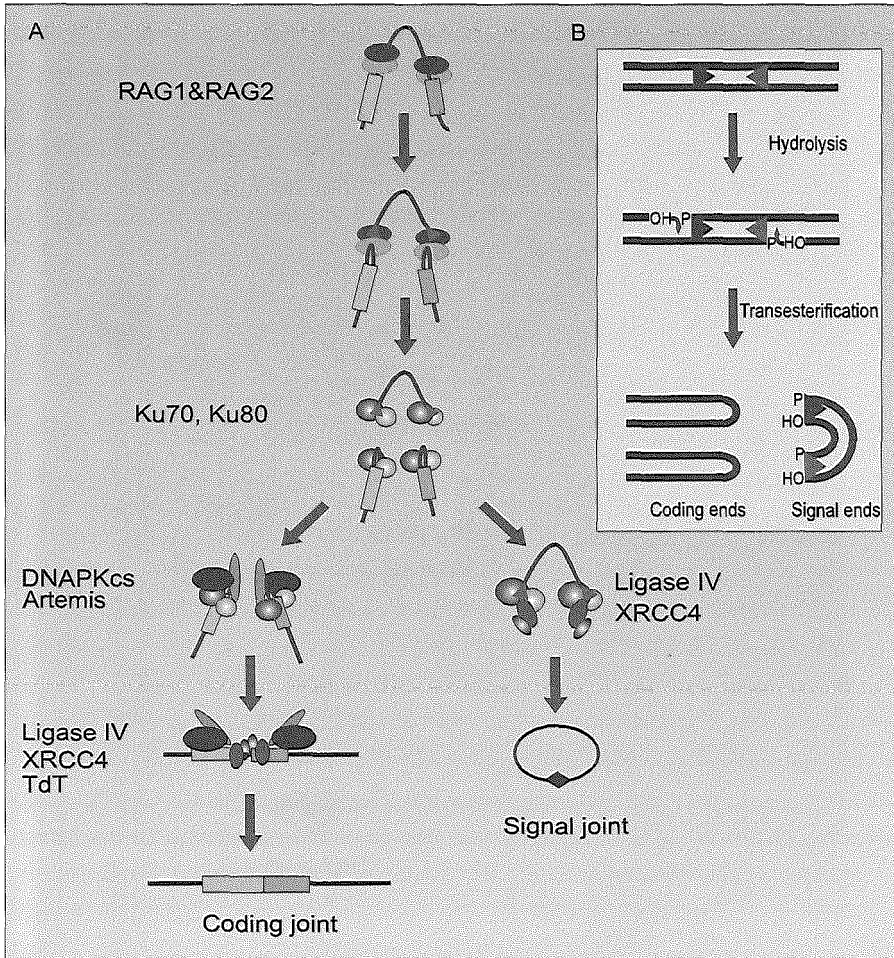
Figure 7. V(D)J recombination. (A) RAG proteins (ovals) initiate the reaction by recognition and binding to the RSSs (triangles). After cleavage and hairpin formation the Ku proteins (circles) bind to the free ends protecting them from degradation and attract DNA-PKcs (dark oval). The hairpins are cleaved by DNA-PKcs/ Artemis (light oval) complex and Ligase IV/XRCC4 complex (small ovals) ligates the break. (B) Representation of the hairpin formation reaction. A nick at 5' end of the heptamer is made by RAG1 and RAG2, leaving 3'-OH on the coding end. The 3'-OH group attacks the opposite strand and coding and signal ends are formed.

stage of the reaction RAG proteins recognize the RSS and make a break at the coding sequence/heptamer border (see Figure 7). The cleavage occurs in two steps: first a nick is introduced at the 5' end of the heptamer sequence leaving a phosphoryl group on the RSS end and the 3' hydroxyl group on the coding end. In the second step the 3' hydroxyl group attacks the opposite

DNA strand to form closed hairpins on the coding ends and blunt phosphorylated RSS ends (see Figure 7B) (McBlane *et al.*, 1995, van Gent *et al.*, 1996). The hairpin ends produced by RAG proteins must be reopened before the coding ends can be joined. This reaction is most likely catalyzed by the DNA-PKcs/Artemis complex. DNA-PKcs probably phosphorylates Artemis, triggering its nuclease activity necessary for hairpin cleavage (Ma *et al.*, 2002). Furthermore, rejoining of signal and coding ends require the Ku heterodimer, which probably protects the ends and recruits other factors like DNAPKcs and Ligase IV/XRCC4 catalyzing the ligation, the last step of V(D)J recombination.

The involvement of end joining proteins in the V(D)J recombination became apparent after analysis of *scid* (severe combined immunodeficiency) mice carrying point mutation in the DNAPKcs gene, followed by generation of mice deficient in Ku70, Ku80, DNA Ligase IV, XRCC4 and Artemis (Bosma *et al.*, 1991, Nussenzweig *et al.*, 1996, Gu *et al.*, 1997, Barnes *et al.*, 1998, Gao *et al.*, 1998, Rooney *et al.*, 2002). The *scid* mice and *DNAPKcs* knockout mice, except irradiation sensitivity and defective DSB repair, display immunodeficiency caused by defects in coding joints formation (Bosma *et al.*, 1991, Gao *et al.*, 1998, Taccioli *et al.*, 1998, Kurimasa *et al.*, 1999). The function of DNA-PKcs in the V(D)J recombination was unclear until the Artemis gene was identified and *in vitro* experiments showed the ability of the DNAPKcs/Artemis complex to open the hairpins (Moshous *et al.*, 2001, Ma *et al.*, 2002). Targeted disruption of Artemis causes immunodeficiency resulting from defective coding end processing, similar to *scid* phenotype (Rooney *et al.*, 2002). The Ku80 and Ku70 mutant mice have impaired formation of both coding and signal joints, suggesting an additional role of the heterodimer, independent of DNA-PK complex (Nussenzweig *et al.*, 1996, Ouyang *et al.*, 1997, Gu *et al.*, 1997, 2000). DNA Ligase IV and XRCC4 deficiency result in embryonic lethality, suggesting greater impairment of NHEJ in comparison to Ku-deficient mice (Barnes *et al.*, 1998, Gao *et al.*, 1998, Frank *et al.*, 1998, 2000).

6.3 Somatic hypermutation

Somatic hypermutation is often described as a fine-tuning process allowing generation of antibodies that tightly bind to antigens. The exact mechanism and proteins involved in this reaction are unknown. The process is thought to occur in three steps: targeting of a nuclease to the site, cleavage of the DNA and repair (Papavasiliou *et al.*, 2002). The DNA break, thought to be a single- or double strand break, would be introduced by yet to be identified factor(s). The break would require processing, possibly involving HR or NHEJ

proteins. In the last step the break could be filled by error prone polymerase and ligated.

Although during this process mutations are introduced throughout all three exons (V, D and J), a mutation "hot spot" region of the V gene, so-called hypermutation domain (HYM domain) has been identified. It is an approximately 2 kb region, localized downstream of the immunoglobulin promoter region. Mutations in the HYM domain (containing up to 50% of all mutations introduced) are mostly point mutations, but also deletions and duplications occur (Jacobs *et al.,* 2001). The evolutionary conserved RGYW motif (where R is A or G, Y is C or T and W is A or T) is the most common target of the hypermutation machinery. Particularly, two triplets AGC and AGT, both serine codons, have been identified as the mutation hot spots (Rogozin *et al.*, 1992). Transitions are introduced more frequently than transversions, with two-fold preference of A over T nucleotides substitution. The frequency of mutations is correlated with the rate of transcription of the locus and is additionally regulated by enhancers (Goyenechea *et al.*, 1997, Bachl *et al.*, 1998, 1999, Papavasiliou *et al.*, 2000).

Although the exact molecular mechanism of somatic hypermutation is not known, two proteins involved in the reaction have been identified.

AID (activation induced cytidine deaminase) was shown to be indispensable for activation of SMH, class switch recombination and gene conversion (Muramatsu *et al.*, 2000, Arakawa *et al.*, 2002). AID is an enzyme belonging to a family of RNA editing enzymes and is thought to regulate the activity of a nuclease introducing the lesion in the first step of somatic hypermutation (Kinoshita *et al.*, 2001). It has also been proposed that AID deaminates the genomic DNA and generate G/U mismatches, which if left unrepaired could lead to transitions after replication. AID could also produce abasic sites that can lead to a single-strand break, followed by misincorporation (Petersen *et al.*, 2002).

In vitro studies of a Burkitt's lymphoma cell line lacking low fidelity DNA polymerase iota (polι) generated evidence of the involvement of polι in SHM. No detectable frequency of mutations was observed in polι deficient cell line after induction for hypermutation (Faili *et al.*, 2002). Although polι clearly participates in somatic recombination the involvement of other error prone translesion synthesis polymerases has not yet been excluded (Gearhart *et al.*, 2001).

There is evidence that a DSB is present at the site of the lesion in the first stage of SHM, although factors involved in this reaction remain elusive (Bross *et al.*, 2000, Papavasiliou *et al.*, 2002). The core components of end-joining machinery, DNAPKcs and Ku proteins, probably do not take part in SHM, since mice lacking those genes do not display defective hypermutation (Bemark *et al.*, 2000, Sale *et al.*, 2001). Rad54 and Rad54B, two HR proteins,

are also not required for proper hypermutation (Bross *et al.*, 2002), leaving the hunt for repair proteins involved in this process still unfinished.

7. Cross links and homologous recombination

Interstrand cross-links (ICLs) are potentially a very toxic type of damage. Left unrepaired, they can block the separation of DNA strands and lead to perturbations in replication and transcription and consequently can cause mutations and DNA rearrangements. Psoralen, MMC, platinium compounds, nitrogen mustards and nitrosoureas are cross-link inducing agents. Their toxicity depends on the cellular uptake efficiency and requirement of metabolic activation (Dronkert *et al.*, 2001). In mammals components of three repair pathways have been implicated in repair of ICLs: nucleotide excision repair (NER), HR and translesion repair.

Proteins involved in recognition of the cross-links have not been identified. NER proteins (ERCC1, ERCC4/XPF) are thought to introduce the first incision on one side of the cross-link, creating a gap or a DSB if the incisions are introduced in close vicinity. Replication over the introduced gap could also create a DSB. When a sister chromatid or a homologous chromosome are available, the DSB could be repaired by the HR machinery. In absence of homologous DNA, translesion repair proteins could fulfill the repair. One of the proposed models suggests that HR factors can perform strand exchange reaction with only one 3' overhang present. This substrate would not be recognized by the NHEJ proteins. The processing of the cross-link could lead to initiation of break-induced replication or creation of a flap DNA, excised latter in the repair process (Dronkert *et al.*, 2001).

The involvement of HR in repair of ICLs emerged from analysis of mutant hamster (CHO) and mouse embryonic stem (ES) cells. It appeared that cells deficient in Rad51 paralogs, Rad54, BRCA1 and BRCA2, are sensitive to ICL agents (Jones *et al.*, 1993, Tebbs *et al.*, 1995, Essers *et al.*, 1997, De Silva *et al.*, 2000, Garcia-Higutiera *et al.*, 2001, Takata *et al.*, 2001). The exact function of the HR proteins in ICL repair remains to be established. Mutants in Rad51 paralogs are more sensitive to cross-linking agents than other HR proteins. They could be important for the strand exchange reaction when no DSB is present. Snm1 and FANC proteins have also been implicated in the repair of cross-links. In humans, mutations in FANC genes lead to an autosomal recessive disorder characterized by increased sensitivity of patient-derived cell lines to cross-linking agents. The precise role of the FANC proteins in this repair process is not defined, although they are essential for chromosomal stability after exposure to ICL agents (Chen *et al.*, 1996, Liu *et al.*, 1997, Clarke *et al.*, 1998). *Snm1* and its homologues *Snm1B* and *Artemis*

(*Snm1C*) have been identified using their homology to previously characterized yeast SNM1 (Richter *et al.*, 1992). The proteins belong to a large family of metallolactamase proteins. They share a sequence homology limited to eight conserved motifs present in all three proteins. Recent reports provide evidence of involvement of Artemis in V(D)J recombination (Moshous *et al.*, 2001, Ma *et al.*, 2002). *SNM1*-deficient mice are MMC sensitive, but the role of this protein in cross-link repair is elusive (Dronkert *et al.*, 2000).

8. Perspective

The interplay between all the processes taking place within the cell becomes more and more surprising. It appears there is no such a thing as a separate, independent reaction that is not connected to other aspects and processes of cellular metabolism. Not only the core reaction is often common between different processes, but also the key proteins are frequently engaged in multiple aspects of the daily cellular survival quest.

DSB repair mechanisms and factors involved in these reactions are perfect examples of the members of cellular interplay. Not only HR is important during meiosis, where it is responsible for creation of genetic diversity in individuals, but it is also involved in error-free repair of DSB. There are indications for a tight connections of HR with replication, where it, most likely, responsible for restart of stalled replication forks. HR is also thought to take part in initiation of genetic diversity of immunoglobulins. Similarly the NHEJ machinery is important not only for repair of IR-induced DNA damage, but is also indispensable in the generation of genetic diversity of imunoglobulins.

The complexity of the cellular communication makes it difficult to define the exact tasks of DSB repair mechanisms within the cell. Before placing them in a broader and interactive cellular context, it is crucial to define the mechanism of action and the function of proteins/genes involved in the processes.

In this thesis we concentrate on the role of three murine DNA repair genes: *Rad54*, *Rad54B* and *Snm1*. We analyze the expression of *Rad54*, its involvement in Sublethal Damage Recovery and we describe the phenotype of mice and cells lacking *Rad54B* and *Snm1*.

Chapter 2

Analysis of mouse *Rad54* expression and its implications for

homologous recombination

Analysis of mouse *Rad54* expression and its implications for homologous recombination

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Homologous recombination is one of the major pathways for repair of DNA double-strand breaks (DSBs). Important proteins in this pathway are Rad51 and Rad54. Rad51 forms a nucleoprotein filament on single-stranded DNA (ssDNA) that mediates pairing with and strand invasion of homologous duplex DNA with the assist of Rad54. We estimated that the nucleus of a mouse embryonic stem (ES) cells contains on average 4.7×10^5 Rad51 and 2.4×10^5 Rad54 molecules. Furthermore, we showed that the amount of Rad54 was subject to cell cycle regulation. We discuss our results with respect to two models that describe how Rad54 stimulates Rad51-mediated DNA strand invasion. The models differ in whether Rad54 functions locally or globally. In the first model, Rad54 acts in *cis* relative to the site of strand invasion. Rad54 coats the Rad51 nucleoprotein filament in stoichiometric amounts and binds to the target duplex DNA at the site that is homologous to the ssDNA in the Rad51 nucleoprotein filament. Subsequently, it promotes duplex DNA unwinding. In the second model, Rad54 acts in *trans* relative to the site of strand invasion. Rad54 binds duplex DNA distant from the site that will be unwound. Translocation of Rad54 along the duplex DNA increases superhelical stress thereby promoting duplex DNA unwinding.

1. Introduction

Homologous recombination is one of the major pathways for DNA repair. Together with non-homologous end-joining (NHEJ), homologous recombination repairs DNA double-strand breaks (DSBs) (Paques and Haber 1999, Sonoda *et al.*, 2001). DSBs are formed by external damaging agents like ionising radiation and a number of chemicals. They also occur during normal chromosome metabolism such as DNA replication, and during specialized events that require DNA rearrangements such as meiosis and assembly of functional immunoglobin and T cell receptor genes. Both in

Saccharomyces cerevisiae and in mammalian cells, NHEJ is relatively more important during the G1 phase of the cell cycle, while homologous recombination plays a prominent role in DSB repair in late S and G2 phases of the cell cycle (Astrom *et al.*, 1999, Takata *et al.*, 1998). Repair of a DSB by NHEJ involves a simple ligation of the ends, potentially after some processing (Lieber, 1999). Homologous recombination on the other hand uses an intact homologous sequence to ensure correct repair of the DSB. The preferred homologous template is the sister chromatid, that is present in the G2 phase of the cell cycle (Liang *et al.*, 1998, Dronkert 2000). Important proteins in homologous recombination are Rad51 and its paralogues, as well as Rad52 and Rad54 (Paques and Haber 1999, Thacker 1999, Sonoda *et al.*, 2001). During homologous recombination the DSB ends are processed to expose long 3 single-stranded DNA (ssDNA) ends. These ssDNA ends are coated with Rad51 to form a Rad51 nucleoprotein filament (Sung, 1995, Baumann, 1996). This Rad51-DNA filament can invade homologous double-stranded DNA (dsDNA). Once paired to its homologous partner, the invading DNA end can be elongated by DNA synthesis to effectively produce continuous sequence over the site of the original break. Subsequently, upon DNA unwinding, the newly-synthesized DNA of both DSB ends anneals and the nicks are ligated. Alternatively, Holliday junctions are formed and resolution of these junctions restores intact DNAs (Haber and Heyer, 2001).

The DNA strand exchange reaction by the Rad51 nucleoprotein filament is stimulated *in vitro* by RPA, the Rad51 paralogues, Rad52, and Rad54 (Sung and Robberson, 1995, Sung, 1997a,b, New *et al.*, 1998, Petukhova *et al.*, 1998, Baumann and West, 1999). RPA, the Rad51 paralogues, and Rad52 are thought to stimulate DNA strand exchange by promoting Rad51 nucleoprotein filament formation. Rad54, on the other hand, most likely stimulates the activity of Rad51 by acting after nucleoprotein filament formation. It appears to support strand invasion and heteroduplex extension of the Rad51 protein filament (Petukhova *et al.*, 1998, Mazin *et al.*, 2000, Solinger and Heyer, 2001, Solinger *et al.*, 2001). The Rad54 protein belongs to the Swi2/Snf2 protein family, whose members contain helicase motifs and are DNA-dependent ATPases (Pazin and Kadonaga, 1997). Rad54 uses the energy of ATP hydrolysis to induce topological changes into DNA (Petukhova *et al.*, 1998, Swagemakers *et al.*, 1998, Tan *et al.*, 1999, Van Komen *et al.*, 2000, Ristic *et al.*, 2001). The architecture of Rad54–DNA complexes, detected by scanning force microscopy, suggests that Rad54 translocates along DNA thereby introducing negative and positive plectonemic supercoils into the DNA that could make the target DNmore accessible for joint molecule formation by Rad51 (Ristic *et al.*, 2001).

Mammalian Rad54 and Rad51 interact *in vitro*, and they can be co-immunoprecipitated from cell extracts after the induction of DNA damage

(Golub *et al.*, 1997, Tan *et al.*, 1999). Both proteins accumulate in DNA damage induced nuclear foci that colocalize (Haaf *et al.*, 1995, Tan *et al.*,1999). These foci are found at the positions of DNA damage and probably represent DNA damage repair sites (Raderschall *et al.*, 1999, Tashiro *et al.*, 2000). The foci are not only formed after treatment of the cells with ionising radiation or DNA interstrand cross-linking agents, they are also observed during S phase (Tashiro *et al.*, 1996, Maizels, 1997, Tashiro *et al.*, 2000). The S phase-induced foci suggest that homologous recombination plays an important role in the repair of collapsed or stalled replication forks. In accordance with foci formation, the expression of *S. cerevisiae RAD51, RAD52,* and *RAD54* genes is induced during S phase and/or after DNA damage induction (Cole *et al.*, 1987, Basile *et al.*, 1992, Averbeck and Averbeck, 1994, Johnston and Johnson, 1995, Walmsley *et al.*, 1997).

Different models have been postulated to describe how Rad54 stimulates Rad51-mediated DNA strand exchange. Knowledge of the relative amounts of Rad54 and Rad51 in the cell could provide some evidence for the mode of operation of these proteins, under the assumption that the cellular levels of the proteins reflect the stoichiometry of their functional complexes. Therefore, we estimated the number of mouse Rad54 protein molecules in the cell and investigated the regulation of the amount of Rad54 protein during the cell cycle. We generated a genomic–cDNA fusion construct which was targeted to the endogenous mouse *Rad54* locus. Expression of this knockin construct yielded the production of a tagged Rad54 protein that was expressed at endogenous levels and could easily be detected. Using mouse embryonic stem (ES) cells containing the knockin construct, we showed that compared to the G1 phase, Rad54 was present at higher amounts during the S and G2 phases in accordance with a prominent role for homologous recombination in the latter phases of the cell cycle. Furthermore, we estimated the number of mouse Rad51 molecules in the cell, and compared this to the number of Rad54 molecules. The results are discussed in the context of alternative modes for Rad54 function in stimulating Rad51-mediated DNA strand invasion.

2. Materials and methods

Identification of exon–intron borders

A mouse *Rad54* cDNA fragment was used to screen a lambda phage and a cosmid library made from a 129/Sv mouse strain (kindly provided by G. Grosveld and N. Galjart, respectively). Genomic fragments hybridizing to the *Rad54* cDNA were subcloned into pBluescript II KS (Stratagene). The location of the intron–exon borders was determined by restriction site mapping, PCR analysis, and DNA sequencing. Exon–intron borders were identified by the presence of consensus splice junctions at sites where the sequence of the

genomic product differed from the *Rad54* cDNA sequence. Intron sizes were determined by direct sequencing or by gel electrophoresis of inter-exon PCR products (Expand Long Template PCR system, Boehringer).

In situ hybridization

Treatment of mouse metaphase spreads of an erythroid cell line, containing a trisomy of chromosome 4 (code red8, N.J. de Both) prior to hybridization was as described (Weeda *et al.*, 1991). A digoxigenin-labeled mouse *Rad54* genomic fragment and a biotin-labeled mouse chromosome 4 specific paint probe (Cambio) were denatured, competed with mouse Cot-1 DNA and hybridized to metaphase spreads as described (Pinkel *et al.*, 1986). To detect the *Rad54* signal, slides were incubated sequentially with sheep-anti-digoxigenin antibodies conjugated to rhodamine and donkey-anti-sheep antibodies conjugated to Texas-red. Chromosome 4 was detected using avidin D-FITC (Vector, USA). Chromosome spreads were washed, dehydrated with ethanol, air-dried, and counterstained with 4,6-diamidino-2-phenylindole (DAPI) in antifade media.

The mouse *Rad54*-knockin construct

A mouse *Rad54–His₆HA* targeting fusion construct was made to obtain expression of tagged *Rad54* from the endogenous locus. To obtain the expression of a *Rad54* allele with a 3'- terminal histidine$_6$-haemagglutinin tag *(His₆HA)*, the *His₆HA* tag (single letter amino acid code RGGSLVPRGSHHHHHHGGYPYDVPDYAS) was cloned behind the *Rad54* cDNA. This tagged cDNA was subcloned into pPGK-p(A) to add a polyadenylation signal. The *Rad54–His₆HA* cDNA was cloned in front of a *hygromycin* resistance gene under the control of a phosphoglycerate kinase (PGK) promoter. Digestion of this construct with *Sfu*I and *Cla*I yielded a fragment containing the 3'-terminal part of the *Rad54–His₆HA* cDNA spanning exons 14 to 18 and the *hygromycin* gene. This fragment was subcloned into the unique *Sfu*I site in exon 4 of a 9 kb *Eco*RI fragment of the *Rad54* genomic sequence containing exons 4–6 in pBluescript II KS. The internal *Sfu*I fragment of *Rad54* cDNA, with exons 4–14, was subcloned into the remaining *Sfu*I site. As a result, exon 4 from the genomic fragment was fused with cDNA exons 4–18 containing the *His₆HA* tag. The construct is schematically depicted in Figure 1B. After linearization, the mouse *Rad54* knockin construct was transfected into *Rad54* ^{neo/+} targeted ES cells (Essers *et al.*, 1997) and clones were selected with *hygromyin* B (200 µg/ml) and expanded. The isolated DNA from the clones was digested with *Stu*I and hybridized with a probe containing exons 7 and 8 to screen for cell lines containing the homologously integrated *Rad54* knockin DNA.

Purification of human Rad51 and Rad54 recombinant proteins

The human *RAD51* cDNA containing a 5'- terminal *His₆* tag was subcloned into pET30A and expressed in *Escherichia coli*. The recombinant protein was purified from the cellular extract using spermidine precipitation as described (Baumann *et al.*, 1996). The pellet containing His₆–Rad51 was resuspended in 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol, and 2 mM imidazole. The supernatant was loaded onto a Ni-NTA column. The column was washed with the same buffer containing 20 mM imidazole and the protein was eluted with buffer containing 200 mM imidazole. Human Rad54
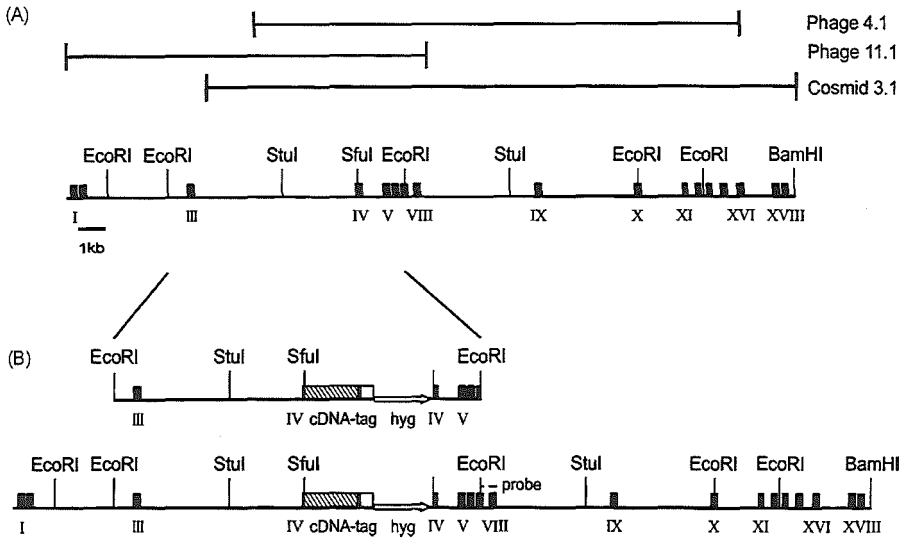
Figure 1. Characterization of the mouse *Rad54* genomic locus and generation of the *Rad54–His$_6$HA* knockin construct. (A) Structure of the genomic *Rad54* locus and genomic phage and cosmid fragments hybridizing to the *Rad54* cDNA. The bottom line represents the approximately 30 kb *Rad54* genomic locus. Boxes indicate the 18 exons (I–XVIII) that make up the *Rad54* cDNA. Shown are the locations of selected restriction sites. Phages 4.1 and 11.1 cover most of the genomic *Rad54* locus, except the 3' end which is covered by cosmid 3.1. (B) Structure of the *Rad54–His$_6$HA* knockin construct. *Rad54* cDNA is represented by a hatched box, the *His$_6$HA* tag by a black box, the poly(A) by a white box, and the *hygromycin* (hyg) selectable marker gene by an arrow. The top line represents the targeting construct and the bottom line the disrupted genomic locus. The lines above exons 7 and 8 indicate the probe used to screen for the presence of the knockin and knockout alleles.

containing an N-terminal His$_6$ tag and a C-terminal HA tag was purified as described before (Swagemakers *et al.*, 1998). The concentrations of the purified His$_6$–Rad51 and His$_6$–Rad54–HA were determined by BioRad dye assay using BSA as a standard. The accuracy of these measurements was within a two-fold range. The proteins were over 90% pure. Purified His$_6$–Rad51 was used to generate polyclonal antibodies in rabbits as described (Harlow and Lane, 1988).

Determination of the amount of Rad51 and Rad54 in ES cells

Wild-type mouse ES cells and *Rad54* $^{-/knockin}$ ES cells were cultured as described before (Essers *et al.*, 1997). To make a protein extract, the cells were trypsinized, counted, and after pelleting dissolved in electrophoresis sample buffer, and sonicated. Immunoblots were made that contained increasing amounts of purified protein and ES cell extracts from increasing number of cells. The blots were hybridized with anti-Rad51 and anti-HA (3F10, Boehringer) antibodies to detect Rad51 and Rad54, respectively. On the blots, the amount of

Rad51 from wild-type ES cells was compared with purified His_6–Rad51 and the amount of Rad54–His_6HA was compared with purified His_6–Rad54–HA using Image Quant.

Determination of the nuclear fraction of Rad51 in ES cells

 A whole cell extract from $Rad54^{-/knockin}$ ES cells was obtained as described above. A nuclear extract was made as described previously (Davies et al., 2001). Immunoblots were made using whole cell extracts and nuclear extracts. The blots were hybridized with anti-Rad51 and anti-HA antibodies to detect Rad51 and Rad54, respectively. On the blots, the amounts of Rad51 and Rad54–His_6HA in the whole cell and nuclear extracts were determined using Image Quant. Because we were unable to detect any Rad54 in the cytoplasm, we infer that Rad54 is a strictly nuclear protein (data not shown). Therefore, the fraction of Rad51 that is nuclear was calculated by dividing the amount of Rad51 protein in the nuclear extract by the amount of Rad51 in the whole cell extract. To correct for differences in the amount of extract loaded on the gel, this fraction was multiplied with the amount of Rad54 in the whole cell extract divided by the amount of Rad54 in the nuclear extract.

Immunostaining and fluorescence activated cell sorting (FACS) analysis

 $Rad54^{+/-}$ ES cells and $Rad54^{-/knockin}$ ES cells were trypsinized and resuspended to single cell suspension in phosphate buffered saline (PBS). They were fixed for 30 min with 0.4% para-formaldehyde on ice. Subsequently, the cells were permeabilized by adding an equal volume of 0.2% Triton-X 100. The cells were incubated with anti-HA antibodies, washed with PBS containing 0.2% Triton-X 100 and incubated with goat-anti-rat-PE antibodies for intracellular detection of the HA tag. After washing, cells were treated with RNAse and incubated with TO-PRO-3 (Molecular Probes) to stain the DNA. The level of fluorescence of TO-PRO-3 and goat-anti-rat-PE in each cell was assessed on a FACSCalibur using Cell Quest (Becton Dickinson). Based on TO-PRO-3 fluorescence and cell shape, cells were assigned towards fractions containing cells in the G1, S, or G2 phase of the cell cycle. For each fraction, the distribution of goat-anti-rat-PE fluorescence was measured and the median was calculated. The fluorescence due to the presence of the HA tag was determined by subtracting the fluorescence in $Rad54^{+/-}$ ES cells, that do not contain an HA tag, from the fluorescence in $Rad54^{-/knockin}$ ES cells. As the absolute levels of fluorescence are dependent on the staining conditions of each experiment, the relative increase in fluorescence in the S and G2 phases of the cell cycle compared to the G1 phase was determined by dividing the HA-specific fluorescence in the S and G2 phases by the fluorescence in the G1 phase. As a negative control, cells were incubated with goat-anti-rat-PE antibodies and TO-PRO-3 only. The experiment was repeated three times and standard deviations were determined.

3. Results

Characterization of the genomic mouse Rad54 locus

 The complete genomic locus encoding mouse Rad54 was isolated from a lambda phage and a cosmid library. Two overlapping phage clones (phages 4.1 and 11.1) and one cosmid clone (cosmid 3.1), covering the Rad54 genomic locus were characterized in detail using restriction enzyme digestion analyses and hybridization with mouse Rad54 cDNA (Figure 1A). Hybridizing

fragments were subcloned into plasmid vectors and sequenced to determine intron–exon borders. The length of the introns was determined by direct sequencing or PCR. The genomic organization of the *Rad54* gene is depicted in Figure 1A. The Rad54 protein was encoded by 18 exons ranging in size from 61 to 286 bp spread over a region of approximately 30 kb.

As shown in Table 1, all sequences around intron–exon and exon–intron junctions were consistent with the consensus splice acceptor and donor signals. The length and the relative position of the mouse *Rad54* exons and introns was remarkably similar to human *RAD54* (Rasio *et al.*, 1997). For both the human and the mouse locus, introns 2–4, 8–10 and 16 are the largest introns. The first exon present in both the human *RAD54* and mouse *Rad54* cDNAs contained the coding sequence for the start codon immediately followed by a splice donor site. We could not exclude the presence of additional untranslated exons upstream of exon 1. The first conserved motif (motif I) of the Snf2/Swi2 protein family (Eisen *et al.*, 1995, Kanaar *et al.*, 1996), which corresponds to the Walker-type A domain of the ATP-binding site, and motif Ia were located in exon 7 of *Rad54*. Motif II, corresponding to the Walker-type B domain of the ATP binding site, was located in exons 8 and 9. The remaining conserved motifs were encoded by exon 9 (motif III), exon 14 (motif IV), exons 15–16 (motif V), and exon 16 (motif VI).

We have previously determined that the human *RAD54* locus is located on human chromosome 1p32 (Kanaar *et al.*, 1996). Since this region is syntenic to both mouse chromosomes 3 and 4, it was important to determine the chromosomal localization of mouse *Rad54*. *In situ* hybridization experiments were performed on mouse metaphase spreads of an erythroid cell line carrying a trisomy of chromosome 4. The hybridization signal generated by the *Rad54* probe showed that the *Rad54* locus is located on mouse chromosome 4, near band C7/D1 (Figure 2).

Expression of tagged mouse Rad54 from the endogenous promoter

We designed a knockin construct for mouse *Rad54* such that a tagged Rad54 protein would be expressed at endogenous levels and with its natural expression properties. This construct was made by fusing exons 4–18 of the *Rad54* cDNA to a 3'-terminal *His$_6$HA* tag, followed by a poly(A) signal and a *hygromycin* selectable marker gene. This fragment was subcloned into exon 4 of a 9-kb *Eco*RI genomic *Rad54* fragment (Figure 1B). The knockin construct was targeted into *Rad54* [+/-] ES cells (Essers *et al.*, 1997). Cells were obtained which contained one *Rad54* knockout allele and one *Rad54* knockin allele. Expressing *Rad54* from the knockin allele resulted in the production of Rad54–His$_6$HA protein from the endogenous *Rad54* promoter.

Table 1.
Structural organization of the mouse *Rad54* gene.

Intron (splice acceptor)	Exon				Intron (splice donor)		
		I	(3 bp)	90	GGATG	GTAAGTGTGA	(82 bp)
GCCTGGCTGCAGGATCACTGCAGGA ACTCACTTTCTGTTCTCCCTTGCAG	91 AGGAG	II	(87 bp)	176	CAGTA	GTGAGTGTTT	(4.5 kb)
TCTCTGGGGTTGTCTAGGTATTGCA ATATAGCTTTTCATTTCTCTCCTAG	177 ACTCC	III	(120 bp)	296	AACAT	GTAAGCCACA	(5.5 kb)
TCGAAGGTTAATAACATTTCAACAG TTTTGAATTTCTGTTTTCCTTTTAG	297 GAAGC	IV	(61 bp)	357	TCAAG	GTAAAATGGA	(1.2 kb)
GAATAAATAAATATAGAAGGTTGGC TTACAGGTTGGTTGCTGATTTCTAG	358 GTCCC	V	(137 bp)	494	GACAA	GTATCACATT	(350 bp)
TGAGTCTGATCATCATGGTTTTGTT TTGTTTTATCTGCTGTATTTCTCAG	495 GGAAA	VI	(70 bp)	564	GAGAG	GTAAAATGAG	(85 bp)
GCTGGGGGCTAAGTAGGCGCCAACA TGGACTAAATGGTGTTACTCATCAG	565 GGAGT	VII	(286 bp)	850	ACTGG	GTATGGAGCC	(220 bp)
GTTTTTCTTTAAGATCTTCTCATGT GTTCTCTGCAACCTTTCTGTTGTAG	851 AAGGA	VIII	(124 bp)	978	ACGAG	GTACTTAACA	(3.8 kb)
ATCGTTTAAGAATATGAAATCATCC TAAGTGAGACTTAATTGTCTTACAG	979 GGCCA	IX	(148 bp)	1127	TTTGG	GTAAGCAATT	(3.8 kb)
AAATCACCCTACCTTACATGCTTTC ATATTCAGTTCTCTCCTCTGTTTAG	1128 GAACT	X	(127 bp)	1255	AACAG	GTGAGAGGCT	(1.2 kb)
TTATTCTGCATCAGTTTCACCTCCC TTGTGCCTTTTCCCATTTCCCCTAG	1256 GTGCC	XI	(72 bp)	1328	TGTAG	GTATGAACTT	(150 bp)
TGTGTGCTCCAGTCTTTTCTATTGA GCTACACCTGCCCTTTTATTTTCAG	1329 GCTGA	XII	(139 bp)	1460	TAATC	GTGAGCCTCC	(550 bp)
AGTATGGTGGTGTTGCGCTTTTTTT TTTTTTTTCTTTTTTTGGTTTTCAG	1461 ATCCA	XIII	(90 bp)	1572	GTCAG	GTGAGCCTCC	(113 bp)
GGTTACTGGGATGACCAATTGTGAG GACTGATAATAGCCACTTTTCCCAG	1573 GTAAG	XIV	(138 bp)	1697	CGAAG	GTAGGGAAGA	(270 bp)
TTCCAATTAATTTATCGTTCTTGGG TCTTGATATTTGGATCTCCCTCAAG	1698 GTACT	XV	(80 bp)	1777	CATCG	GTAAGTATGT	(300 bp)
TTCCAAGTGTGTGCATCCTATGCTC TCCTTCTTGTTTCTTTTCTTCCCAG	1778 AGCCC	XVI	(182 bp)	1960	TATCT	GTAAGTATGT	(900 bp)
CCTCAGCATCTACAGGAATGGTTGA GTTCTACATTTCATCCCATTTCTAG	1961 GCAGG	XVII	(159 bp)	2120	GACAG	GTATGTGGAG	(90 bp)
GACCAAGCACAGAAATCCGTAGTGA CCACAACTCTGTCCTCTGACCCCAG	2121 ATTGC	XVIII					

The nucleotide sequence of each intron–exon–intron junction is shown. All acceptor and donor sequences are in accordance with the consensus sequence $(Py)_n$ NCAG/G and (C,A)AG/GTPuAGT, respectively (Senapathy *et al.*, 1990). Exon numbering is as in Figure 1. The size of each intron and exon is given in parentheses.

This offered two major advantages. The tagged protein mimicked exactly the endogenous expression and could easily be detected by immunoblotting or immunofluorescence using anti-HA antibodies. We have previously shown that the relative protein levels of the *Rad54* wild-type allele and the *Rad54* knockin allele are indistinguishable and that the tagged Rad54 protein rescues the DNA repair defect of Rad54-deficient cells (Tan *et al.*, 1999).
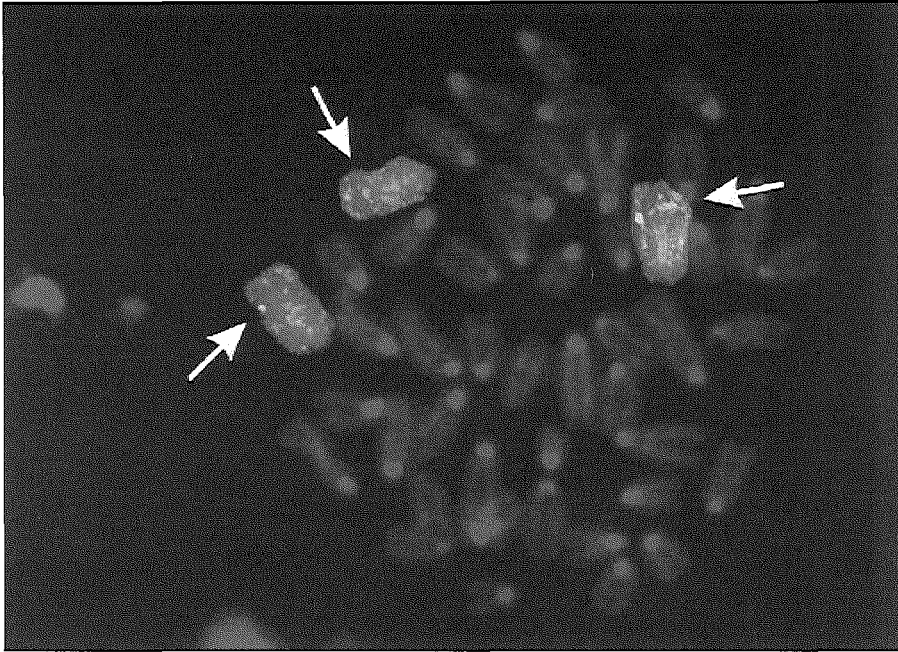
Figure 2. Chromosomal localization of mouse *Rad54* by *in situ* hybridization. Metaphase spreads of mouse chromosomes were hybridized to a digoxigenin-labeled *Rad54* genomic probe and a biotinylated chromosome 4 specific paint probe. The *Rad54* hybridization signal, indicated by the arrows, was detected on chromosome 4, near band C7/D1. DAPI counterstaining of the chromosome spread revealed the chromosome banding pattern.

Amount of Rad54 in ES cells

To estimate the amount of Rad54 in mouse ES cells, we compared the amount of Rad54–His$_6$HA protein in cell extracts from an exponentially growing population of *Rad54* $^{-/knockin}$ ES cells with purified His$_6$–Rad54–HA on immunoblots using anti-HA antibodies (Figure 3A). Two fmol of purified protein was comparable with the amount of Rad54 protein in 1.0×10^4 cells. Therefore, one cell contained about 1.2×10^5 molecules of Rad54–His$_6$HA protein. The protein level in wild-type cells is twice as high as in heterozygous cells (Tan *et al.*, 1999, Tashiro *et al.*, 2000). Therefore, wild-type ES cells contain, on average, approximately 2.4×10^5 Rad54 molecules per cell.

The Rad54 and Rad51 proteins cooperate closely during homologous recombination. Therefore, it is of interest to determine the relative amounts of the two proteins in cells. We prepared filters containing different amounts of purified His$_6$–Rad51 and cell extracts from wild-type ES cells and hybridized these with anti-Rad51 antibodies. Nineteen fmol of purified protein was comparable with the amount of Rad51 protein in 1.7×10^4 ES cells. Thus, one
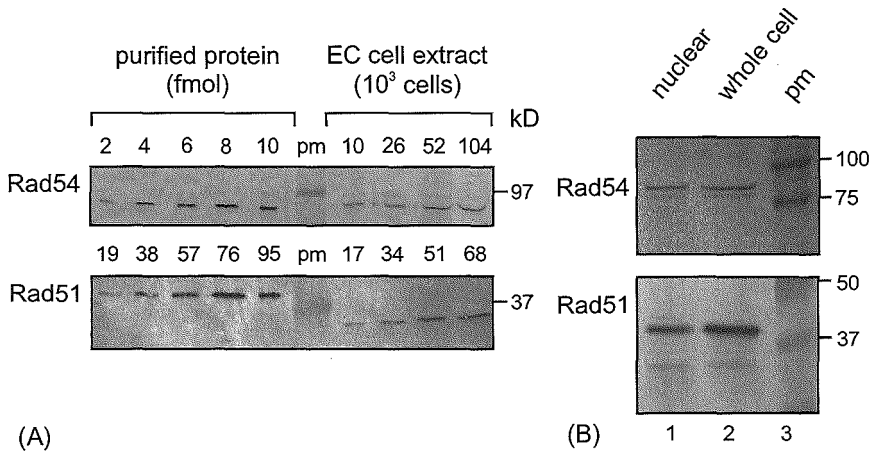
Figure 3. Amount of Rad54–His$_6$HA and Rad51 proteins in mouse ES cells and nuclei.
(A) The number of Rad54–His$_6$HA and Rad51 protein molecules per ES cell was estimated by quantitative immunoblotting using purified His$_6$–Rad54–HA and His$_6$–Rad51 as references. The blots were hybridized with anti-HA and anti-Rad51 antibodies, respectively. The sizes of the prestained marker (pm) bands visible on the blots are indicated on the right. (B) The fraction of Rad51 that is nuclear was determined by comparison of the amounts of Rad51 and Rad54–His$_6$HA in a whole cell extract (lane 2) and a nuclear extract (lane 1). The sizes of the prestained marker bands (lane 3) are indicated on the right. The membrane has been cut horizontally to facilitate separate hybridizations with anti-HA and anti-Rad51 antibodies.

wild-type ES cell contained about 6.7 x 10^5 molecules of Rad51 protein. Rad51 protein is detected both in the cytoplasm and in the nucleus, while Rad54 is a nuclear protein (Tan *et al.*, 1999, Davies *et al.*, 2001, Kraakman-van der Zwet *et al.*, 2002). To determine the amount of Rad51 protein in the nucleus, we made nuclear extracts from ES cells and compared the amounts of Rad51 and Rad54 in the nuclear extracts with the amounts in whole cell extracts. We found that about 70% of the Rad51 pro-tein was nuclear, which means that ES cell nuclei contain about 4.7 x 10^5 molecules of Rad51 (Figure 3B). Taking into account that the accuracy of all these measurements is about two-fold, the level of Rad51 in the nucleus is in the same range as the level of Rad54.

Cell cycle regulation of the amount of Rad54 in ES cells

Previously, it has been determined that *RAD54* mRNA levels in HeLa cells are three times higher during late G1 phase than in the other phases of the cell cycle (Kanaar *et al.*, 1996). Using the ES cells containing Rad54–His$_6$HA protein expressed under the control of the endogenous promoter, we now compared Rad54 protein levels in the different phases of the cell cycle. Using FACS analysis on *Rad54* $^{+/-}$ and *Rad54* $^{-/knockin}$ ES cells, we measured
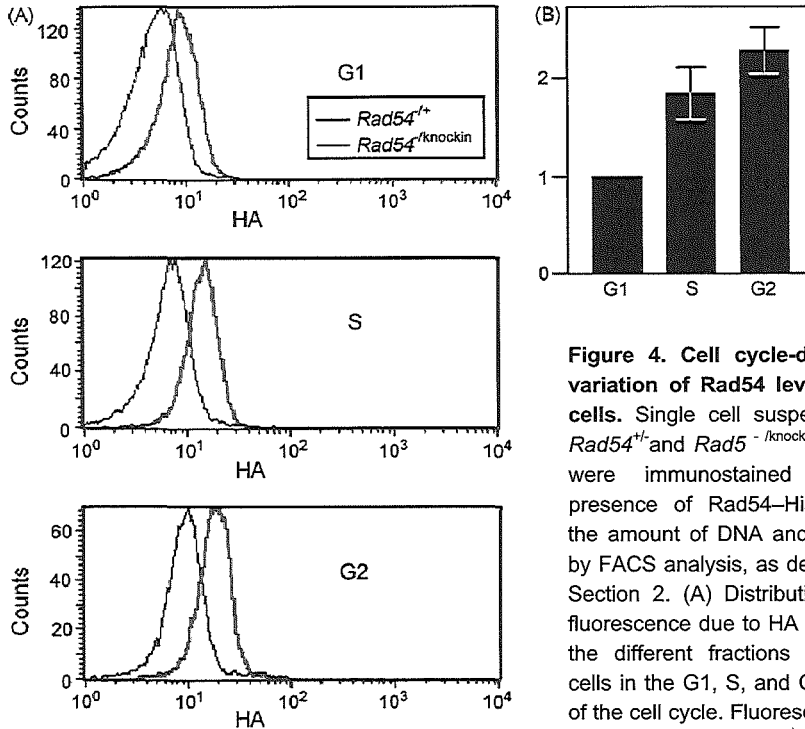
Figure 4. Cell cycle-dependent variation of Rad54 levels in ES cells. Single cell suspensions of $Rad54^{+/-}$ and $Rad5^{-/knockin}$ ES cells were immunostained for the presence of Rad54–His$_6$HA and the amount of DNA and analyzed by FACS analysis, as described in Section 2. (A) Distribution of the fluorescence due to HA staining in the different fractions containing cells in the G1, S, and G2 phases of the cell cycle. Fluorescence was measured in $Rad54^{+/-}$ ES cells (black curves) that did not contain Rad54–His$_6$HA, and in $Rad54^{-/knockin}$ ES cells (gray curves) that contained Rad54–His$_6$HA. (B) Relative amounts of Rad54–His$_6$HA protein in the different phases of the cell cycle. The amount was calculated by subtracting the median of the fluorescence due to anti-HA antibodies in $Rad54^{+/-}$ ES cells from the median in $Rad54^{-/knockin}$ ES cells. The amount of protein in G1 phase was used as a reference to enable comparison among the three independent experiments. The error bars indicate the standard deviation.

both the fluorescence due to staining with anti-HA antibodies and the DNA content. DNA content and cell shape were used to divide the cells in fractions representing cells in either the G1, S, or G2 phases of the cell cycle. Staining with anti-HA antibodies gave a higher level of fluorescence in the $Rad54^{-/knockin}$ ES cells, that contained Rad54–His$_6$HA, than in $Rad54^{+/-}$ ES cells, that did not contain Rad54–His$_6$HA (Figure 4A). The background level of fluorescence obtained by omission of the primary antibodies was similar in $Rad54^{+/-}$ and $Rad54^{-/knockin}$ ES cells (data not shown). The difference in HA-specific fluorescence between these cell lines is a measure for the amount of Rad54–His$_6$HA protein. Using the HA-specific G1 fluorescence as a reference, we calculated that the amount of Rad54–His$_6$HA protein in the S and G2 phases of the cell cycle is about twice as high as the amount in the G1 phase (Figure 4B).

4. Discussion

We determined the genomic organization of mouse *Rad54* and generated a gene targeting knockin construct that encodes a C-terminal His_6HA tagged Rad54 protein (Figure 1). Using mouse ES cells containing this knockin construct at the endogenous locus, we estimated the number of Rad54 molecules in the cell and the cell cycle variation in Rad54 protein levels. ES cells contain approximately 2.4×10^5 Rad54 molecules per cell in an unsynchronized population and three times as many Rad51 molecules. Similar to Rad51, mammalian Rad54 expression varies throughout the cell cycle (Flygare *et al.*, 1996, Chen *et al.*, 1997). The Rad54 protein level increases two-fold from G1 to S phase.

Rad54 and Rad51 protein levels and cell cycle regulation of Rad54

With 2.4×10^5 molecules of Rad54 per mouse ES cell and 6.0×10^9 bp in the diploid genome of the mouse, Rad54 is present at one molecule per 2.5×10^4 bp. Exponentially growing diploid yeast cells contain 7000 Rad54 molecules per cell, which amounts to one molecule of Rad54 per 3.4×10^3 bp (Clever *et al.*, 1999). Thus, yeast cells contain 7.4 times as many Rad54 protein molecules relative to their genome size compared to mammalian cells. This is consistent with the observation that certain sub-pathways of homologous recombination are much more efficient in yeast than in mammalian cells. In both yeast and mammalian cells, the amount of Rad54 protein is reduced by two-fold when only one functional *RAD54* gene is present, suggesting control of the amount of protein at the transcriptional level (Clever *et al.*, 1999). Nevertheless, heterozygous cells have no obvious phenotypes.

We observed that the amount of Rad54 protein in ES cells is about two times higher in the S and G2 phases of the cell cycle than in the G1 phase (Figure 4). Similarly, mouse Rad51 is present in greater amounts in the S and G2 phases, compared to the G1 phase (Yamamoto *et al.*, 1996). This is consistent with the induction of mammalian *Rad54* and *Rad51* mRNA expression in late G1 (Kanaar *et al.*, 1996, Flygare *et al.*, 1996, Yamamoto *et al.*, 1996). *S. cerevisiae RAD54* expression is also increased in late G1 (Cole *et al.*, 1987, Johnston and Johnson, 1995). The increased level of these recombination proteins in the S and G2 phases corresponds to the important role of homologous recombination during replication and to the Rad54-dependent increased resistance of cells to DSB-inducing agents in the G2 phase (Takata *et al.*, 1998).

The importance of cell cycle regulated expression of the proteins involved in homologous recombination is emphasized by the dysregulation of recombination reactions due to ectopic expression of these proteins.

Moderate overexpression of mammalian *RAD51* (1.6–3-fold) leads to increased spontaneous and DSB-induced recombination (Xia *et al.*, 1997, Vispe *et al.*, 1998, Lambert and Lopez, 2000, Arnaudeau *et al.*, 2001). Gene conversion with or without crossover, which is dependent on Rad51 and Rad54, is especially stimulated as opposed to single-strand annealing which can occur independent of these proteins (Lambert and Lopez, 2000, Arnaudeau *et al.*, 2001). Furthermore, *RAD51*-overexpressing cells are less sensitive to ionising radiation than wild-type cells (Vispe *et al.*, 1998, Yanez and Porter, 1999). Overexpression of human *RAD52* also induces spontaneous, intrachromosomal recombination and leads to increased resistance to ionising radiation and methyl methanesulfonate (Park, 1995, Liu and Maizels, 2000).

However, overexpression of recombination proteins does not always lead to increased recombination. A high level of overexpression of *RAD51* and/or *RAD52* (4–10-fold) in Chinese hamster ovary cells leads to reduced DSB-induced recombination frequencies (Kim *et al.*, 2001). Overexpression of *RAD51* in a fibrosarcoma cell line yields a decreased plating efficiency, reduced cell growth, and increased levels of apoptosis shortly after the induction of *RAD51* overexpression (Flygare *et al.*, 2001). Transient overexpression of *RAD51* or *RAD52* in fibrosarcoma cells has also been shown to increase spontaneous recombination, but reduce DSB-induced recombination (Kim *et al.*, 2001). These differences in the effects of overexpression could be dependent on the cell type used, for example, due to differences in p53 status or apoptotic potential.

Models for the cooperation of Rad51 and Rad54 in homologous recombination: cis versus trans action of Rad54

From genetic data on the frequency of homologous recombination events in wild-type and Rad54-deficient yeast and mammalian cells, it is clear that Rad54 is important for those pathways of homologous recombination that involve DNA strand invasion rather than single-strand annealing (Liefshitz *et al.*, 1995, Ivanov *et al.*, 1996, Shinohara *et al.*, 1997, Dronkert *et al.*, 2000). *In vitro*, the yeast and human Rad54 proteins have been shown to stimulate Rad51-mediated DNA strand exchange reactions (Petukhova *et al.*, 1998) (P. Sung, personal communication). This stimulation is maximal when Rad54 is added after formation of the Rad51 nucleoprotein filament and before or together with the homologous dsDNA molecule (Mazin *et al.*, 2000, Solinger *et al.*, 2001). Both the ATPase and DNA supercoiling activity of Rad54 are stimulated by Rad51 or a Rad51 nucleoprotein filament (Mazin *et al.*, 2000, Van Komen *et al.*, 2000).

These data suggest that Rad54, after binding to the preformed Rad51 nucleoprotein filament, starts its activity on dsDNA. Different hypotheses have

been put forward concerning the number of Rad54 molecules binding to the Rad51 nucleoprotein filament (Mazin *et al.*, 2000, Ristic *et al.*, 2001). Purified yeast Rad54 is a homodimer that oligomerizes in the presence of DNA (Van Komen *et al.*, 2000). Complexes of human Rad54 on DNA contain three to six molecules of Rad54 (Ristic *et al.*, 2001). It could be that just one or a few of these Rad54 oligomers bind to the Rad51 nucleoprotein filament and are sufficient to stimulate DNA strand invasion by promoting unwinding of the homologous dsDNA. Alternatively, the whole Rad51 nucleoprotein filament might be covered with Rad54 molecules.

We distinguish two fundamentally different models for the action of Rad54. In the first, Rad54 functions in *cis* relative to the site of strand invasion (Figure 5A), i.e. Rad54 is bound to that part of the dsDNA that will be unwound to enable strand invasion by the Rad51 nucleoprotein filament (Mazin *et al.*, 2000). In the second model, Rad54 functions in *trans* relative to the site of strand invasion. In this case, Rad54 acts on dsDNA distant from the site that will be unwound (Figure 5B). In the *trans*-acting model, Rad54 has a DNA translocation activity that introduces supercoils into the dsDNA (Ristic *et al.*, 2001). The Rad51 nucleoprotein filament on the broken chromosome will provide frictional torque that will constrain the translocating Rad54 protein from rotating around the DNA. The result of *Rad54* movement along the DNA under those conditions is that positive supercoils will accumulate in the chromosomal domain ahead of the translocating protein complex and negative supercoils behind it (Liu and Wang, 1987). The negative supercoiling will lower the energy required to unwind the DNA and therefore it will facilitate invasion of the ssDNA contained in the Rad51 nucleoprotein filament into the homologous template DNA (Figure 5B). To perform this function, Rad54 and the Rad51 nucleoprotein filament bind the dsDNA at different positions. Hence, we refer to this model as the *trans*-acting model. Although a low ratio of Rad54-to-Rad51 is sufficient in this model, it is not a requirement.

In the *cis*-acting model the Rad54 protein would have a more structural role, as opposed to the dynamic nature of Rad54 action in the *trans*-acting model. The *cis*-acting model requires that the Rad51 nucleoprotein filament is covered with Rad54 over much of its length by the binding of Rad54 to Rad51 (Figure 5A) (Mazin *et al.*, 2000). The coverage of the Rad51 nucleoprotein filament with Rad54 requires similar amounts of Rad51 and Rad54 protein. The Rad51/Rad54 nucleoprotein the ssDNA in the filament. Rad54 could then directly unwind the dsDNA it is associated with (Figure 5A). How Rad54 would unwind the dsDNA in this model is not immediately clear, as helicase activities have never been detected. After unwinding, the Rad54 could help DNA strand invasion by keeping the dsDNA unwound.

The high rate of ATP hydrolysis by Rad54, which is further increased by the presence of the Rad51 nucleoprotein filament, supports a dynamic
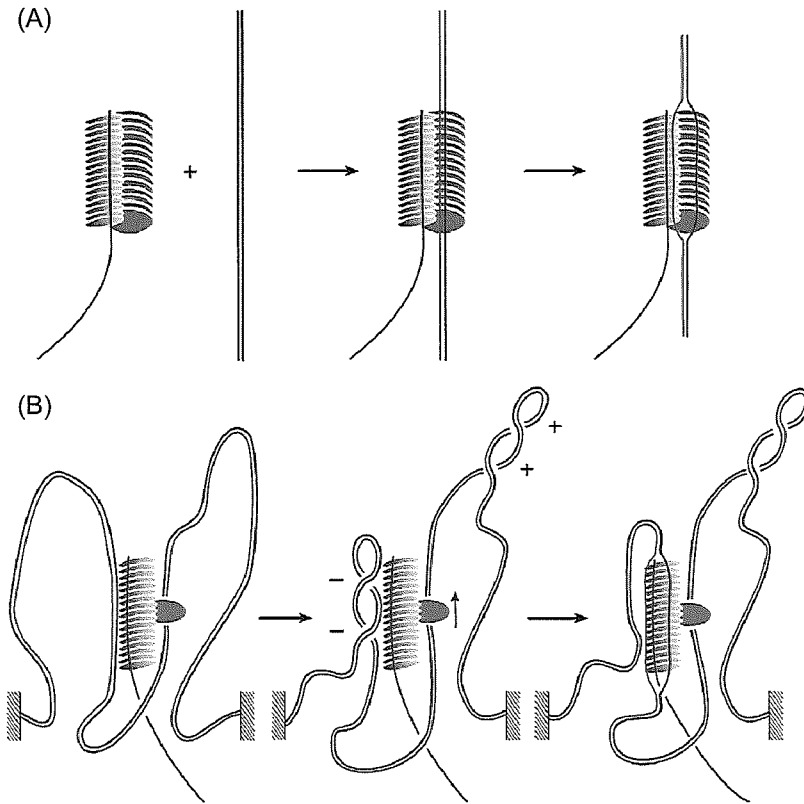
(A)

(B)

Figure 5. Models for stimulation of Rad51-mediated DNA strand invasion by Rad54. Rad51 is represented by the small light green ovals while Rad54 proteins are depicted as larger dark green ovals. The ssDNA in the Rad51 nucleoprotein filament is indicated by the single black line and homologous duplex DNA is represented by the black and gray lines. (A) The *cis*-acting model for Rad54 action. Rad54 interacts more or less stoichiometricly with the Rad51 nucleoprotein filament. After Rad51-mediated interaction of the ssDNA with the homologous duplex DNA, Rad54 stimulates DNA strand invasion by acting locally. It directly promotes unwinding of the duplex DNA to which the Rad51 nucleoprotein filament is bound. (B) The *trans*-acting model for Rad54 action. A chromosomal duplex DNA domain is indicated by the black and gray lines connecting the hatched areas. Rad54 interacts with the Rad51 nucleoprotein filament at sub-stoichiometric amounts. DNA translocation of Rad54 causes the introduction of negative supercoils behind the moving protein complex (indicated by minus signs) and positive supercoils ahead of it (indicated by plus signs), because the complex is prevented from moving around the DNA due to the frictional torque provided by the Rad51 nucleoprotein filament. The negative supercoiling lowers the energy required to unwind the duplex DNA and therefore it facilitates invasion of the ssDNA contained in the Rad51 nucleoprotein filament into the homologous duplex DNA. Unwinding causes the conversion of negative writhe into negative twist, but for simplicity, the twist component in duplex DNA is not indicated. In this model, Rad54 functions at a distance relative to the site of strand invasion. In contrast to the *cis*-acting model a low ratio of Rad54-to-Rad51 is sufficient in the *trans*-acting model, but it is not a requirement.

function of Rad54, which is more apparent in the *trans*-acting model (Petukhova *et al.*, 1998, Swagemakers *et al.*, 1998, Mazin *et al.*, 2000, Van Komen *et al.*, 2000). The stimulation of ATP hydrolysis and supercoiling activity of Rad54 by the Rad51 nucleoprotein filament does not require homology between the ssDNA in the filament and the dsDNA upon which Rad54 acts, which also supports the *trans*-acting model. Our observation that the amounts of nuclear Rad51 and Rad54 are similar, unfortunately, still leaves room for either model.

Ratio between Rad54 and Rad51: *in vivo* and *in vitro*

The Rad51 protein is approximately three times more abundant in ES cells compared to Rad54. However, approximately one-third of the Rad51 protein molecules is located in the cytoplasm, which implies that there is twice as much Rad51 in the nucleus compared to Rad54 (Figure 3). In yeast, Rad51 and Rad54 are also present in similar amounts (Mazin *et al.*, 2000). *In vitro*, yeast Rad51 performs DNA strand exchange reactions which are stimulated by Rad54 (Petukhova *et al.*, 1998, Petukhova *et al.*, 1999, Mazin *et al.*, 2000, Van Komen *et al.*, 2000, Solinger *et al.*, 2001). The ratio of Rad54-to-Rad51 at which product formation is optimal varies from 1:1 to 1:50 (Mazin *et al.*, 2000, Solinger *et al.*, 2001). The specific activity of the proteins used in these assays is unknown, which could explain some of the differences. Another reason for this large difference in optimal stoichiometry could be the types of ssDNA used in the different DNA strand exchange assays. Using linear ssDNA molecules of several kilobases long, an increasing concentration of Rad54 causes the induction of networks of DNA molecules, probably due to DNA strand exchange of one linear ssDNA molecule with several circular dsDNA molecules (Petukhova *et al.*, 1998, Solinger *et al.*, 2001). Because the networks are not resolved in the gel electrophoresis assay used to detect DNA strand exchange, the formation of networks would decrease the number of products detected. Therefore, in this assay, optimal product formation will occur with a low amount of Rad54 compared to Rad51. In contrast, in assays using linear 90 nt DNA molecules, too short to perform joint molecule formation with several dsDNA molecules at the same time, an optimal ratio of 1:1 for Rad51:Rad54 has been found (Mazin *et al.*, 2000).

The results of the DNA strand exchange reactions *in vitro* are not conclusive as to whether the *cis*-or the *trans*-acting model should be preferred. The low optimal ratio of Rad54-to-Rad51 in a number of *in vitro* DNA strand exchange reactions is consistent with the translocation function for Rad54 in the *trans*-acting model (Petukhova *et al.*, 1999, Solinger *et al.*, 2001). However, as discussed above, this low ratio could be due to DNA network formation which limits the detection of joint molecules in the *in vitro* reaction. In alternative *in vitro* DNA strand exchange reactions, a high optimal

ratio of Rad54-to-Rad51 has been found, which is compatible with the *trans*-acting model and required for the *cis*-acting model (Mazin *et al.*, 2000). However, the use of short ssDNA molecules in these reactions hampers extrapolation to the *in vivo* situation, because long ssDNA molecules are most probably the proper substrate *in vivo*. *In vivo* network formation of DNA molecules will most likely not occur as frequently as *in vitro* (White and Haber, 1990, Sugawara and Haber, 1992) because only one or a few stretches of homologous dsDNA are present in the cell, in contrast to the many homologous dsDNA molecules present in the test tube. Assuming similar lengths of ssDNA tails in ES cells as found in yeast cells, the high nuclear ratio of Rad54-to-Rad51 in ES cells is not expected to hinder homologous recombination *in vivo*.

Acknowledgements

Chapter 3

Sublethal damage recovery in *Rad54*-deficient embryonic

stem cells

Sublethal damage recovery in *Rad54*-deficient embryonic stem cells

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Sublethal damage recovery (SLDR) is believed to result from repair of potentially lethal damage. It is observed as improved survival of cells when the same radiation dose is given in multiple fractions (split-dose) as compared to a single dose. To test if absence of *Rad54*, a gene involved in DSB repair by homologous recombination, influences the SLDR, we determined the SLDR capacity of wild type and *Rad54*-deficient mouse embryonic (ES) cells. Although SLDR was not fully abolished in *Rad54* mutant cells, in comparison to wild type cells a difference in the kinetics of SLDR was observed. We propose that the change in the recovery pattern in absence of *Rad54* could be due to a selective increase of the sensitivity of the normally relatively resistant S-phase of the cell cycle.

1. Introduction

Response to radiation therapy, translated to radiation sensitivity of cells and tissues, can differ between treated individuals. One possible explanation for a range of response severities to the treatment might be abolished or reduced ability of cells to repair the damage introduced to DNA by ionizing radiation.

Among many different types of ionizing radiation-induced damage DSBs are extremely toxic. Since approximately forty DSBs are produced after treatment with only 1 Gy of ionizing radiation (IR), the protective role of DSB repair mechanisms and their capacity to repair the damage are particularly important issues in radiation treatment. Screening patients for defects in DSB repair may be important for optimization of radiotherapy, since it could allow adjustment of the treatment for each individual, depending on their ability to repair DNA damage introduced by ionizing radiation.

A classical way of assessing cellular radiation sensitivity is to establish cell survival curves (Steel *et al.*, 1997). This method is applicable only to cells which are able to divide for at least six generations after treatment and form colonies of more than 50 cells. An increasing steepness of such curves representing survival efficiency as function of radiation dose indicates an

increase in radiosensitivity. For cells that differ only by presence/absence of certain DNA repair genes, an increasing overall steepness of the cell survival curves may represent loss of DNA repair. It can be derived that cell survival should decrease exponentially with dose, meaning that equal fractions of cells are killed per unit of dose, which would reflect in straight lines on a survival versus (log-scale) dose plot. Usually, however, cell survival after treatment with sparsely ionizing types of radiation (X- or γ-rays) is non-linear and shows a downward deflection, implying that irradiation becomes progressively more effective with increasing dose. The most commonly accepted view is that part of the damage becomes lethal only if it "interacts" with additional damage produced by continued irradiation. This type of damage has been termed sublethal damage (SLD). Giving a certain dose in two or more equal fractions with time intervals varying from minutes to hours, depending on cell type can diminish SLD. This straightens the survival curve, but leaves it with additional non-zero slope, indicating that not all the damage can be recovered by splitting the dose. The capacity to perform such Sublethal Damage Recovery (SLDR) differs between cell types, which could be a useful feature for modification of radiotherapy, since this parameter might be different in normal and tumour tissues.

SLDR has been also referred to as Sublethal Damage Repair, implying repair of DNA damage. Recently, it has been shown that disruption of DSB repair mechanisms affects SLDR to different degrees in different cell types (Utsumi et al., 2001, Myint et al., 2002). In this context, but also to further define the role of *Rad54* in homologous recombination (HR) (See Chapter 1), we evaluated overall radiosensitivity and SLDR of wild type and *Rad54⁻/⁻* embryonic stem (ES) cells. Previously we have shown that loss of *Rad54* increases overall radiosensitivity of the cells (Essers et al., 1997). Here we describe split dose experiments to determine the kinetics of SLDR at iso-effective doses. We find that SLDR is not fully abolished in *Rad54 ⁻/⁻* ES cells. We suggest that the absence of *Rad54* selectively increases the sensitivity of the relatively resistant S-phase of the cell cycle.

2. Materials and methods

Cell survival assay and split dose experiments
        The ES cells used in this study were previously described by Essers et al., 1997. Irradiations were performed using a ^{137}Cs source. In split-dose experiments iso-effective single doses and the first fraction of the split-dose were given at time zero. The second fractions of the split dose were given at different intervals up to 24 h. Data shown are the average of three independent experiments, where each time point was determined in duplicate.
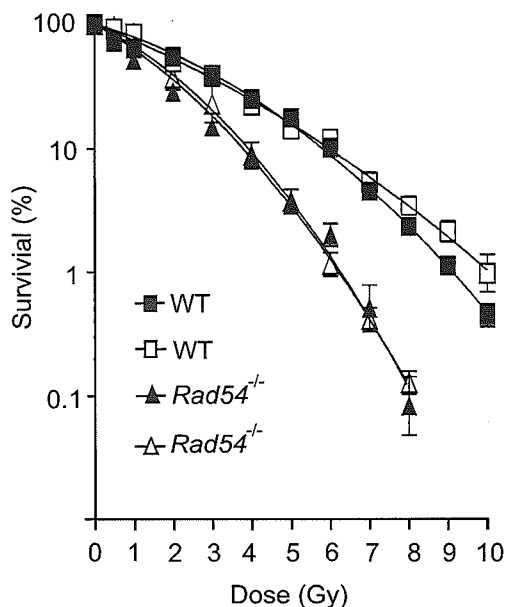
Figure 1. Single dose ionizing radiation survival curves of wild type and *Rad54*-/- ES cells. Two wild type (wt) and two independently derived *Rad54* -/- cell lines were treated with increasing doses of γ -irradiation, allowed to form individual colonies, fixed, stained and counted. The percentage of surviving cells was measured by their colony-forming ability and is plotted as a function of the γ -ray dose.

Flow cytometric analysis

60-70% confluent ES cells were irradiated with a single dose of 12 Gy using a [137] Cs source. At different time points after treatment cells were collected and fixed with 70% ethanol. After a minimum of 2 h on ice cells were washed with PBS and resuspended in 400 μl PBS containing 0.1% Triton X-100, 0.1 mg/ml propidium iodide and 0.1 mg/ml RNase. Cells were incubated overnight and analyzed on a Facscan (Becton Dickinson).

3. Results

Cell survival curves

The IR sensitivity of wild type and *Rad54*-/- mouse ES cells was determined using clonogenic survival assays. Figure 1 shows survival curves of two wild type and two independently obtained *Rad54*-/- cell lines. The wild type cell lines showed a similar average sensitivity. The two Rad54 mutant cell lines were almost superimposable and demonstrated a significantly increased IR sensitivity compared to the wild type cell lines. The relative dose resistance indicative for SLDR and expressed as a downward deflection of the survival curve, however, did not seem to be decreased.
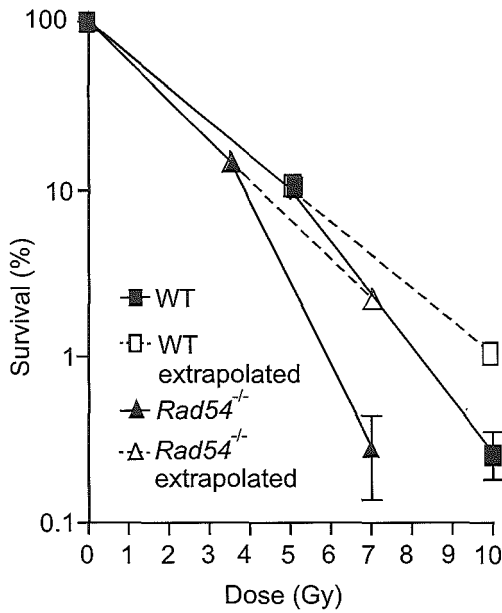
Figure 2. Single and split-dose survival curves of wild type and Rad54^{-/-} ES cells. (A) Two-point single γ-ray dose survival for wild type (wt) and Rad54^{-/-} cells determined within the split dose experiment depicted in panel B. Open symbols represent linear extrapolations showing hypothetical maximal split-dose survival. (B) Split-dose kinetics for wild type (wt) and Rad54^{-/-} cells. Doses were chosen at approximately iso-survival level determined from single dose survival curves, 10 Gy (2 x 5 Gy) for wild type and 7 Gy (2 x 3.5 Gy) for Rad54^{-/-} cells. Split-dose survival is expressed as Recovery Ratio, relative to cell survival after single administration of the irradiation dose (time zero).
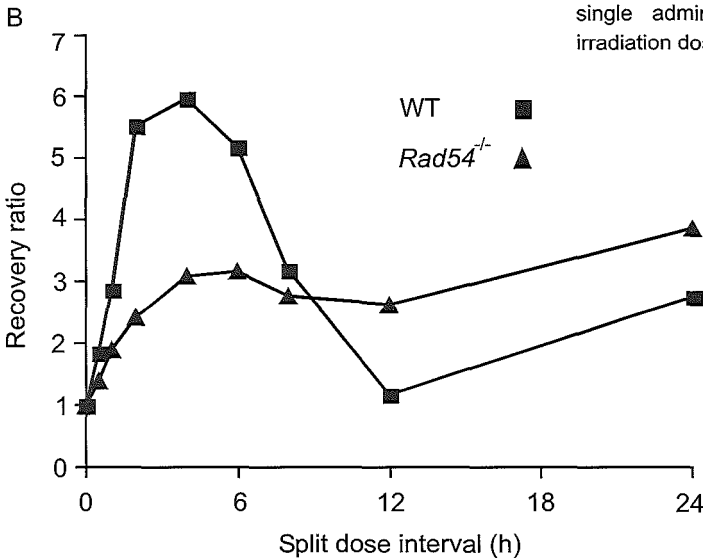
Sublethal damage recovery

To determine SLDR Figure 1 was used to choose iso-effective radiation doses for wild type and Rad54^{-/-} cells. Cell survival after giving a dose in two equal fractions with an interval allowing complete recovery of SLD should in theory be equal to that of two independent fraction doses. It is, therefore, represented by the square of surviving fraction after one fraction dose. To

control for subtle variations in experimental conditions additional single fraction dose irradiation at time zero were included in the SLDR experiment. Figure 2A shows single dose survival for total and fractional doses.

The reference survival values for wild type (10 Gy) and *Rad54* $^{-/-}$ cells (7 Gy) were both slightly smaller than those shown in Figure 1, but matched the intended iso-survival conditions. Expected cell survival after split dose recovery is indicated in Figure 2A by linear extrapolation of the first segment of the two point survival curves. SLDR is usually expressed as ratio between split-dose and single dose survival, which is termed the recovery ratio (RR). Based on data obtained from Figure 2A, the expected RR is 3.8 and 7.7 for wild type and *Rad54* $^{-/-}$ cells, respectively.

The time dependence of split dose survival was determined using intervals ranging from 0.5 to 24 h between the fractions of 5 Gy and 3.5 Gy for wild type and *Rad54* $^{-/-}$ cells, respectively (Figure 2B). The recovery time course for the wild type cell line was characteristic for rapidly cycling cells. After an initial rapid increase in RR, the second fraction irradiation at 4 h revealed the first sign of cells accumulating in late G2/M, the most sensitive phase of the cell cycle, as manifested by a decease in RR. After 12 h split dose survival efficiency approached that of a single dose of 10 Gy and after 24 h it still did not reach the attained maximum. The SLDR pattern for *Rad54* $^{-/-}$ cells was clearly different. RR increased slower and attained a lower maximum, which was a factor of two lower than for wild type cells (Figure 2B). However, the data show that *Rad54* $^{-/-}$ cell line retained a significant capacity to repair SLD.

The half times for reaching the maximum recovery, approximately 1.5 h, were similar for both cell lines. Strikingly, RR of *Rad54* $^{-/-}$ cells showed hardly any tendency to decrease again, which points to a change in cell cycle characteristics, either in duration of cell cycle phases or in the associated differences in IR sensitivity. These split-dose kinetics were not specific for the two cell lines used, since similar results were observed with independently obtained wild type and *Rad54* $^{-/-}$ ES cells. These data indicate that the observed differences in SLDR are due to absence of *Rad54* rather than a fortuitous mutation in the cell lines.

Cell cycle distribution

The apparent loss of cyclic pattern in SLDR of *Rad54* $^{-/-}$ cells suggests a possible change in cell cycle progression, although an overall change in cell cycle did not seem likely, since both wild type and *Rad54* $^{-/-}$ cell exhibited similar growth rates in cell culture. To analyze the cell cycle phase distribution of wild type and *Rad54* $^{-/-}$ cells in response to IR we used flow cytometry under the same conditions as used in SLDR experiments. Cell lines were irradiated
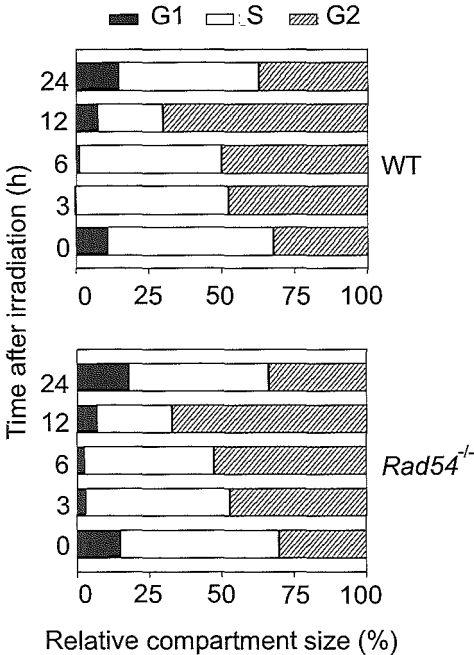
Figure 3. Cell cycle distribution of wild type and *Rad54* [-/-] ES cells before and after γ-irradiation. Wild type (wt) and *Rad54*^{-/-} cells were irradiated with 5 and 3.5 Gy, respectively, covering the range of intervals used in SLDR experiment. Shown is the mean of two independent experiments.

with split doses and the second fraction dose was delivered at four different time points (see Figure 3). Cell cycle distributions of both cell lines were similar, with a dominant S phase fraction (approximately 56%) and small G1 compartment (about 16%). The cell cycle progression patterns were also very similar. 3 h after irradiation the G1 compartment was empty, in both cell lines indicating there is no delay at the G1/S transition. Between 6 and 12 h around 70% of the cells accumulated in G2, most likely arrested at the G2/M border. At 12 h some cells were appearing in G1 and after 24 h the distribution was similar to the one before irradiation. This data indicates no effect of *Rad54* deficiency on cell cycle distribution. Therefore, the change in SLDR kinetics of *Rad54* mutant cells is likely due to changes in the cell cycle-specific radiosensitivity of these cells.

4. Discussion

Both DSB repair pathways, HR and NHEJ, have been recognized to contribute to repair of ionizing radiation damage (Gu *et al.*, 1997, Nussenzweig *et al.*, 1997, Yosida *et al.*, 2002, Essers *et al.*, 2000, see Chapter 1). Interestingly, the relative contribution of these pathways differs during animal development in mammals. While HR defective *Rad54*-deficient murine ES cells and embryos are hypersensitive to IR, adult animals are not (Essers *et al.*, 1997, 2000). In adult mice HR appears to play a backup role for the repair of IR-induced DNA damage, since its contribution to survival of adult mice is revealed only when NHEJ is impaired. *Rad54*^{-/-}*scid* mice are more IR sensitive than *scid* mice. The contribution of HR to IR protection is

best seen in rapidly proliferating cells. Irradiated *Rad54^{-/-}scid* double mutant mice die because of complete bone marrow failure (Essers *et al.*, 2000).

Contribution of HR to repair of IR induced DNA damage has been also demonstrated in chicken DT40 cells, where the irradiation sensitivity of the mutant cells is specifically increased in S phase (Takata *et al.*, 1998).

A radiobiological distinction between *Rad54^{-/-}* DT40 cells and ES cells is a considerable amount of remaining SLDR activity in ES cells, which is totally abolished in DT40 cells. The complete loss of SLDR accompanied by fully straight and steeper cell survival curves observed by Utsumi *et al.*, in *Rad54^{-/-}* DT40 cells led to the conclusion that SLDR is associated exclusively with HR. Recent and similar results with *Ku80*-deficient mouse embryonic fibroblasts, however, demonstrate also an association of SLDR with NHEJ (Myint *et al.*, 2002). In contrast with DT40 cells, *Rad54^{-/-}* ES cells show relative low dose resistance and measurable SLDR despite an increased overall sensitivity. Since large proportion of fast cycling cells resides in relatively resistant late S and early G2 and arrest temporarily at the G2/M border after irradiation, the second fraction hits the cells in the those relatively radiosensitive phases. This is manifested by lower RR caused by increased cell kill during G2/M phases of the cell cycle.

Loss of this specific pattern in *Rad54^{-/-}* cells might indicate a change in cell cycle distribution, but since in our experiments wild type and *Rad54* mutant cells cycled similarly, we propose that absence of *Rad54* causes a change in cell cycle related differences in radiosensitivity. Our experiments support findings of Takata *et al.*, who demonstrated a selective increase in IR sensitivity of S-phase synchronized *Rad54^{-/-}* DT40 cells. Similar observations were also made by Liu and co-authors in *XRCC2*-deficient hamster cell line.

In general, heterogeneity of radiosensitivity within a cell population leads to less curvature in the survival curve because the most resistant compartment will selectively increase overall survival at higher doses (Zagars *et al.*, 1987, Denekamp *et al.*, 1989). A selective increase in sensitivity of the most resistant compartment may, therefore, give the impression of a more strongly bended survival curve, even if SLDR capacity is unchanged or even lowered. This may explain the observed discrepancy between the expected (Figure 2A) and observed recovery ratio's for wild type and *Rad54^{-/-}* cells. In late S/early G2 SLD may be predominantly repaired by HR, whereas in G1/early S this may be predominantly NHEJ (Takata *et al.*, 1998; Rothkamm *et al.*, 2001). The SLDR observed in *Rad54^{-/-}* cells may, therefore, represent SLDR by NHEJ. However, it cannot be excluded that S-phase cells have retained some SLDR proficiency. In fact, considering the pronounced S/G2 compartments in ES cells the effect of deletion of *Rad54* seems smaller than might have been expected. This would suggest that there are additional HR (sub)pathways involved in DSB repair in ES cells. Our results imply that differences in cell

proliferation (i.e size of S-phase compartment) may affect the degree of radiosensitivity by *Rad54* deletion. This substantiates our abovementioned observations at the whole animal level, i.e. the finding that adult *Rad54* defective animals do not necessarily show increased radiosensitivity, and that the effects of *Rad54* deletion are best seen with rapidly proliferating cells (Essers *et al.*, 2000).

Our results may have an as yet remote consequence at the clinical level. Differences in cellular radiosensitivity, possibly as a result of varying DNA repair defects, are presumed to be a major source of heterogeneity in normal tissue sensitivity of tumor patients receiving radiation therapy. Although identification of repair defects is expected to aid in individualizing therapy, application of this knowledge may not be straightforward. Conventionally many tumors are treated with a course of 30-35 daily fractions of 2 Gy. There is a great deal of interest, however, in schedules with much shorter intervals. Although it is well accepted that cells are not easily synchronized in a conventional clinical course due to efficient cell cycle redistribution, the effects described in this study may indicate that general models used for iso-effect calculations at shorter intervals may not be adequate for tissues containing rapidly turning over critical cell populations among patients with various repair defects.

Chapter 4

Overlapping and specific roles of *Rad54* and *Rad54B* in

DNA-damage repair

Overlapping and specific roles of *Rad54* and *Rad54B* in

DNA-damage repair

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Genes of the Rad52 epistasis group are shown to be involved in homologous recombination both in yeast and mammals. We previously demonstrated that the mouse *Rad54* gene is required for homologous recombination in embryonic stem (ES) cells and contributes to resistance to ionizing radiation (IR) and the interstrand crosslink inducing agent mitomycin C (MMC). We find that at the cellular level *Rad54B* deficiency results in a milder phenotype then *Rad54* deficiency with respect to ionizing radiation sensitivity. Like *Rad54*$^{-/-}$ ES cells, *Rad54B*$^{-/-}$ cells are MMC sensitive. By contrast to *Rad54*$^{-/-}$ cells, *Rad54B*$^{-/-}$ cells display no defect in homologous recombination as measured by gene targeting. However *Rad54*$^{-/-}$ *Rad54B*$^{-/-}$ cells are extremely deficient in homologous gene targeting. At the animal level we find that just as single mice mutants, *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ animals are not IR sensitive, but they are extremely MMC sensitive. The involvement of the Rad54 paralogues in meiosis is revealed by an abnormal staining pattern of Rad51 during prophase I in meiotic spreads of *Rad54*$^{-/-}$, *Rad54B*$^{-/-}$ and *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ spermatocytes. We propose that *Rad54* and *Rad54B* function in different subpathways of homologous recombination that can have overlapping roles.

1. Introduction

The integrity of the genome is continuously challenged by endogenous and exogenous agents that damage the DNA. Double-strand breaks (DSBs), damage that affects both strands of DNA helix, are potentially very dangerous for the cell. If left unrepaired, DSBs can lead to cell cycle arrest, apoptosis or illegitimate DNA rearrangements such as translocations, inversions or

deletions (Hoeijmakers, 2001). These rearrangements can cause inactivation of tumor suppressor genes, activation of oncogenes or loss of heterozygosity, thereby contributing to cell dysfunction, cell death or carcinogenesis. In addition to potentially harmful DSBs introduced by DNA damaging agents or due to the processing of stalled replication forks, DSBs are also critical beneficial intermediates in processes that involve DNA rearrangements such as V(D)J recombination and meiosis (Modesti *et al.*, 2001).

Homologous recombination (HR) is an error free mechanism that is well suited for repair of DSBs. In the process of HR an identical or near identical sequence of the sister chromatid or homologous chromosome is used as a template to heal the DSB (Symington, 2002). Recombinational repair is a complex pathway requiring a number of proteins of the Rad52 epistasis group including Rad51 and Rad54 (Kanaar *et al.*, 1998, Modesti *et al.*, 2001). Rad51 is the key player in this process because it is critical for the search for homology and performs strand exchange between recombining DNAs (Baumann *et al.*, 1998). Rad54 is an important accessory factor for Rad51 (Tan *et al.*, 2003). A number of biochemical characteristics of Rad54 have been well defined for different species ranging from yeast to humans (Clever *et al.*, 1997, Golub *et al.*, 1997, Petukhova *et al.*, 1998, Swagemakers *et al.*, 1998, Petukhova, 1999, Van Komen *et al.*, 2000, Mazin *et al.*, 2000, Ristic *et al.*, 2001, Solinger *et al.*, 2001). Rad54 is a double-stranded (ds) DNA-dependent ATPase with ability to change DNA topology and chromatin structure (Alexeev *et al.*, 2003, Alexiadis *et al.*, 2003, Jaskelioff *et al.*, 2003). Rad54 has been implicated to participate throughout the whole duration of the HR reaction by first stabilizing the Rad51 nucleoprotein filament, subsequently by stimulating Rad51-mediated joint molecule formation and chromatin remodeling. Finally, in the last stage of the reaction it could displace Rad51 from DNA (Tan *et al.* 2003). Genetic analysis of *Rad54* has been performed in a number of species including yeast, *Drosophila* and mice. Mice lacking *Rad54* are viable (Essers *et al.*, 1997). The only detectable phenotype of the mice is their sensitivity to the cross-linking agent mitomycin C (MMC) (Essers *et al.*, 2000). This observation supports the possible involvement of *Rad54* and HR in cross-link repair. The contribution of HR to repair of ionizing irradiation (IR)-induced DSBs in adult animal is revealed only when non-homologous end joining (NHEJ), another DSB mechanism that is mechanistically different from HR, is impaired. Severe combined immune deficiency mice (*scid*) are IR sensitive due to a defect in NHEJ (van Gent *et al.*, 2001). *Rad54$^{-/-}$scid* mice are more IR sensitive than *scid* mice (Essers *et al.*, 2000). *Rad54*-deficient cells show increased sensitivity to IR, MMC and methanesulfonate, which is consistent with a defect in DSB repair. Levels of HR, measured by gene targeting, are also down in *Rad54$^{-/-}$* embryonic stem

(ES) cells, showing the requirement of *Rad54* for efficient homologous integration in this cell type (Essers *et al.*, 1997).

In yeast a homologue of *RAD54* - *RDH54/TID1* has been identified (Dresser *et al.*, 1997, Klein, 1997). *Rad54* and *Rad54B* belong to Swi2/Snf2 family of proteins implicated in chromatin remodelling. Both proteins contain seven conserved helicase motifs, but neither Rad54 nor Tid1 display helicase activity (Pazin *et al.*, 1997, Petukova *et al.*, 1998, Swagemakers *et al.*, 1998). The proteins have similar biochemical properties. Both are dsDNA dependent ATPases and the rate of ATP hydrolysis is comparable between Rad54 and Tid1. Yeast Rad54 and Tid1 promote Rad51-mediated joint molecule formation and have ability to modify DNA topology (Petukhova *et al.*, 1998, 2000). Both Rad54 and Tid1 interact with Rad51 (Dresser *et al.*, 1997, Petukhova *et al.*, 1998, 2000). In addition Tid1 promotes co localization of Rad51 and DMC1 during meiosis, but both proteins are important for disassembly of Rad51/DMC1 foci (Shinohara *et al.*, 2000). In yeast there is a functional overlap in between both proteins, but Rad54 is more important in mitosis, using sister chromatid as a template, while Tid1 is important in meiosis directing the recombination towards homologous chromosome (Klein, 1997, Shinohara *et al.*, 1997, Arbel *et al.*, 1999).

Recently, a human gene, termed *Rad54B*, sharing a significant homology to *Rad54* has been isolated (Tanaka *et al.*, 2000). Human Rad54B binds both ssDNA and ds DNA. It is a dsDNA-dependent ATPase with weaker ATPase activity than human Rad54 (Tanaka *et al.*, 2002). Human Rad54B is a nuclear protein and it colocalizes with Rad51, Rad54 and Brca1 in from of foci (Tanaka et al., 2000).

Here we report the cloning of mouse *Rad54B* gene and the generation of *Rad54B* deficient mice. We show that *Rad54B^{-/-}* and *Rad54^{-/-}Rad54B^{-/-}* ES cells are IR and MMC sensitive. We demonstrate that *Rad54B^{-/-}* display wild type levels of HR, but absence of both the *Rad54* and the *Rad54B* genes drastically influences the homologous gene targeting efficiency in ES cells, suggesting overlapping roles of both proteins in homologous recombination. Just as adult *Rad54^{-/-}* mice, neither *Rad54B^{-/-}* nor *Rad54^{-/-}Rad54B^{-/-}* animals are IR sensitive. However, adult *Rad54^{-/-}Rad54B^{-/-}* mice are extremely MMC sensitive, much more than *Rad54^{-/-}* mice. Since in *Saccharomyces cerevisiae* genes of the Rad52 epistasis group are involved in meiotic recombination, we also examine the role of *Rad54* and *Rad54B* in meiosis (Klein, 1997, Shinohara *et al.*, 1997, Shinohara *et al.*, 2000). Using immunohistochemistry we have analyzed spermatocyte spreads of *Rad54^{-/-}*, *Rad54B^{-/-}* and *Rad54^{-/-}Rad54B^{-/-}* mice and found abnormalities in distribution of Rad51 during prophase of the first meiotic division.

2. Materials and methods

Isolation of the mouse *Rad54B* cDNA

Two degenerate oligonucleotides based on the chicken *Rad54* amino acid sequence were used to perform RT-PCR on RNA isolated from mouse spleen (Kanaar *et al.*, 1996). A 123-bp DNA fragment, termed fragment A, was obtained that shared a significant homology with *Rad54*. The gene encoding this open reading frame (ORF) was named *Rad54B*. The fragment A was used as a probe (Probe 1) to screen mouse thymus (Stratagene, Cat#935303) and testis (Clontech, Cat#ML1020b) cDNA libraries. One of the positive clones (fragment B) contained 758 bp of the *Rad54B* cDNA and included a poly(A)+ tail. To obtain the complete sequence of the *Rad54B* gene the mouse testis library was rescreened with a 256-bp fragment of the 5' cDNA of human *Rad54B*. A cDNA clone that hybridized to this probe was isolated and sequenced (fragment C). It contained a putative start codon that fitted into the context of a Kozak translation initiation sequence. The missing sequence between obtained fragments was amplified by RT-PCR from mouse testis mRNA. The complete cDNA was assembled from four overlapping fragments. It contained an ORF of 2658 bp with the potential to code for a 886 amino acid protein.

Generation of a *Rad54B* disruption construct and *Rad54B* knockout mice

A DNA fragment derived from the mouse *Rad54B* cDNA was used to screen a genomic library made of 129 mouse strain DNA (Stratagene, Cat. #946308). DNA of a positive phage was isolated and the insert was subcloned into pBluescript KS. Three exons were mapped and a restriction map of five enzymes (EcoRI, BglII, HindIII, BamHI, XbaI) was determined. A 2.2 kb fragment of the genomic *Rad54B* sequence between EcoRI and BglII sites was replaced with the 1.1 kb XhoI-HindIII fragment of pMC1Neo (Stratagene) carrying the neomycin resistance (neo) gene under control of the tk promoter. A 9 kb EcoRI and 1.1 kb BglII-XbaI fragment of *Rad54B* flanked the neo gene. Targeted integration of the construct would eliminate 28 highly conserved amino acids. The isogenic targeting construct was electroporated into E14 ES cells. Colonies surviving G418 selection were screened by DNA blot analysis using EcoRI digested DNA and probe A (see Figure 2). One of 238 clones showed a fragment of a size expected for targeted integration. The DNA of this clone was further analyzed by DNA blotting using several restriction enzymes and both upstream (A) and downstream (B) probes (Figure 2). ES cells of this clone were injected into blastocysts and gave rise to 17 chimerical males, which were backcrossed with BDF1 females.

Expression of *Rad54B*

Expression of *Rad54B* in different tissues was examined by RNA blotting. The human Multiple Tissue Northern Blot (Clontech, cat.# 7759-1) was hybridized with *Rad54B* probe B and was exposed for 10 days. Expression of *Rad54B* in mouse testis was analyzed by isolation of total RNA using Sigma Tri reagent, product number T9424. 15µg of total RNA per lane was loaded on gel. RNA blotting was performed according to standard procedures.

Chromosomal localization of *Rad54B*

FISH on a murine erythroid cell line containing a trisomy of chromosome 4 (code red8, N.J. de Both) was used to determine the localization of *Rad54B*. Treatment of metaphase spreads prior to hybridization was described previously (Weeda *et al*, 1991).

A PAC clone containing *Rad54B* genomic DNA fragment was labeled with digoxigenin and used in combination with chromosome 4 specific telomeric probe labeled with biotin. Together with mouse Cot-1, the DNA probes were hybridized to metaphase spreads as described (Pinkel *et al.*, 1986). To detect the *Rad54B* signal metaphase spreads were incubated with sheep-anti-digoxigenin-rhodamine and donkey-anti-sheep-texas-red, the telomeric probe was detected using avidin D-FITC. Slides were dehydrated with ethanol, air dried and counter-stained with DAPI in antifade media.

Homologous targeting of *Rad54* and *CTCF* loci

Targeting and analysis of the *Rad54* and *CTCF* loci were done as described previously (Zhou *et al.*, 1995). The *Rad54*-pur targeting construct has been described (Essers *et al.*, 1997), *CTCF* targeting construct was kindly provided by H.Heath and Niels Galjard. Targeted integration events into the *Rad54* locus were distinguished from random integration by DNA blot analysis using probe flanking targeting construct. PCR was performed on genomic DNA of the clones transfected with the *CTCF* targeting construct. Homologous events were scored by appearance of a 5 kb PCR product, while PCR specific for the *Rad54B* locus was performed as an internal control for presence of DNA in the samples. Both PCR reactions were mixed and run on agarose gel. The χ^2 test was used to evaluate the significance between observed HR frequencies. To quantitate the efficiency of homologous gene targeting by FACS analysis, ES cells were transfected with a *RAD54-GFP* knockin construct containing a puromycin selectable marker. One week after puromycin selection, plates were trypsinized, resuspended to single cell suspensions in
phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde. After permeabilization with 0.1% triton X-100 and RNase treatment cells were analyzed in a Becton- Dickinson FacsCalibur on a green fluorescence (FL1) versus forward scatter (FSC-H) plot. GFP positive and GFP negative cells appear in separate populations, above and below the diagonal line respectively. Results were also plotted in a fluorescence (FL1) histogram and gene targeting efficiency was determined using Modfit.

Cell survival assays

The sensitivity of ES cells of indicated genotype to increasing doses of ionizing radiation was examined by their colony-forming ability as described (Essers et al. 1997). The measurements were performed in triplicate. To determine the sensitivity of Rad54$^{-/-}$Rad54B$^{-/-}$ cells to MMC, the cells were incubated in medium containing increasing concentrations of the cross-linking agent for 1 hour. Subsequently, cells were washed with PBS and fresh medium was added. Cells were grown for 10 days, after which colonies were stained and counted. The MMC survival experiments were performed in quadruplicate.

IR and MMC treatment of mice

Two-to-four months old *Rad54*$^{-/-}$, *Rad54B*$^{-/-}$, *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ littermates of both genders were irradiated with a dose of 7 Gy (^{137}Cs source) and monitored for 21 days. After this period surviving animals were euthanized. *Rad54B*$^{-/-}$, *Rad54*$^{-/-}$ and *Rad54*$^{-/-}$*Rad54*B$^{-/-}$ mice were injected with various doses of MMC and were monitored for 14 days. Females and males were treated with 15, 10, 7.5, 5, 2.5 and 1 mg of MMC per kg bodyweight.

Micronucleus assay

100 µl of peripheral blood was collected by orbital puncture. The micronucleus assay was performed according to Hayashi et al., 1990. Slides preheated to 70° C were coated with acridine orange (1 mg/ml) and covered with a coverslip. 500 polychromatic erythrocytes were scored for the presence of micronuclei using an Axioplan fluorescence microscope.

Immunocytochemistry of meiotic chromosomes

Testes were isolated from one-, two- and five- months old 129/Bl6 mice and processed as previously described (Peters et al., 1997). Immunofluorescence was performed as described (Baarends et al., 2003). Mouse monoclonal anti-SCP3 was kindly provided by C. Heyting, Wageningen, The Netherlands. The anti-Rad54 antibody was as described (Essers et al., 2002).

3. Results

Isolation of a cDNA encoding a *Rad54* paralogue

With the use of degenerate oligonucleotide primers we isolated a mouse cDNA encoding a *Rad54* paralog, named *Rad54B*. The mouse *Rad54B* cDNA consists of a 2658 bp ORF with the potential to code for a 886 amino acid protein with a predicted molecular weight of 102.95 k. Amino acid sequence comparison of mouse Rad54 and Rad54B revealed a 33.1% sequence identity that extended over the entire length of the proteins. Mouse Rad54B showed 80% of the predicted amino acid sequence identity with human Rad54B. Sequence comparison indicated that both mammalian Rad54 paralogues are more closely related to the *S. cerevisiae* Rad54 than to *S. cerevisiae* Rad54 paralogue Rdh54/Tid1 (see Figure 1).
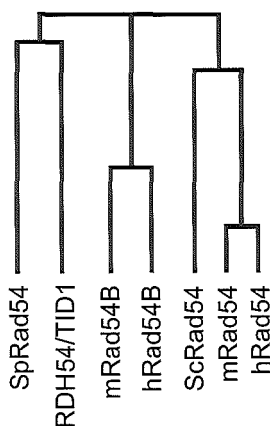

Figure 1. Dendrogram displaying evolutionary relationship among Rad54 proteins in different species. Mouse Rad54B is closely related to human Rad54B, both mouse Rad54 and mouse Rad54B are evolutionary closer to *S. cerevisiae* Rad54 than Rdh54/Tid1.

Chromosomal localization and expression pattern of *Rad54B*

To determine the chromosomal localization of the mouse *Rad54B* gene a PAC clone containing the *Rad54B* locus was used for fluorescent *in situ* hybridization analysis (Figure 2A). The *Rad54B* signal was observed on chromosome 4 near band A2, a region syntenic to human chromosome 8q21 (Vissinga *et al*., 1999) where the human *Rad54B* is localized (Hiramoto *et al*., 1999). Both mouse *Rad54* and *Rad54B* are localized on chromosome 4 (Essers *et al*., 2002), but the distance between the genes must be sufficient for efficient meiotic cross-over to occur since *Rad54$^{-/-}$Rad54B$^{-/-}$* mice are obtained according to Mendelian segregation (data not shown, see below).

Subsequently, we determined the *Rad54B* expression pattern. RNA blot analysis revealed that *Rad54B* is expressed in all the tissues tested (Figure 2B). The highest expression level was observed in tissues containing a large proportion of proliferating cells such as thymus, testis and colon, similar to the expression pattern of *Rad54* (Kanaar *et al*., 1996).
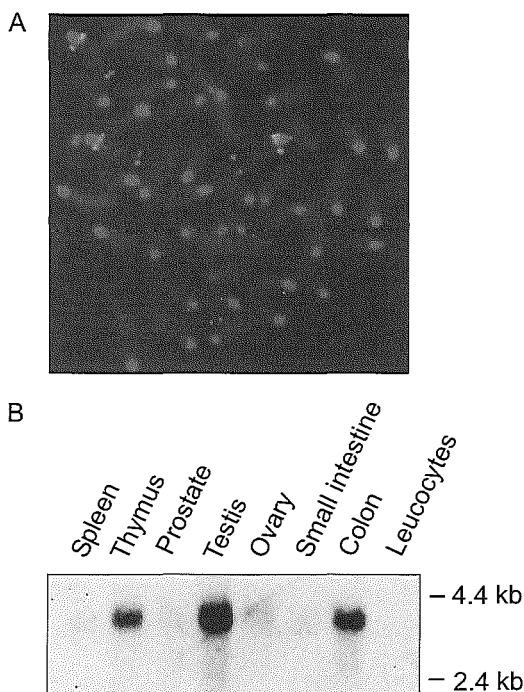
Figure 2. Chromosomal localization and expression pattern of *Rad54B*.
(A) Chromosomal localization of mouse *Rad54B* by fluorescent *in situ* hybridization. Metaphase spreads of mouse chromosomes were hybridized to a digoxigenin-labeled *Rad54B* genomic probe and a biotinylated chromosome 4 specific sub-telomeric probe. The *Rad54B* hybridization signal, in green, was detected on chromosome 4 near band A2. The chromosome 4 specific probe is in red. DAPI counterstaining of the chromosome spread revealed the chromosome banding pattern. (B) Expression of *Rad54B* in different human tissues. An RNA blot containing approximately 2 µg of poly (A)$^+$ RNA isolated from the indicated tissues was hybridized with a probe derived from the human *Rad54B* cDNA.

— 4.4 kb

— 2.4 kb

Spleen Thymus Prostate Testis Ovary Small intestine Colon Leucocytes

Generation of *Rad54B* disrupted ES cells and mice

Screening of a phage mouse genomic library (129 strain) with probe A yielded a clone spanning the 3' part of the *Rad54B* gene. Using oligonucleotides derived from the cDNA sequence, 3 exons were mapped on genomic DNA. A 2 kb BamHI fragment was subcloned, sequenced and subjected to detailed restriction analysis with 5 nucleases (Figure 3A). An approximately 2.2 kb fragment between EcoRI and BglII sites was replaced with a neomycin resistance gene (Neo) under tk promoter. Targeted integration of the construct results in deletion of a sequence of 28 highly conserved amino acids spanning the last helicase motif on the carboxy-terminal end of Rad54B. The targeting construct was electroporated into wild type ES cells. After selection clones were identified by DNA blotting using a probe outside the targeting construct (probe A) as shown in Figure 3A and 3B. A targeted clone was further analyzed using several restriction enzymes (data not shown).

The targeted clone was propagated, checked for correct karyotype and injected into blastocysts, which resulted in 17 chimeric males that were backcrossed to BDF1 females. Heterozygote animals were further backcrossed to 129Sv or C57BL6 mice and bred to obtain *Rad54B*$^{-/-}$ mice. To confirm the complete inactivation of the *Rad54B* gene, RNA blot analysis was carried out. In samples derived from wild type and *Rad54B*$^{+/-}$ testes, both the 5' and 3' probes detected the *Rad54B* transcript corresponding to a 2.3 kb band. No signal was detected in *Rad54B*$^{-/-}$ RNA sample, while a 0.6 kb band of the GAPDH transcript, used as internal control, was present in all samples (Figure 3C).

Interbreeding of F1 *Rad54B* heterozygous animals results in F2 offspring in which all three genotypes are detected in Mendelian ratio. *Rad54B*$^{-/-}$ mice are healthy and do not display any growth abnormalities up to six months of age. *Rad54B*$^{+/-}$ animals were crossed in order to obtain 3.5 day old embryos cultured for isolation of *Rad54B*$^{-/-}$ ES cells. Two independent *Rad54B*$^{-/-}$ ES cell lines, in 129 and 129/bl6 genetic background were isolated.

Further *Rad54B*$^{+/-}$ mice were intercrossed with *Rad54*$^{+/-}$ animals to obtain *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mice. Similarly to *Rad54*$^{-/-}$ and *Rad54B*$^{-/-}$ mice, *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ animals were obtained in Mendelian ratio and did not display an overt phenotype up to six moths of age. *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ ES cell line in 129/bl6 genetic background was isolated from blastocysts obtained from *Rad54*$^{+/-}$*Rad54B*$^{+/-}$ crosses.

Frequencies of targeted integration in *Rad54B* deficient cells

Previously we have shown the involvement of mammalian *Rad54* in homologous recombination by demonstrating that the efficiency of homologous gene targeting is reduced 5-10 fold in *Rad54*$^{-/-}$ ES cells (Essers
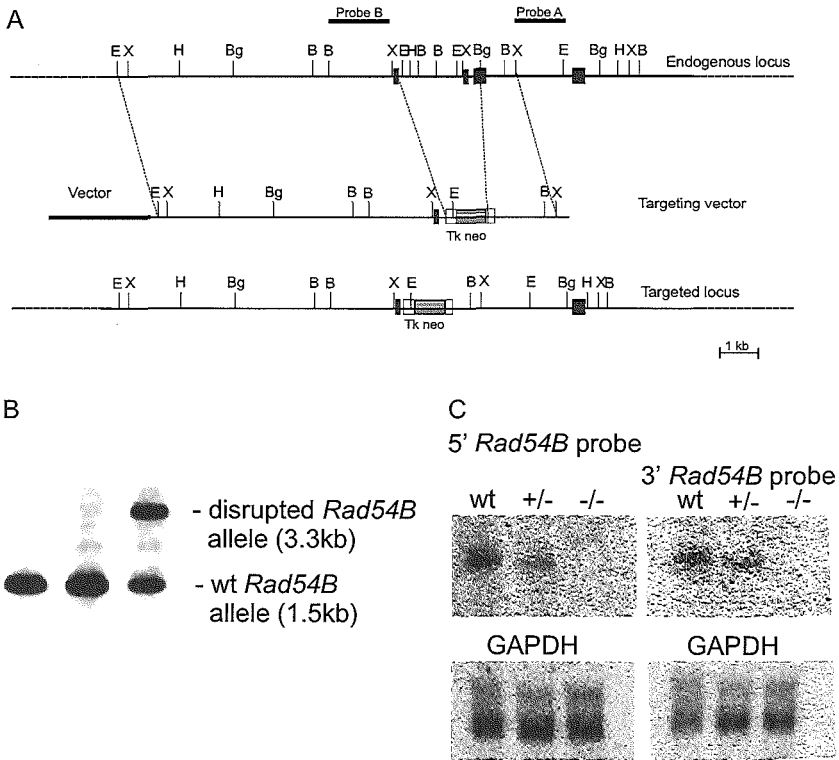
Figure 3. Characterization of part of the *Rad54B* genomic locus and generation of mouse ES cells carrying a disrupted *Rad54B* allele. (A) Part of the *Rad54B* genomic locus and structure of the targeting construct. Exons are indicated by black boxes. Shown are the locations of selected restriction sites, EcoRI (E), BamHI (B), BglII (Bg), HindIII (H), XbaI (X). The positions of two different probes, named A and B, are indicated. (B) DNA blot analysis of G418 resistant ES clones using probe A and EcoRI digested DNA. The wild type (wt) allele yields a 1.5 kb band while the disrupted allele results in a 3.3 kb band. (C) RNA blot analysis of *Rad54B* in mice carrying the disrupted allele. Total RNA (15µg) isolated from testes of wild type (wt), *Rad54B⁺/⁻* and *Rad54B⁻/⁻* males was probed with 5' and 3' *Rad54B* cDNA probes. Human cDNA GAPDH probe served as a loading control.

et al., 1997). To test whether *Rad54B* is involved in homologous recombination as well, we examined the capacity of wild type, *Rad54B⁻/⁻* and *Rad54⁻/⁻Rad54B⁻/⁻* cells for gene targeting. Cells were transfected with linearized constructs targeted to *Rad54* or *CTCF* loci. Both constructs carried puromycin resistance selectable marker flanked by 4-6 kb regions of homology. Homologous integration events into the *Rad54* and *CTCF* loci were detected by DNA blotting and PCR, respectively (Figure 4A, Table 1 and data not shown). Interestingly, we found that the efficiency of homologous recombination as measured by gene targeting is not reduced in *Rad54B⁻/⁻*

ES cell genotype	Targeted locus	
	Rad54	*CTCF*
WT	69% (87/126)	60% (54/90)
Rad54B^{-/-}	64.7% (178/275)	54% (61/113)
Rad54^{-/-}	2.1%[a,c] (6/284)	21.3%[a] (36/169)
Rad54^{-/-} *Rad54B*^{-/-}	<0.17%[b] (0/560)	2.1%[b] (7/332)

[a] The differences in targeting efficiency between wild type (WT) and *Rad54*^{-/-} cell lines are statistically significant for both targeting constructs (p<0.005 by χ^2 analysis)

[b] The differences in targeting efficiency between *Rad54*^{-/-} and *Rad54*^{-/-}/*Rad54B*^{-/-} cell lines are statistically significant for both targeting constructs (p<0.005)

[c] Previously reported (Niedernhofer *et al.*, 2001)

Table 1. Efficiency of homologous recombination in wild type, *Rad54*^{-/-}, *Rad54 B*[-/-] and *Rad54*^{-/-}*Rad54B*^{-/-} ES cells as measured by homologous gene targeting. ES cells of the indicated genotype were electroporated with the indicated gene targeting constructs. After selection under the appropriate conditions individual clones were isolated and expanded. Genomic DNA from the clones was isolated. For clones electroporated with *Rad54* targeting construct genomic DNA was digested with the appropriate restriction enzyme, electrophoresed through an agarose gel and transfered to a nylon membrane. Membranes were hybridized with radiolabeled probes that discriminated between homologously and randomly integrated targeting construct. For the clones electroporated with the *CTCF* targeting construct genomic DNA was used for PCR reactions that discriminated between random and homologous integration events. The percentage of clones containing homologously integrated targeting construct relative to the total number of analyzed clones is indicated. Absolute numbers are indicated in parentheses.

cells as compared to wild type cells (Table 1). However, the involvement of *Rad54B* in homologous recombination was revealed in absence of *Rad54*. Hardly any homologous integration events were detected in *Rad54*^{-/-}*Rad54B*^{-/-} ES cells.

We also examined the capacity of wild type and mutant cells for gene targeting using a GFP-based recombination assay. ES cells were electroporated with a construct designed to introduce a GFP-tag in the mouse *Rad54* locus. This targeting construct is referred to as *Rad54*-GFP knockin construct. Targeted integration of the construct within the *Rad54* locus results in expression of GFP-tagged Rad54 from the endogenous *Rad54* promoter. Random integration of the *Rad54*-GFP construct does not activate GFP expression (data not shown). Homologous integration of the *Rad54*-GFP knockin construct can be scored in individual cells as green fluorescence using FACS analysis, as confirmed by DNA blot analysis of genomic DNA of
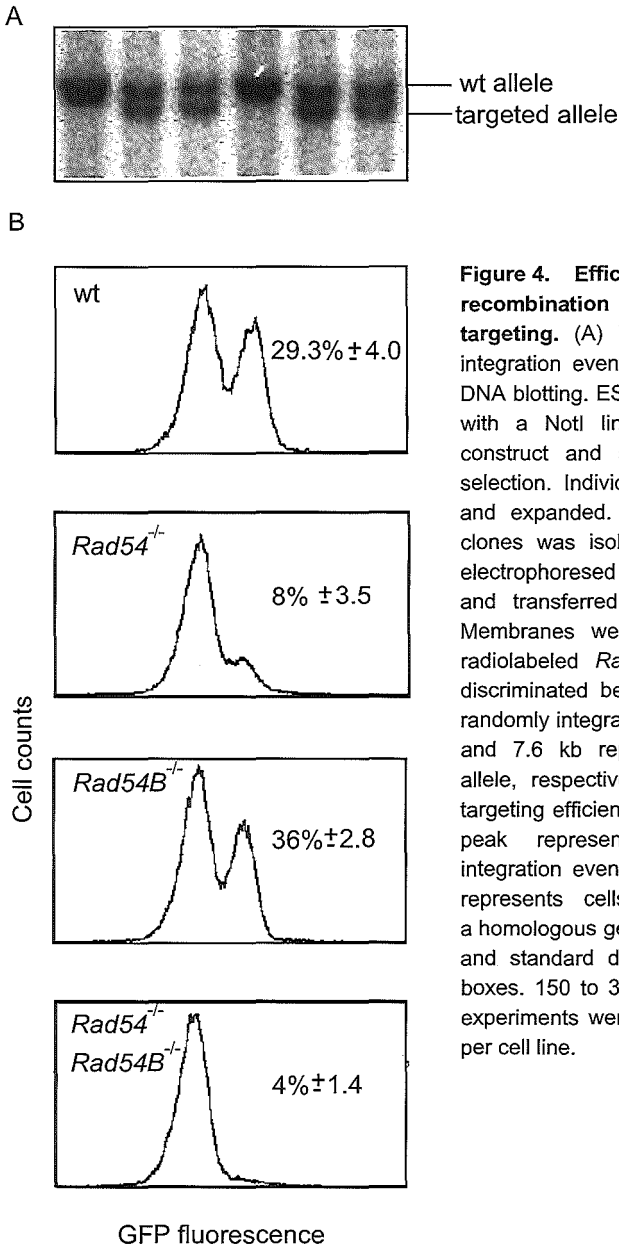
A

— wt allele

—targeted allele

B

wt

29.3% ± 4.0

Rad54$^{-/-}$

8% ± 3.5

Rad54B$^{-/-}$

36% ± 2.8

Rad54$^{-/-}$
Rad54B$^{-/-}$

4% ± 1.4

Cell counts

GFP fluorescence

Figure 4. Efficiency of homologous recombination as determined by gene targeting. (A) Detection of homologous integration events in the *Rad54* locus by DNA blotting. ES cells were electroporated with a NotI linearized *Rad54* targeting construct and subjected to puromycine selection. Individual clones were isolated and expanded. Genomic DNA from the clones was isolated, digested with StuI, electrophoresed through an agarose gel and transferred to a nylon membrane. Membranes were hybridized with α-^{32}P radiolabeled *Rad54* exon7/8 probe that discriminated between homologously and randomly integrated constructs. Bands of 9 and 7.6 kb represent wt and targeted allele, respectively. (B) Determination of targeting efficiency by FACS analysis. Left peak represents signal of random integration events. The peak on the right represents cells that have undergone a homologous gene targeting. Frequencies and standard deviations are depicted in boxes. 150 to 300 clones were analyzed, experiments were performed 2 to 3 times per cell line.

individually sorted and expanded green cells (data not shown). Using this assay, transfection of the *Rad54*-GFP construct into the wild type ES cell lines from both 129Sv and C57bl/6 genetic background resulted in targeting efficiencies of approximately 30% (data not shown). This efficiency is similar
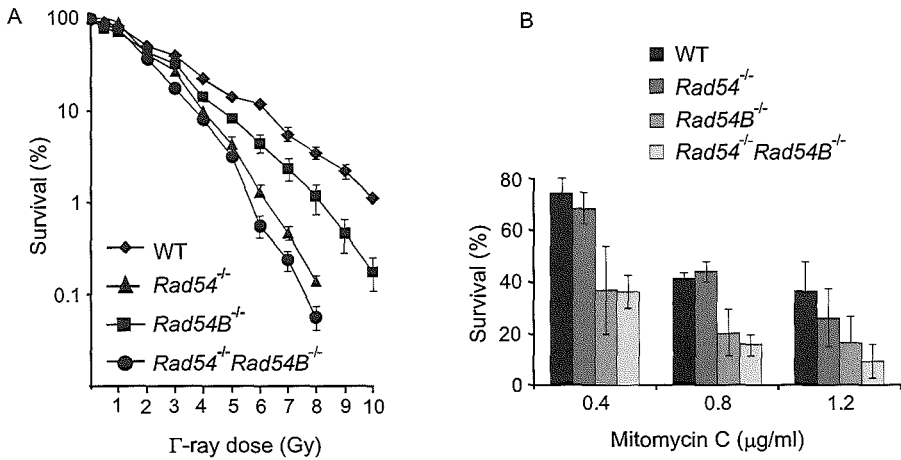
Figure 5. Effect of ionizing radiation and MMC on wild type, *Rad54B*$^{-/-}$, *Rad54*$^{-/-}$ and *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ ES cells. (A) Clonogenic survival of wild type and mutant ES cells after increasing doses of γ-rays. The percentage of surviving cells measured by their colony-forming ability is plotted as function of the γ-ray dose. (B) Clonogenic survival of wild type, *Rad54*$^{-/-}$, *Rad54B*$^{-/-}$, *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ ES cells after treatment with increasing concentrations of MMC. ES cells were treated with MMC for 1 hour, washed with PBS and grown for 10 days in fresh medium then fixed, stained and counted. The percentage of surviving cells was calculated relative to untreated cells. Cloning efficiencies of untreated cells varied between 10-30%. The experiment was performed four times and the error bars represent standard error of the mean.

to that determined by DNA blotting analysis (Niedernhofer *et al.*, 2001). We conclude that the difference in genetic background between 129Sv and C57bl/6 does not result in a significant difference in the efficiency of targeted gene replacement at the *Rad54* locus. As a negative control we used *ERCC1*$^{-/-}$ ES cells that are completely deficient in targeted gene replacement (Niedernhofer *et al.*, 2001). Transfection of the *Rad54*-GFP construct to *ERCC1*$^{-/-}$ cells resulted in 0.7 % green cells, which is similar to the background level in this assay (data not shown). Similarly to the data obtained by DNA blotting analysis, no targeting defect was observed in *Rad54B*$^{-/-}$ cell line, while the *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mutant was severely impaired in gene targeting by homologous recombination (see Figure 4B).

We also investigated the levels of spontaneous and MMC-induced SCEs in *Rad54B*$^{-/-}$ and *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ ES cells, because this assay reflects homologous recombination between completely identical DNA molecules. In contrast to *Rad54*$^{-/-}$, where the frequency of SCE decreases after MMC treatment (Dronkert *et al.*, 2000), *Rad54B*$^{-/-}$ cells displayed the same
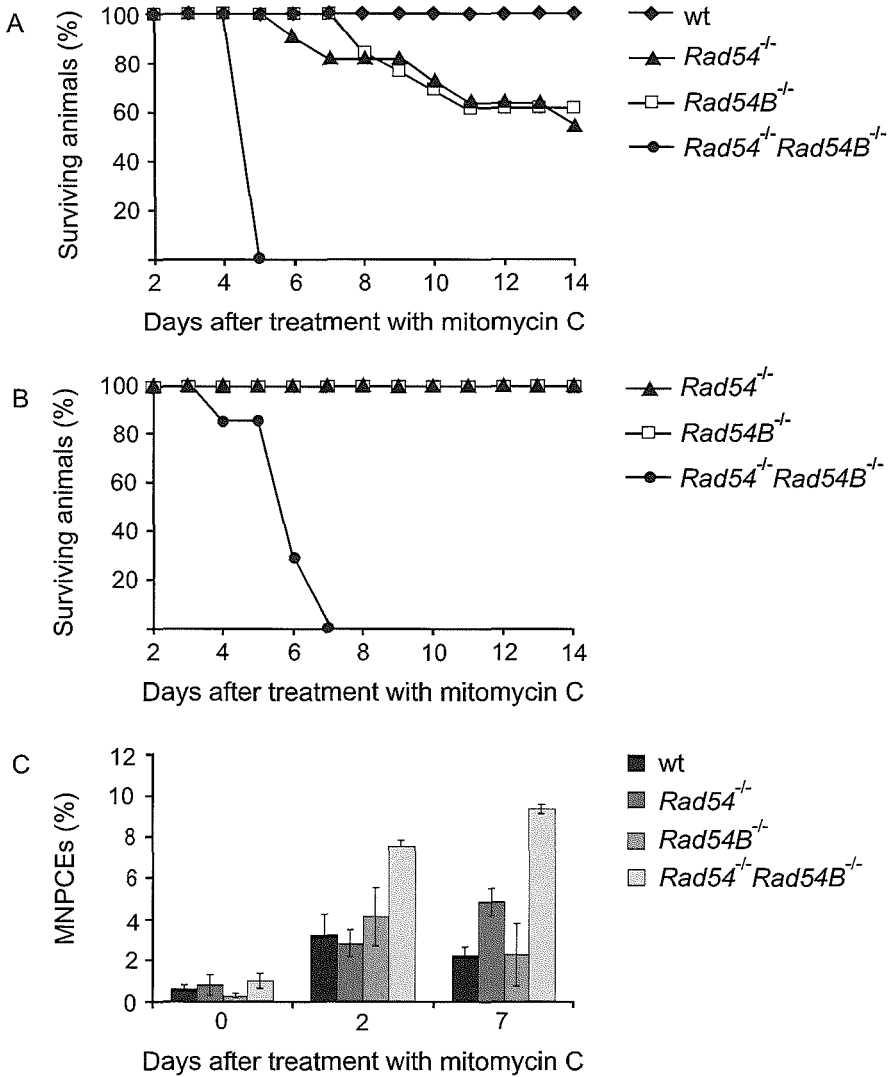
Figure 6. MMC sensitivity of *Rad54/Rad54B* mice. (A) Survival curve of *Rad54B*$^{-/-}$ (n=13), *Rad54*$^{-/-}$ (n=11) and *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ (n=4) males after single intraperitoneal injection of 7.5 mg of MMC per kg of bodyweight. (B) Survival curve of *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mice after treatment with 5 mg of MMC per kg of bodyweight. Two *Rad54*$^{-/-}$, two *Rad54B*$^{-/-}$ and seven *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ males were used in this experiment. (C) Measurement of MMC induced chromosomal aberrations in *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mice using peripheral blood micronucleus assay. Plotted are percentages of micronuclei-containing polychromatic erythrocytes per 500 polychromatic erythrocytes at day two and seven after treatment with 2.5 mg MMC per kg of bodyweight. Data represent average from three independently treated animals. The standard error is indicated.

frequency of SCE as wild type cells (data not shown), whereas the levels of SCE in *Rad54*^{-/-}*Rad54B*^{-/-} cells were comparable to those of *Rad54*^{-/-} cells.

Rad54B-deficient cells are sensitive to ionizing radiation and MMC.

To determine whether the contribution of *Rad54B* to homologous recombination impinges on the ability of the cell to repair DNA damage we examined the effect of ionizing radiation and MMC on the sensitivity of *Rad54B*^{-/-} and *Rad54*^{-/-}*Rad54B*^{-/-} ES cells. It has been previously reported that *Rad54* [-/-] ES cells were 2-3 fold more sensitive to ionizing radiation than wild type cells (Essers *et al.*, 1997). Here we show that *Rad54B*^{-/-} cells are 1.5 fold more irradiation sensitive in comparison to wild type cells. Interestingly, *Rad54B*^{-/-} cells were more sensitive to MMC than *Rad54*^{-/-} cells. *Rad54*^{-/-} *Rad54B*^{-/-} cells were very sensitive to both agents (Figure 5A, B). Interestingly inactivation of both genes did not dramatically increase the IR sensitivity of *Rad54*^{-/-}*Rad54B*^{-/-} cells in comparison to the sensitivity of *Rad54*^{-/-} cells, suggesting a minor role for *Rad54B* in repair of IR-induced DNA damage. In contrast, the sensitivity of *Rad54*^{-/-}*Rad54B*^{-/-} cells to MMC did not seem to be drastically increased in comparison to the sensitivity of *Rad54B* ES cells, pointing towards important function of *Rad54B* in repair of MMC-induced DNA damage in ES cells.

Rad54^{-/-}*Rad54B*^{-/-} mice are extremely sensitive to MMC

In order to establish the contribution of *Rad54B* to repair of DSBs in adult animals *Rad54*^{-/-}, *Rad54B*^{-/-} and *Rad54*^{-/-}*Rad54B*^{-/-} mice were irradiated and treated with MMC. Similarly to *Rad54*^{-/-} mice, neither *Rad54B*^{-/-} nor *Rad54*^{-/-}*Rad54B*^{-/-} mice were sensitive to IR. All the 2-4 months old littermates survived the treatment with 7Gy. It has been previously reported that *Rad54*-deficient mice are hypersensitive to MMC (Essers *et al.*, 2000). *Rad54B*^{-/-} and *Rad54*^{-/-}*Rad54B*^{-/-} mice were injected peritoneally with different doses of MMC and monitored for 14 days. The latency periods of *Rad54B*^{-/-} mice were comparable to those of *Rad54*^{-/-} mice at doses 15 and 10mg of MMC per kg of bodyweight (data not shown). In agreement with our previous observation, mutant females appeared to be less sensitive to MMC than the males (Essers *et al.*, 2000). Here we find *Rad54B*^{-/-} females and *Rad54*^{-/-}*Rad54B*^{-/-} females survived dose of 7.5 and 2.5 mg of MMC per kg of bodyweight, respectively. We show the survival curves of wild type, *Rad54*^{-/-}, *Rad54B*^{-/-} and *Rad54*^{-/-} *Rad54B*^{-/-} males treated with 7.5 and 5 mg MMC per kg of bodyweight (Figure 6A, B). While both single mutant males survived the dose of 5 mg of MMC per kg of bodyweight, none of the *Rad54*^{-/-}*Rad54B*^{-/-} did. The *Rad54*^{-/-}*Rad54B*^{-/-} males survived only the treatment with a very low dose of 1 mg of MMC per kg of bodyweight. We conclude that *Rad54*^{-/-}*Rad54B*^{-/-} mice are hypersensitive to MMC.

We also tested the effect of MMC on blood cells using the peripheral blood micronucleus assay. Six to eight weeks old animals of all genotypes were exposed to a single dose of 2.5 mg of MMC per kg of bodyweight. The dynamics of induction and decrease in numbers of micronuclei in polychromatic erythrocytes were similar in cells derived from wild type, *Rad54B*$^{-/-}$ and Rad54$^{-/-}$ mice. A significant increase in the frequency of micronuclei-containing polychromatic erythrocytes was observed in *Rad54*$^{-/-}$ *Rad54B*$^{-/-}$ mice, which is consistent with hypersensitivity of these mice to MMC (Figure 6C).

Abnormal distribution of Rad51 during meiosis in *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ spermatocytes.

Using immunohistochemistry we analyzed the distribution of Rad51 and its colocalization with Scp3 on meiotic chromosomes. Surface-spread meiotic chromosome prepared from wild type, one *Rad54*$^{+/-}$*Rad54B*$^{+/-}$, three *Rad54*$^{-/-}$, two *Rad54B*$^{-/-}$ and two *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mice were double stained with fluorescent rabbit monoclonal anti-Rad51 and anti-Scp3 antibodies. During leptotene and zygotene the distribution of both Rad51 (in red) and Scp3 (in green) was similar in spermatocytes of all genotypes. In leptotene Rad51 is positioned along the chromosomes and Scp3 accumulates at the forming synaptonemal complex. In control and *Rad54B*$^{-/-}$ nuclei, Rad51 signal was partially localized on the pachytene chromosomes, combined with an overall staining of the nucleus. In *Rad54*$^{-/-}$ and *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ spermatocytes Rad51 signal was concentrated in abnormal "blobs", on the chromosomes only (see Figure 7). The defect was even more pronounced in diplotene, were instead of a homogenously stained wild type nucleus, we observed long Rad51 stretches of different length in *Rad54*$^{-/-}$ and *Rad54*$^{-/-}$ *Rad54B*$^{-/-}$ cells. The abnormalities were most severe in diplotene *Rad54*$^{-/-}$ *Rad54B*$^{-/-}$ cells. The single *Rad54* $^{-/-}$ and *Rad54B*$^{-/-}$ cells displayed a less severe phenotype. Number of scored abnormalities in *Rad54B*$^{-/-}$ nuclei was significantly lower than in *Rad54*$^{-/-}$ nuclei, suggesting a minor role of *Rad54B* in meiosis (see figure 7B).

3. Discussion

Involvement of *Rad54* and other members of the *Rad52* epistasis group of genes in HR has been well established in prokaryotes and eukaryotes. Yeast lacking *RAD54* are sensitive to IR and the alkylating agent methyl methanesulfonate (MMS), consistent with the DNA repair phenotype (Friedberg *et al.*, 1995, Shinohara *et al.*, 1997). Defects in spontaneous and
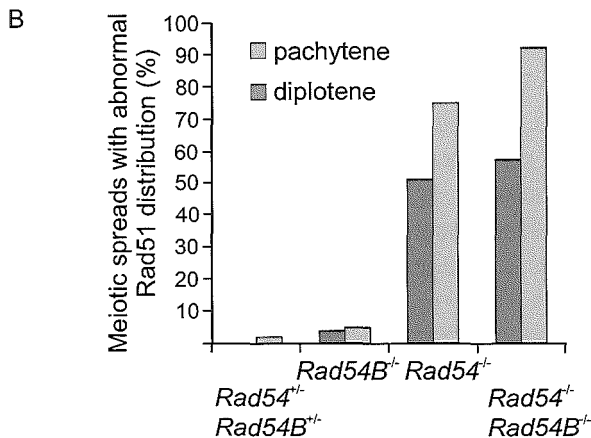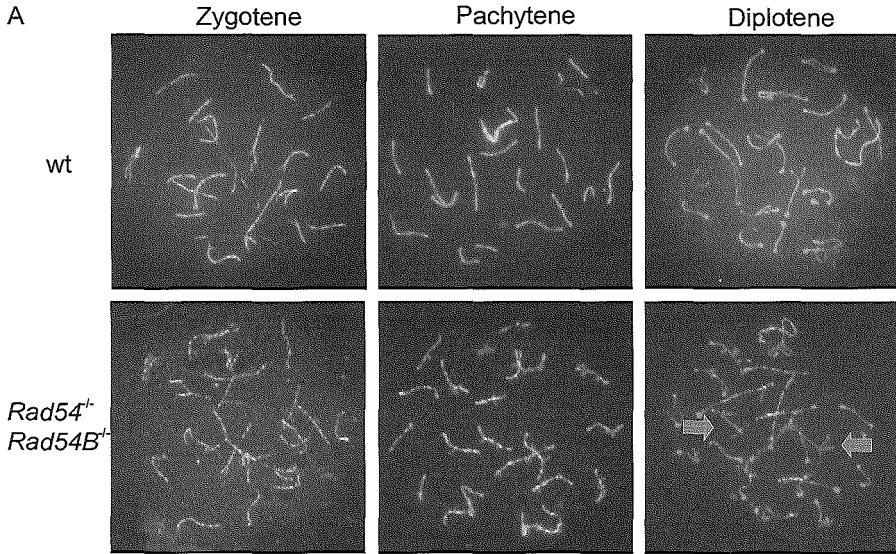
A

B

Figure 7. Analysis of meiotic chromosome spreads from *Rad54*-proficient and *Rad54*-deficient mice. (A) Localization of Rad51 and Scp3 on meiotic chromosomes from mouse spermatocytes. Localization of Scp3 and Rad51 as detected by immunofluorescence is shown in spermatocytes derived from wild type (upper panel) and *Rad54$^{-/-}$Rad54B$^{-/-}$* (lower panel) mice. In leptotene and zygotene no aberrant Rad51 and/or Scp3 staining was observed on the meiotic chromosomes from the *Rad54$^{-/-}$Rad54B$^{-/-}$* mice. Abnormalities, scored as abnormal Rad51 distribution, were visible in pachytene and diplotene chromosomes derived from in the *Rad54$^{-/-}$Rad54B$^{-/-}$* mice, while no abnormalities were present in the controls. (B) Quantification of aberrant Rad51 staining in *Rad54$^{+/-}$Rad54B$^{+/-}$*, *Rad54B$^{-/-}$*, *Rad54$^{-/-}$* and *Rad54$^{-/-}$Rad54B$^{-/-}$* meiotic spreads. Percentage of cells with aberrant Rad51 distributions indicated. In total 100 meiotic spreads per data point were analyzed. These abnormalities were never seen in wild type meiotic spreads.

induced mitotic recombination in *rad54* mutants and reduced frequency of formation of viable spores support a role of *RAD54* in mitosis and meiosis, respectively (Saeki *et al.*, 1980, Game *et al.*, 1983, Resnick *et al.*, 1987). In yeast, a *RAD54* homologue *RDH54/TID1* has been identified (Shinohara *et al.*, 1997, Klein, 1997). The two share homology on amino acid level in the seven conserved ATPase domains and the N-terminal regions. Single *RDH54/TID1* mutants do not display sensitivity to MMS while RAD54/RDH54/TID1 double mutants are more sensitive than *RAD54* mutants (Shinohara *et al.*, 1997). In contrast to *RAD54*, required for intrachromosomal recombination, *RDH54/TID1* is needed for interchromosomal recombination during meiosis (Arbel *et al.*, 1999). The reduction of interchromosomal recombination is even more pronounced in *RAD54/ RDH54/TID1* double mutants, suggestive of a possible overlap between *RAD54* and *RDH54/TID1* (Shinohara *et al.*, 1997, Klein, 1997). Compared to wild type cells, *Rad54*^{-/-} mouse embryonic stem (ES) cells are two- to four- fold more sensitive to IR, MMS and MMC. Additionally HR, measured by targeted integration, is five- to ten- fold reduced in *Rad54*^{-/-} ES cells. *Rad54* deficiency in adult mice is demonstrated only by their sensitivity to cross-linking agent MMC (Essers *et al.*, 1997). Lack of IR-induced DSB repair phenotype in the adult animal can be explained by a difference in contribution of HR to repair spontaneous and IR-induced DSBs in different stages of animal development (Essers *et al.*, 2000). The phenotype of *Rad54B*^{-/-} mice and the role of murine *Rad54B* in HR have not been described. In this work we analyze the expression of the gene, its chromosomal localization and we characterize mice and ES cells lacking *Rad54B*.

Recently we localized the *Rad54* locus to mouse chromosome 4 near band C7/D1 (Essers *et al.*, 2002). *Rad54B* was localized to the same chromosome near band A2. RNA blot experiments showed that *Rad54B* expression is increased in tissues with fast proliferating cells, like colon, but also in organs of lymphoid and germ cell development. A very similar pattern has been observed for *Rad54* (Kanaar *et al.*, 1996).

Sensitivity to DNA-damaging agents is a hallmark of yeast strains deficient in genes of the *Rad52* epistasis group. In mammalian systems, *Rad54*^{-/-} DT40 and mouse ES cells display similar phenotypes in terms of IR and MMC sensitivity (Bezzubova *et al.*, 1997, Essers *et al.*, 1997). The first clue about possible involvement of *Rad54B* in DNA repair came from our survival data where *Rad54B*^{-/-} ES cells appeared to be mildly IR sensitive, significantly less though than *Rad54*^{-/-} ES cells (see Figure 5A). Interestingly, *Rad54B*^{-/-} cell line was significantly more sensitive to MMC than *Rad54*^{-/-} cells, which may point to more important role of *Rad54B* in repair of cross-link intermediates than *Rad54* in ES cells (see Figure 5B). In contrast to *Rad54*^{-/-}

cells, homologous recombination levels as measured by targeted integration and SCE were not affected in Rad54B$^{-/-}$ ES (Table 1 and data not shown).

Disruption of Rad54B in mice does not interfere with embryonic and neonatal development. Rad54B$^{-/-}$ mice are viable, fertile and not sensitive to IR, similarly to Rad54$^{-/-}$ mice. MMC sensitivity of adult animals was comparable between Rad54$^{-/-}$ and Rad54B$^{-/-}$ mice, with males being more sensitive to this agent as observed previously (Essers et al., 2000). Since yeast RAD54 homologue RDH54/TID1 mutant has a meiotic phenotype, exhibited by defects in sporulation and spore viability (Klein, 1997, Shinohara et al., 1997), we tested possible involvement of murine Rad54B in meiosis. Consistent with normal offspring production of Rad54B$^{-/-}$ mice, hardly any abnormalities scored as Rad51 distribution during meiotic prophase I, were observed in spermatocytes isolated from these mice, indicative of non-essential function of Rad54B in meiosis (see Figure 7). Our data weakens the suggestion of Tanaka et al., (2002), that Rad54B is a functional paralogue of RDH54/TID1 in mammals.

Generation of Rad54$^{-/-}$ Rad54B$^{-/-}$ double knockout mice provided more information about a possible role of Rad54B in DSB repair via HR. Disruption of both Rad54$^{-/-}$ and Rad54B$^{-/-}$ leads to more severe phenotypes on cellular level in respect to sensitivity to IR and MMC. Rad54$^{-/-}$ Rad54B$^{-/-}$ ES cells are significantly more sensitive to these agents than Rad54$^{-/-}$ and Rad54B$^{-/-}$ cells. These observations suggest partially overlapping functions of both genes, with Rad54 being the main player in repair of IR-induced DNA and Rad54B being possibly more important for repair of cross-link intermediates. The defect in HR is much more severe in Rad54$^{-/-}$ Rad54B$^{-/-}$ ES cells, since hardly any targeting events were scored in these cells in contrast to Rad54$^{-/-}$ and Rad54B$^{-/-}$ cells. Thus, again this result suggests functional overlap between Rad54$^{-/-}$ and Rad54B$^{-/-}$. Although Rad54 can mask absence of Rad54B, Rad54B is not able to take over all the functions of Rad54 (see Table 1). SCE were not affected in Rad54$^{-/-}$ Rad54B$^{-/-}$ splenocytes and were comparable to those in Rad54$^{-/-}$ cells, indicating that Rad54B is not required for this process. Rad54$^{-/-}$ Rad54B$^{-/-}$ mice showed extreme sensitivity to MMC, supporting a possible involvement of the genes in ICL repair (see Figure 5) (Dronkert et al., 2001). Clear abnormalities were seen in the distribution of Rad51 during prophase of the first meiotic division in Rad54$^{-/-}$ and Rad54$^{-/-}$ Rad54B$^{-/-}$ spermatocytes. An abnormal Rad51 distribution pattern during pachytene and diplotene suggests a defect in Rad51 removal from chromosomes in absence of Rad54 and Rad54B. Our observations are in agreement with biochemical data of Solinger et al. (2002), stating that Rad54 protein dissociates Rad51 from nucleoprotein filaments formed on double-stranded DNA. Surprisingly, this defect does not seem to have a significant functional relevance in vivo,

since the general reproduction features of both *Rad54B*^{-/-} and *Rad54*^{-/-} *Rad54B*^{-/-} mice are not influenced by the defect.

Our data provide evidence for *Rad54B*^{-/-} involvement in HR. Although the phenotype of *Rad54B*^{-/-} cells and mice is less severe than one of *Rad54*^{-/-} mutants, depletion of both genes leads to more defects on cellular and animal level, suggesting synergism of both genes. Gene targeting experiments and immunohistochemistry on meiotic spermatocytes suggest that the genes may have a partially redundant role. *Rad54* and *Rad54B* might be important for different subpathways of recombination or be responsible for repair of different subgroups of lesions introduced by a wide range of damaging agents.

Acknowledgments

Chapter 5

Somatic hypermutation does not require Rad54 and Rad54B-mediated homologous recombination

Somatic hypermutation does not require Rad54 and Rad54B-mediated homologous recombination

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Secondary diversification of immunoglobulin (Ig) genes occurs through somatic hypermutation (SHM) in B cells of the germinal center (GC). The GC reaction is associated with a high frequency of DNA double-strand breaks (DSB) in the hypermutation domain of Ig genes. Homologous recombination (HR) is a prominent DSB repair pathway. Among the proteins involved in HR are the Rad-54 paralogues, Rad54 and Rad54B. To investigate whether Rad54/Rad54B-mediated HR is involved in SHM, we determined the ratio of mutated versus non-mutated VλPCR products from memory (IgM⁻,IgD⁻,Vλ1⁺) and GC(PNA^high ,Vλ1⁺) B cells, the mutation load, the mutation frequency, the base exchange pattern and the distribution of somatic mutations along the rearranged Vλ light chain (VλLC) genes. All these parameters of SHM were unaltered in memory and GC B cells lacking one or both *Rad54* paralogues. Thus, our data indicate that Rad54 and Rad54B-mediated HR is not essential for SHM. In addition, the finding that the ablation of *RAD51* paralogues causes an increase in SHM argues against a direct involvement of HR in promoting SHM.

1. Introduction

Primary and secondary diversification of immunoglobulin (Ig) genes by V(D)J recombination and somatic hypermutation (SHM), respectively, underlie distinct molecular mechanisms that act sequentially in B cell development to shape the enormous diversity of antibody (V) genes. While V(D)J recombination is mediated by the RAG recombinase and is restricted to B cell precursors of the fetal liver and the adult bone marrow, SHM is restricted to antigen-activated B cells of the germinal center (GC), which forms in secondary lymphatic organs, such as spleen, lymph nodes and Peyer's

patches (Rajewsky, 1992). GC B cells receive signals that induce the activation-induced cytidine deaminase (AID). AID is expressed in B cells of the germinal center and is absolutely required for SHM and Ig class switch recombination (Muramatsu *et al.*, 2000, Revy *et al.*, 2000).

SHM is an efficient process that allows the introduction of predominantly point mutations at an estimated mutation rate of 10^{-3} base pairs per generation, which is six orders of magnitude higher than the spontaneous mutation rate of eukaryotic cells. Most mutations locate within the hypermutation domain, a region of about two kilo bases (kb) downstream of Ig promoters, encompassing the rearranged VDJ and VJ segments of Ig heavy and light chain genes, respectively. Mutations are not introduced at random but locate predominately within the complementary determining regions (CDR) of antibody V genes that encode the antigen-contact sites of the Ig variable domains (for review see (Jacobs and Bross, 2001). This site preference is due to mutational hot spots, where about 50–60% of all mutations are found. These hot spots are intrinsic to the SHM process and are mutated in functional and non-functional V genes. The RGYW motif and its inverse complement WRCY (R for a purine base A or G, Y for a pyrimidine base C or T, and W for a weak hydrogen bond A or T) have been identified as hot spots of SHM (Rogozin *et al.*, 1992). In particular, the AGCW and AGTW tetramers, which encompass the AGC and AGT serine codon, have been identified as preferred hot spots and are, compared to the other four serine codons (TCN), preferentially used to encode the serines within the CDR (for review see (Neuberger and Milstein, 1995, Storb, 1996).

This codon bias highlights the importance of evolving and maintaining hypermutable Ig genes. Besides the codon bias, an A/T bias is characteristic for hypermutated Ig genes. In the coding strand of hypermutated Ig genes, A nucleotides are replaced about three- to four-fold more frequently than T nucleotides (Milstein *et al.*, 1998). The strand- biased A mutations are likely to be established by the preferential targeting of an error prone DNA polymerase to one DNA strand. Recently, the DNA polymerase η and ζ have been implicated in the process of SHM and the DNA polymerase η has been proposed to establish the A/T bias (Rogozin *et al.*, 2001, Zeng *et al.*, 2001, Matsuda *et al.*, 2001, Diaz *et al.*, 2001).

It has been demonstrated that DNA double-strand breaks (DSB) are potential intermediates or frequent by products of SHM. These DSB occur preferentially at mutational hot spots (Bross *et al.*, 2000, Papavasiliou *et al.*, 2000), for review see: (Jacobs and Bross, 2001). This suggests that hot spot mutations relate to an error prone DSB repair pathway. Three main DSB repair pathways are known, homologous recombination (HR), non-homologous end joining (NHEJ) and single-strand annealing (SSA), each of which can be divided further into subpathways (for review see van Gent *et al.*,

2001). Which of these main pathways controls the repair of the DSB found in hypermutating Ig genes is not known. However, the involvement of NHEJ and SSA is unlikely. SHM is unaffected in *Scid* mice, which harbor a mutant of the catalytic subunit of the DNA-dependent protein kinase (DNA PK$_{cs}$) and are, therefore, NHEJ deficient (Bemark *et al.*, 2000). SSA is a specialized form of homology-dependent DSB repair that only operates between directly repeated sequences and results in deletion of one of the repeats and the intervening sequence. These characteristics are inconsistent with a role of SSA in SHM. On the other hand, DSB in hypermutating Ig genes might be repaired by some form of HR that would have to be error prone. Consistent with the possible involvement of HR in SHM, it has been shown that DSB in hypermutating Ig genes occur in the late S/G2 phase of the cell cycle when the repair template DNA is available (Papavasiliou *et al.*, 2000).

The effects of genes involved in HR on SHM have been investigated in chicken cells. Chickens possess a limited number of Ig coding genes. In the light chain (LC) system only a single V-, J- and C-gene segment and in the heavy chain (HC) system a single V, 16 D and a single J gene segment exist. Despite these limitations, chickens use upstream-lying Vλ pseudogene segments to insert sections of these pseudogenes into the functional Vλ region by gene conversion (Reynaud *et al.*, 1987, Thompson and Neiman, 1987). Ablation of HR genes *XRCC2*, *XRCC3* or *RAD51B* in the chicken DT40 B cell lymphoma line causes a decrease in gene conversion and, remarkably, a marked increase in SHM (Sale *et al.*, 2001). Sale *et al.* (2001) propose that in chicken DT40 B cells only a small proportion of the lesions in the IgV genes are subjected to templated repair, which makes use of upstream pseudogenes leading to detectable gene conversion. However, a larger proportion of these lesions are subject to sister chromatid-based recombinational repair and are, therefore, not detected in chicken DT40 B cells. They propose that in the absence of XRCC2, XRCC3 or RAD51B proteins, a lesion would be resolved by some kind of error prone sister-chromatid recombinational repair process that would lead to SHM instead of error-free gene conversion.

Here we have analyzed the effect of the mouse HR genes *Rad54* and *Rad54B*, on SHM.These *Rad54* paralogues are members of the SNF2/SWI2 family of proteins that in general affect protein-DNA interaction in an ATP-dependent manner (Eisen *et al.*, 1995, Petukhova *et al.*, 1999). Both *Rad54* paralogues are involved in HR and associate via their N-terminal domain with Rad51, a key player in HR that mediates formation of joint molecules between the broken DNA and the intact template DNA (Essers *et al.*, 1997, Tanaka *et al.*, 2000). To repair a DSB, HR can utilize two different templates in the cell, the sister-chromatid or the homologous chromosome. In yeast, Rad54 is critical for sister-chromatid-based homologous recombinational repair rather

than for homologous chromosome-based repair of DSB (Arbel et al., 1999). In mouse cells, Rad54 has also been shown to be involved in DSB repair using the sister-chromatid as a repair template (Dronkert et al., 2000). Whether Rad54B is involved in homologous chromosome-based and/or sister-chromatid-based DSB repair is not yet known. Rad54-promoted HR has previously been excluded ashaving an effect on SHM (Jacobs et al., 1998). Therefore, a possible redundancy in the HR pathway provided by *Rad54B* could explain the lack of an effect of *Rad54* on SHM. Evidence for redundancy between the *Rad54* paralogues in HR has been obtained. HR, as measured by efficiency of homologous gene targeting, is markedly reduced in *Rad54*^{-/-} *Rad54B*^{-/-} mouse embryonic stem cells as compared to either single mutant (J. Wesoly, R. Kanaar et al., in preparation). Based on this knowledge, we here determined the percentage of mutated Vλ genes, mutation frequency, mutation load, distribution of mutations, and base exchange pattern in the rearranged λLC locus of GC and memory B cells obtained from *Rad54B* - and *Rad54/Rad54B* - deficient mice.

2. Results and discussion

2.1 Analysis of hypermutation in Rad54/Rad54B - deficient B cells

A previous study has shown that SHM is unaffected in memory and GC B cells derived from *Rad54*^{-/-} mice (Jacobs et al., 1998). The subsequent cloning of a *Rad54* paralogue, *Rad54B,* suggested that *Rad54* function could be redundant. To determine whether Rad54B protein is involved in SHM, we analyzed the SHM of Ig genes in B cells from either *Rad54B* single - or *Rad54/Rad54B* double-deficient mice by sequencing the V region of the rearranged λ LC locus from Vλ[+], memory (IgM[-], IgD[-]) and Vλ[+], GC (PNA [high]) B cells of these mice. Mice were immunized with 100 µg NP-chicken gamma globulin (NP-CG) and killed 10 days later. Immunization with NP-CG favors the outgrowth of Vλ- expressing B cells (Cumano and Rajewsky, 1986). The presence of Vλ[+], IgM[-], IgD[-] memory B cells in *Rad54B* single - and *Rad54/ Rad54B* double-deficient mice indicates that class switch recombination (CSR) is Rad54/Rad54B independent.

PCR amplification and sequencing of the VJ rearranged λ gene segments allows the determination of mutations along the rearranged Vλ ½ gene segments from sorted *Rad54B*-deficient and *Rad54/Rad54B*-deficient B cells. As noted previously, the single cell PCR approach omits cloning of any
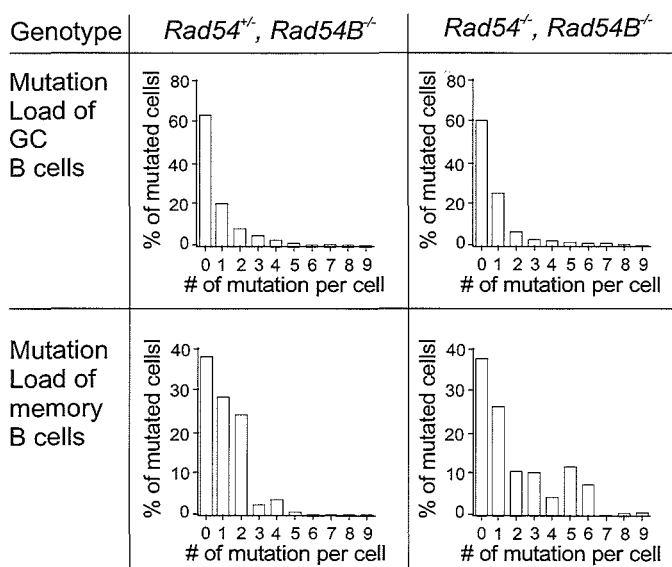
Genotype	*Rad54*^{+/-}, *Rad54B*^{-/-}	*Rad54*^{-/-}, *Rad54B*^{-/-}
Mutation Load of GC B cells		
Mutation Load of memory B cells		

Figure 1. Mutation load of GC and memory B cells in *Rad54B*-and *Rad54/Rad54B*-deficient mice. Percentage of PCR products with a given number of point mutations found per analyzed B cell.

PCR products and, due to the direct sequencing of the PCR fragments, it is also essentially not obscured by Taq polymerase error (Jacobs *et al.*, 1998). The Taq error frequency of our assay was 0.018%, as determined by amplifying and sequencing rearranged Vλ ½ segments obtained from GC B cells derived from activation- induced cytidine deaminase (AID)-deficient mice (Muramatsu *et al.*, 2000) (Table 1).

In *Rad54B* single- and *Rad54/Rad54B* double-deficient memory B cells the percentages of mutated PCR products were 63% (71/113) and 65% (45/69), respectively. These values are similar to previous studies where 59% (13/22) of the PCR products analyzed from memory B cells deficient for *Rad54* were found to be mutated (Jacobs *et al.*, 1998). In addition, these values do not deviate from that determined previously in wild-type and other repair-deficient mutants (Jacobs *et al.*, 1998). The percentages of mutated PCR products in GC B cells from *Rad54B* single- and *Rad54/Rad54B* double-mutant mice were 36% (47/129) and 42% (39/93), respectively (Table 1). Taking only the mutated PCR fragments into account, the actual mutation frequencies in the Vλ ½ gene segments of GC and memory B cells derived from *Rad54B* single- and *Rad54/Rad54B* double-deficient mice are 0.77% and 0.72% for GC and 0.92% and 1.15% for memory B cells, respectively

Tabela 1. Mutation frequencies og Vλ ½ gene segments from GC and memory B cells from *Rad54B* single and *Rad54/Rad54B* double deficient mice.

	GC B cells	Memory B cells	GC B cells	Memory B cells
Genotype	*Rad54$^{+/-}$ B$^{-/-}$*	*Rad54$^{+/-}$ B$^{-/-}$*	*Rad54$^{-/-}$ B$^{-/-}$*	*Rad54$^{-/-}$ B$^{-/-}$*
% of mutated PCR products	36% (47/129)	63% (71/113)	42% (39/93)	65% (45/69)
Mutation frequency[a]	0,28% (95/33,669^{bp})	0,58% (130/22,600^{bp})	0,30% (73/24,273^{bp})	0,75% (135/18,009^{bp})
Actual mutation frequency[b]	0,77% (95/12,267^{bp})	0,92% (130/14,200^{bp})	0,72% (73/10,179^{bp})	1,15% (135/11,745^{bp})

[a] Based on the percentage of mutation found in all PCR products sequenced
[b] Based on the percentage of mutations found in mutated PCR products only. The Taq error frequancy was determinated by analyzing GC B cells from AID deficient mice and is 0,02% in our assay.

(Table 1). Also these values do not deviate from that previously determined in DSB repair-proficient mice using the same methodology (Jacobs *et al.*, 1998). In line with these observations, also the mutation load in GC and memory B cells was unaffected (see Figure 1). As Rad54 and RAD54 cooperate with Rad51 in mediating strand invasion in HR they might be involved in establishing the A/T bias by favoring a strand-specific invasion. Therefore, the base exchange pattern of the mutations found in GC and memory B cells of the respective mutants were determined. However, like *Rad54* (Jacobs *et al.*, 1998), *Rad54B* single- and *Rad54/Rad54B* double-deficient B cells displayed a clear A/T bias (Figure 2).

In conclusion, by analyzing the percentage of mutated Vλ genes in GC and memory B cells, the mutation frequency, the actual mutation frequency, the mutation load and the base exchange pattern in Vλ -expressing GC and memory B cells we found that the process of SHM appears normal in *Rad54* (Jacobs *et al.*, 1998), *Rad54B* single- and *Rad54/Rad54B* double-deficient mice. Thus, neither Rad54 nor Rad54B are critical for the molecular process underlying SHM of Ig genes, clearly indicating that Rad54/Rad54B-mediated HR is not involved in SHM. However, our findings do not exclude a Rad54/Rad54B-independent HR repair pathway governing the repair of the DNA lesions found in hypermutating Ig genes. Rad51 - containing nucleoprotein

| Genotype | *Rad54⁺/⁻* | | *Rad54B⁻/⁻* | | | *Rad54⁻/⁻* | | *Rad54B⁻/⁻* | | |

Base Exchange Pattern in CC B cells

n=95	to				%
from \	A	G	C	T	
A		16	4	13	35
G	18		6	1	26
C	1	13		6	21
T	6	4	7		18

n=73	to				%
from \	A	G	C	T	
A		8	4	8	27
G	10		5	3	25
C	1	6		15	30
T	6	2	5		18

Base Exchange Pattern in memory B cells

n=130	to				%
from \	A	G	C	T	
A		29	16	9	42
G	26		9	9	34
C	0	3		18	16
T	4	4	3		8

n=135	to				%
from \	A	G	C	T	
A		18	13	16	35
G	31		9	8	36
C	1	6		18	18
T	6	1	8		11

Figure 2. Base exchange pattern in the Vλ ½ gene segments from GC and memory B cells from *Rad54B*-single- and *Rad54/Rad54B*-double-deficient mice. The strand bias typically for Ig genes hypermutation is defined by a preferential mutation of A rather than T bases in the coding strand and is evident in both *Rad54B*-single- and *Rad54/Rad54B*-double-deficient mice.

filaments are still capable of strand invasion, albeit in the absence of Rad54 proteins with a lower efficiency (for review see van Gent *et al.*, 2001). A recent report has demonstrated a marked shift from gene conversion to SHM in a chicken DT40 B cell lymphoma cell line lacking either one of the *Rad51* paralogues *XRCC2, XRCC3* or *RAD51B* (Sale *et al.*, 2001). Sale *et al.* (2001) proposed that normally in wild-type chicken DT40 cells a minority of DNA lesions are repaired via detectable gene conversion with a pseudogene, while the majority of DNA lesions are repaired by "silent" sister-chromatid-based HR. In the absence of efficient HR, due to the lack of *Rad51* paralogues HR might become perverted in such a way that these lesions are repaired in an error-prone manner involving an error-prone translesion DNA polymerase. This suggests the possibility that, in chicken cells, the role of HR is to suppress SHM, rather than to promote it. However, because RAD54 and RAD54B in mammalian cells are involved in HR, an alternative interpretation of these and our data in the context of mammalian SHM would be that HR does not play a role in SHM at all. Based on these findings an error-prone repair process is proposed that depends on AID and involves NBS1 and γH2AX. NBS1 and γH2AX were recently shown to co-localize in an AID-dependent manner in class switch-induced cells (Petersen *et al.*, 2001).

Although not addressed in detail, the presence of class-switched (Vλ1$^+$, IgM$^-$, IgD$^-$) memory B cells in all mutant mice analyzed indicates that CSR takes place in *Rad54B* single- and *Rad54/Rad54B* double-deficient B cells.

3. Materials and methods

Mice

Rad54-deficient mice have been described elsewhere (Essers *et al.*, 1997). *Rad54B*-deficient mice have recently been generated. *Rad54B* mutant mice lack Rad54B protein. The genotyping of Rad54B knockout will be published along with the characterization of the *Rad54/Rad54B* double-mutant mice (J.W. and R.K., in preparation). Single- and double-mutant mice were genotyped independently in Rotterdam and Basel. AID-deficient mice were kindly provided by the group of Prof. T. Honjo, Japan (Muramatsu *et al.*, 2000) and maintained at the Basel Institute for Immunology.

Immunization

Mice were immunized with NP-CG. For this, NP(28)-CGG® (Biosearch Technologies) was resuspended at 1 mg/ml in PBS, and an equal volume of Alu-Gel-S® (Serva) was added, mixed andincubated over night at 4°C. Of this suspension 0.2 ml [100 µg NP(28)-CGG] was injected i.p into the mice.

Isolation of single non-GC, GC and memory B cells

For the isolation of viable (propidium iodide negative, PI $^-$), V $^+$, CD19$^+$, GC (PNA ^{high}) and non-GC (PNA ^{low}) B cells, spleens were histoectomized and single-cell suspensions were prepared. Cells were stained with a PE-conjugated Vλ –specific antibody (LS-136) (Reth *et al.*, 1979), PNA-FITC and CD19-bio. CD19-bio was revealed indirectly with streptavidin-allophycocyanin. Single, viable, Vλ^+, CD19$^+$, PNA^{high} (GC) or Vλ^+, CD19$^+$, PNA^{low} (non-GC) were sorted. Isolation of single, viable Vλ^+, CD19$^+$, IgM $^-$, IgD$^-$ memory B cells was done as described using a FACStar^{TM} (Becton Dickinson) (Jacobs *et al.*, 1998).

Primers, single-cell PCR, and sequencing

The strategy to amplify and sequence rearranged Vλ½ genes from Vλ^+ -expressing B cells has been described in detail (Jacobs *et al.*, 1998).

Chapter 6

The Artemis homologue *Snm1* contributes to ionizing radiation resistance in mice

The Artemis homologue *Snm1* contributes to ionizing radiation resistance in mice

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The toxicity of interstrand DNA crosslink (ICL)-inducing agents and ionizing radiation (IR) to proliferating cells is the basis for their frequent application in anti-cancer therapies. Previously, we have shown that the mouse *Snm1* gene is important for the cellular response to ICLs. However, its exact function is unknown. Recently, the *Snm1* paralogue *Artemis* (*Snm1C*) has been shown to be involved providing IR resistance and in double-strand break (DSB) processing during V(D)J recombination via non-homologous end joining (NHEJ). NHEJ defective mice are hypersensitive to IR. Here, we find that *Snm1*$^{-/-}$ mice were mildly hypersensitive to IR. Furthermore, *Snm1*-deficiency sensitized *Rad54*$^{-/-}$ mice to IR. *Rad54* is involved in homologous recombination (HR), a pathway that can operate as an alternative to NHEJ in DSB repair. Previously, we have shown that NHEJ-deficient *DNA-PKcs* mutant mice also sensitize *Rad54*$^{-/-}$ mice to IR. However, inspection of the immune system of *Snm1*$^{-/-}$ mice did not reveal an overt immuno-deficiency. Thus, unlike genes required for NHEJ, *Snm1* is not required for V(D)J recombination and, by implication, NHEJ. By contrast to *Rad54*, we found no evidence for involvement of *Snm1* in HR. We propose that *Snm1* is not directly required for DSB repair, but instead, is required to repair a common, yet unidentified, subset of DNA lesions introduced by IR and certain ICLs.

Introduction

Interstrand crosslinks (ICLs) inducing agents and ionizing radiation (IR) radiation are frequently used in treatment of cancer due to their high toxicity to proliferating cells. To gain a full understanding of how ICLs and IR-induced damage is processed *in vivo* and to unravel their biological effects it is important to identify genes involved in these processes. Most data concerning the genes involved in ICL repair comes from studies of yeast, while considerably less is known about ICL repair genes in mammalian systems. A number of genes important for the cellular response to ICLs have been identified through genetic screens for interstrand crosslink-sensitive *Saccharomyces cerevisiae* mutants. The gene *SNM1*, for Sensitive to

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Nitrogen Mustard, is one of them (Henriques *et al.*, 1980, Dronkert *et al.*, 2001). Yeast cells lacking *SNM1* are sensitive to a number of cross-linking agents and mildly sensitive to UV (Ruhland *et al.*, 1981). Sequence homologues of *S. cerevisiae SNM1* have been identified in mammals (Dronkert *et al.*, 2000). *Snm1* deficient mice are viable, fertile and do not display major abnormalities if not challenged with DNA damaging agents (Dronkert *et al.*, 2000). The only phenotypic manifestation of *Snm1* deficiency in murine embryonic stem (ES) cells is their sensitivity to mytomicin C (MMC), supporting the possible involvement of the protein in the ICL repair in mammals (Dronkert *et al.*, 2000). However, the role of *Snm1* in ICL repair remains illusive. Low expression in ES cells and toxicity of the protein in overexpression systems have hindered analysis of its exact functions at the cellular and biochemical level (Dronkert *et al.*, 2000, Richie *et al.*, 2002, M.Dronkert unpublished results). Although one study has reported colocalization of Snm1 with the DNA double-strand break (DSB) processing Rad50 complex in MMC-induced foci, we have only observed these structures in cells undergoing apoptosis (M. Dronkert unpublished results, Richie *et al.*, 2002). Possibly *Snm1* could be involved in activation of MMC, recognition of ICLs or even play a role in a MMC response regulatory pathway.

The mild phenotype of *Snm1* deficient mice and cells can be explained by the presence of *Snm1* paralogues, *Snm1B* and *Snm1C*. These paralogues have also been identified in humans. The regions of the highest homology between, both, murine and human paralogues are restricted to carboxy-terminal part of the proteins. Eight motifs are particularly conserved in all proteins, although there are no indications of their exact function (Dronkert *et al.*, 2000, Callebaut *et al.*, 2002).

Based on the amino acid sequence the Snm1 proteins were assigned to a very large group of proteins called metallo-β-lactamase family. Members of this family include enzymes of wide range of activities such as sulfatases, glyoxylases, hydrolases and RNA processing enzymes (Callebaut *et al.*, 2002). Recently, one member of this family has been shown to be involved in DNA metabolism. In 2001 Moshous *et al.* identified *hSnm1C*, also termed *Artemis*, as a gene mutated in patients with T-B-severe combined immunodeficiency associated with increased cellular radiosensitivity (RS-SCID). Biochemical studies have shown that Artemis forms a complex with DNA-PKcs and that it has nuclease activity involved in hairpin opening during hairpin opening during V(D)J recombination (Ma *et al.*, 2002).

DNA-PKcs is a kinase involved in DSB repair by non-homologous end joining (NHEJ) (Hoeijmakers, 2001, Jackson 2002). Radiosensitivity of patient-derived cell lines and mouse embryonic fibroblasts (MEFs) lacking Artemis could be explained by defect in irradiation induced damage repair by NHEJ,

although direct evidence for the involvement of Artemis in this process remains to be obtained.

An alternative DSB repair pathway to NHEJ is homologous recombination (HR). *Rad54,* together with other members of *Rad52* epstasis group, participate in DSB repair via HR. Cells deficient in *Rad54* are sensitive to DNA-damaging agents, such as IR and MMC and display defects in HR as measured by homologous gene targeting efficiency, DNA-damage induced sister chromatid exchange (SCE) and have aberrant repair of site-specific induced DSB (Essers *et al.*, 1997, Dronkert *et al.*, 2000). While *Rad54^{-/-}* mice are not sensitive to IR, they are MMC sensitive, suggesting an involvement of *Rad54* and HR in ICL repair (Essers *et al.*, 1997, 2000). The contribution of HR to repair of IR-induced damage differs in stages of mouse development. While HR is extremely important for repair of IR-induced DSBs in rapidly proliferation cells and in embryo, NHEJ appears to be the dominant IR-induced DSB repair pathway in adult animals. Its contribution to IR resistance can be detected only when NHEJ is impaired (Essers *at al.*, 2000).

In this chapter we investigate the response of mice and cells derived from *Snm1^{-/-}* and *Rad54^{-/-}Snm1^{-/-}* mice to IR. Interestingly, we find that *Snm1* deficiency sensitizes *Rad54^{-/-}* mice to IR. We test the levels of HR and NHEJ in cells derived from *Rad54^{-/-}Snm1^{-/-}* mice to establish the possible involvement of *Snm1* in DSB repair.

Materials and methods

Irradiation and pathological analysis of mice

Two to four months old female and male *Snm1^{+/-}*, *Snm1^{-/-}* and *Rad54^{-/-}Snm1^{-/-}* littermates were irradiated with dose of 7 Gy using a ^{137}Cs source. After 21 days of monitoring surviving animals were euthanized. Tissues were fixed in 4% phosphate buffered formalin and embedded in paraffin. Four micron sections were stained with haemetoxilin and eosin and analyzed using a light microscope.

Immunofluorescence

Primary MEFs were grown till passage 3 to 7. Irradiation treatment of 10 Gy (^{137}Cs source) followed three days after seeding of the cells. Slides were fixed with 2% paraformaldehyde at different time points after irradiation and washed twice with 0.1% Triton-X100 in phosphate-buffered saline (PBS). Cells were incubated with anti-γH2AX antibody (Upstate Technology, #07-164) for 90 minutes, washed twice with 0.1% Triton-X100/PBS and incubated with secondary antibody (Alexa488, Molecular probes) for 60 min. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole. Experiments were performed twice with duplicate time points.

Flow cytometric analysis

Spleen, thymus and bone marrow cells from two to four months old wild type, *Rad54^{-/-}Snm1^{-/-}* and *Rad54^{-/-}Snm1^{-/-}* mice were isolated as previously described (Hendriks *et al*, 1996).

One milion cells were stained using fluorescent antibodies and scored with a FACSscan flow cytometer (Beckon Dickinson, Sunnyvale,CA). The following antibodies were obtained from Pharmingen (San Diego, CA): fluorescein isothiocyanate-conjugated anti-B220/RA3-6B2, biotinylated anti-IgM, anti-CD8, phycoerythrin (PE)-conjugated anti-CD4. PE-conjugated anti-IgD was purchased from Southern Biotechnology Associates (Birmingham, AL), Tricolor-conjugated streptavidin (Caltag Labolatories, CA) was used as a secondary antibody.

Sister chromatid exchanges

SCEs in wild type, $Rad54^{-/-}$, $Snm1^{-/-}$ and $Rad54^{-/-}Snm1^{-/-}$ splenocytes were analyzed. Metaphase spreads were also analyzed for the presence of gross chromosomal abnormalities. Splenocytes were treated with MMC (0, 0.1, 0.2 µg/ml) for 1 h, washed with PBS and cultured in medium containing 10 µM BrdU. After 23 hours cells were treated with colcemid (10 µl/ml) for 0.5 h, collected, resuspended in 0.075 M KCl. Subsequently, cells were fixed by 3 washes with methanol-acetic acid (3:1) and metaphase spread slides were made. To visualize SCEs, slides were dipped in 0.07 M Na_2HPO_4/KH_2PO_4 (pH 5.2, 87° C), washed with 0.07 M Na_2HPO_4/KH_2PO_4 (pH 6.5, RT) and stained with acridine orange for 5 min. After washing with phosphate buffer (pH 6.5, RT) cells were mounted in 0.07 M Na_2HPO_4/KH_2PO_4 (pH 6.5) with a cover slip, and exposed to UV (254 nm, 10 min). Metaphase spreads were analyzed using a fluorescence microscope (Leitz Orthoplan).

Results

Ionizing radiation sensitivity of $Rad54^{-/-}Snm1^{-/-}$ deficient mice. At least two pathways are responsible for repairing IR-induced DSB: error-free HR and error-prone NHEJ. In lower organisms HR is a major repair pathway of IR-induced DSBs, HR-deficient yeast mutants are extremely irradiation sensitive (Symington, 2002). In contrast, in higher organisms repairs most DSBs induced by IR are repaired by NHEJ, although HR also contributes to the repair process. While HR-deficient $Rad54^{-/-}$ mice are not IR sensitive, mice deficient in components of the NHEJ machinery are (Bosma et al., 1991 Taccioli et al., 1998). Since $Snm1$ shares homology with $Snm1C/Artemis$, which is involved in NHEJ, we tested $Snm1^{-/-}$ mice for their sensitivity to IR. Two to four months old littermates were irradiated with 7 Gy and monitored for 21 days. While all wild type animals were alive three weeks post irradiation, only 66% and 28% of $Snm1^{+/-}$ and $Snm1^{-/-}$ with $SNM1C/Artemis$ involved in NHEJ, we tested $Snm1^{-/-}$ mice for their sensitivity mice, respectively, survived the treatment. To test whether parallel impairment of DSB repair through HR would lead to enhanced IR sensitive phenotype of $Snm1^{-/-}$, $Rad54^{-/-}Snm1^{-/-}$ mice were generated. The additional deficiency in $Rad54$ resulted in death of all $Rad54^{-/-}Snm1^{-/-}$ animals within 14 days after irradiation treatment (see Figure 1).

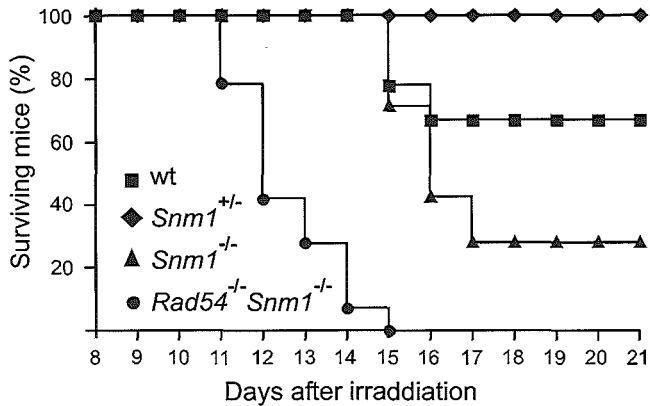**Figure 1. Effect of ionizing radiation on *Rad54^{-/-}Snm1^{-/-}* mice.** Irradiation sensitivity of two to four months old *Rad54^{-/-}Snm1^{-/-}* mice exposed to 7 Gy of whole body γ-irradiation. Six wild type, nine *Snm1^{+/-}*, seven *Snm1^{-/-}* and fourteen *Rad54^{-/-}Snm1^{-/-}* mice were used for the experiment.

Pathological analysis of *Rad54^{-/-}Snm1^{-/-}* mice. In order to determine if the sensitivity of *Rad54^{-/-}Snm1^{-/-}* mice to IR was due to global or selective toxicity of this agent, we performed a pathological analysis of tissues obtained from wild type, *Snm1^{-/-}*, *Rad54^{-/-}Snm1^{+/-}* and *Rad54^{-/-}Snm1^{-/-}* mice. Animals were euthanized at different days post irradiation and analyzed for histological abnormalities in a number of tissues. The IR treatment did not affect heart, kidney liver and lung. Moderate to severe radiation-induced damage, estimated by presence of apoptotic cells in the tissues, was observed in the intestines of treated animals. However, the bone marrow was dramatically affected by the IR treatment. Complete depletion of bone marrow cells was observed in animals of all genotypes four days after the irradiation. By day twelve the cellularity of the bone marrow had nearly completely recovered in control animals (Figure 2). While the bone marrow of *Snm1^{-/-}* mice was only partially recovered, with a few islands of bone marrow cells that could lead to full repopulation of the tissue, no repopulation was detected in *Rad54^{-/-}Snm1^{-/-}* mice (Figure 2). We conclude that *Rad54^{-/-}Snm1^{-/-}* mice do not display global radiation toxicity but their radiosensitivity results from effects of bone marrow failure.
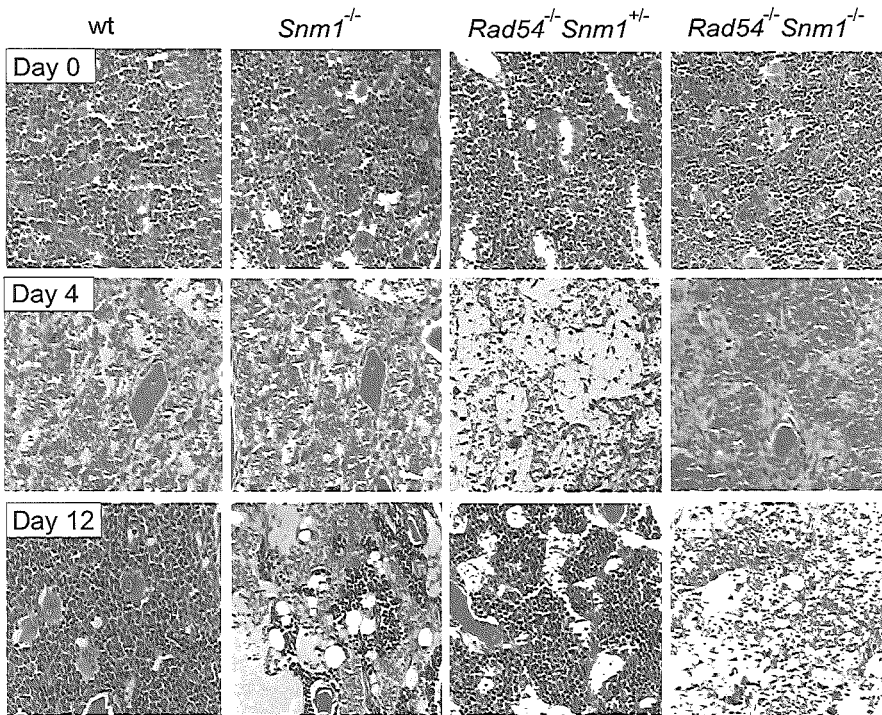
Figure 2. Histological appearance of bone marrow after ionizing radiation exposure. Two to four months old *Rad54⁻/⁻Snm1⁻/⁻* mice were irradiated with 7 Gy and euthanized at 0, 2, 4, 9 and 12 days post irradiation. Haematoxylin/eosin-stained sections of bone marrow at day 0, 4 and 12 after irradiation are shown.

γ**H2AX foci formation in *Rad54⁻/⁻Snm1⁻/⁻* primary MEFs.** One of the first cellular responses to introduction of DSBs, is the phosphorylation of histone H2AX within 1 to 3 minutes after treatment with a DSB-inducing genotoxic agent (Rogakou *et al.*, 1998). We examined the kinetics of these foci in the *Rad54⁻/⁻Snm1⁻/⁻* mutant cells. Wild type, *Rad54⁻/⁻*, *Snm1⁻/⁻* and *Rad54⁻/⁻Snm1⁻/⁻* primary MEFs were irradiated with 10 Gy and fixed at different time points after irradiation. Wild type, *Snm1⁻/⁻* and *Rad54⁻/⁻* cells displayed similar kinetics in disappearance of γH2AX foci, with approximately 30% of positive cells, 24 hours after the irradiation treatment. Interestingly, over 54% of foci *Rad54⁻/⁻ Snm1⁻/⁻* cells were still positive for γH2AX foci at the same time point, suggesting that these cells might have more difficulty with removal of the irradiation-induced DNA damage. These results could explain the more pronounced irradiation sensitivity of the *Rad54⁻/⁻Snm1⁻/⁻* mice in comparison to *Snm1⁻/⁻* mice.
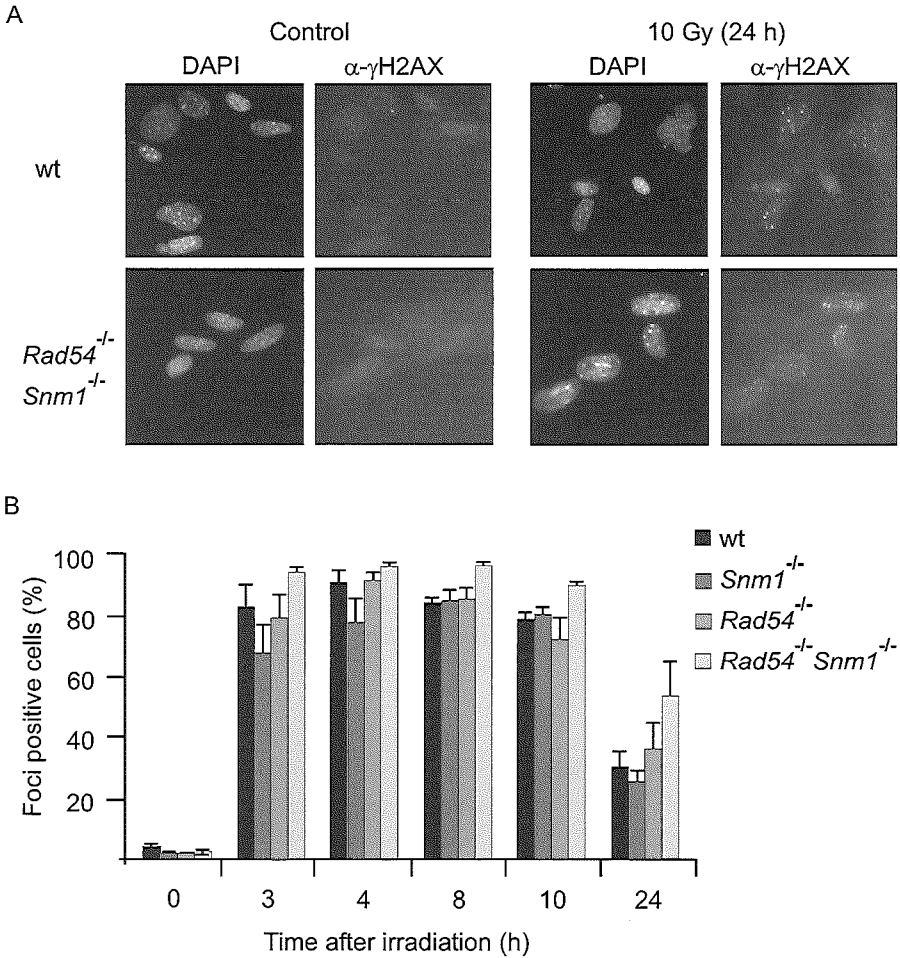
Figure 3. Ionizing radiation-induced γH2AX foci in primary *Rad54^-/-^Snm1^-/-* MEFs. (A) Wild type and *Rad54^-/-^Snm1^-/-* primary MEFs without and 24 hours after treatment with 10 Gy are shown. Left panel shows DAPI stained nuclei, while the right panel displays α-γH2AX staining. (B) Quantitative analysis of γH2AX foci in primary *Rad54^-/ Snm1^-/-* MEFs. The experiment was performed twice, with each time point scored in duplicate. Three hundred nuclei were counted per time point.

Homologous recombination and non-homologous end joining in *Rad54^-/-^Snm1^-/-* mice and cells. To investigate whether the IR sensitivity and difficulty with processing of DNA damage are caused by a defect in one of the DSB repair pathways, *Rad54^-/-^Snm1^-/-* cells and mice were assayed for levels of HR and defects in NHEJ.

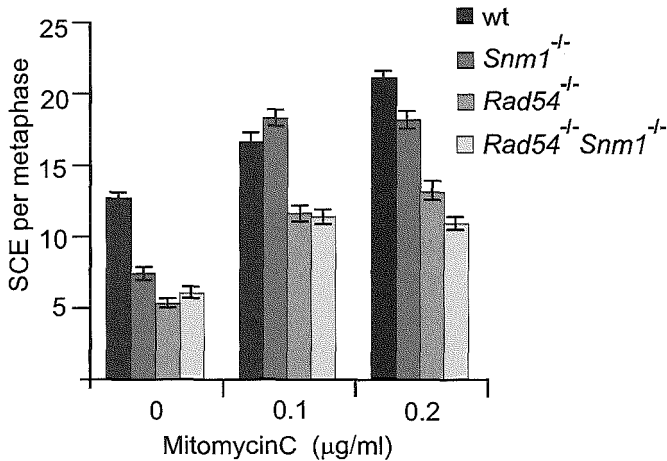**Figure 4.** **Induction of sister chromatid exchanges in *Rad54^{-/-}Snm1^{-/-}* splenocytes.** Wild type, *Rad54^{-/-}*, *Snm1^{-/-}* and *Rad54^{-/-}Snm1^{-/-}* splenocytes were analyzed for the frequency of SCEs. Cell were either mock treated or treated with 0.1 or 0.2 µg/ml of MMC for one hour in presence of BrdU. After 24 hours cells were collected and metaphase spreads were prepared. Fifty metaphases per sample were analyzed. The error bars represent standard error of the mean

To analyze HR efficiency we measured spontaneous and MMC-induced levels of sister chromatid recombination in *Rad54^{-/-}Snm1^{-/-}* splenocytes (Sonoda *et al.*, 1999). Wild type and HR-deficient *Rad54^{-/-}* cells served as controls (Dronkert *et al.*, 2000). The level of SCE, either spontaneous or DNA damage-induced, in *Snm1^{-/-}* cells did not significantly differ from that observed in wild type cells. In the absence of *Rad54*, the increase in SCE induced by DNA damage is less than in wild type cells (Dronkert *et al.*, 2000). The same effect was observed in *Snm1^{-/-}* cells in absence of *Rad54*, excluding the possibility of masking role of *Rad54* in this process in absence of *Snm1* (see Figure 4). Levels of HR measured by gene targeting were also not affected in *Snm1* deficient ES cells (J.Essers, personal communication).

To investigate whether *Snm1* is necessary for the processing of DSBs during V(D)J recombination, as it is the case for many proteins required for DSB repair by NHEJ, we analyzed the expression of immunoglobulin and TCR genes in *Snm1^{-/-}* and *Rad54^{-/-}Snm1^{-/-}* mice using flow cytometry. Bone marrow, thymus and spleen of these mice were examined for presence of premature and mature B and T cells. The B cell population in bone marrow and spleen was analyzed on the basis of surface expression of B220, IgM and
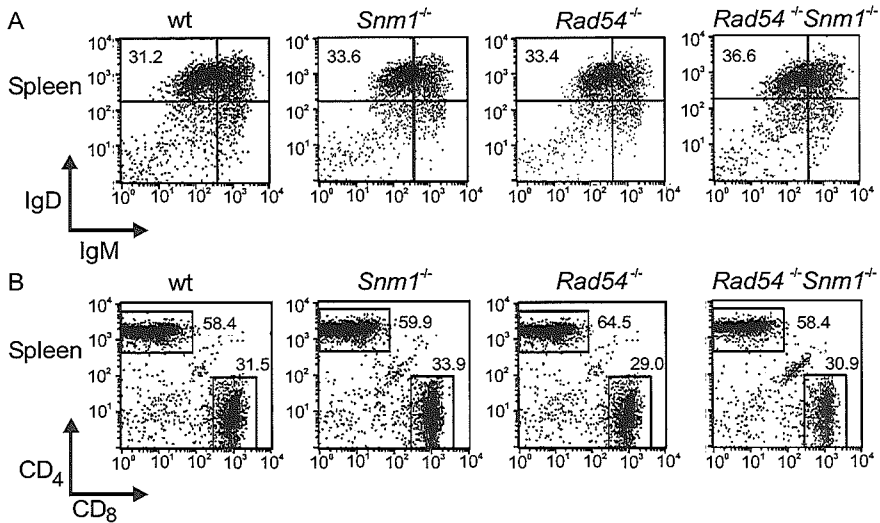
Figure 5. Flow cytometry analysis of mature B and T lymphocytes of *Rad54⁻ᐟ⁻Snm1⁻ᐟ⁻* mice. (A) Surface IgM-IgD profiles of splenic B cells. Percentages of B220⁺ mature B cells (IgM^low^IgD^high^) are given. Data are displayed as dot plots of all gated viable B220⁺ cells from 1x10^6 total events.(B) Surface CD8-CD4 profiles of splenic T cells. Percentages of mature CD3⁺ cells positive for CD8 or CD4 are indicated. Data are displayed as dot plots of all gated CD3⁺ cells from 1x10^6 total events. Lymphocytes were gated on the basis of forward and side scatter.

IgD, which allows for a classification of B cell progenitors. No differences were observed in levels of various B cell progenitors among the genotypes, suggesting that absence of *Snm1* did not affect completion of immunoglobulin V(D)J recombination (Figure 5A).

Similarly, the absence of *Snm1* did not affect the differentiation process leading to mature single-positive T cells, since examination of thymus, bone marrow and spleen of *Snm1⁻ᐟ⁻* and *Rad54⁻ᐟ⁻Snm1⁻ᐟ⁻* mice did not reveal quantitive differences in numbers of premature and mature T cells in these tissues (Figure 5B). We conclude that Snm1 is not absolutely required for NHEJ-mediated processing of DSBs during V(D)J recombination.

Discussion

The hypersensitivity of yeast cells mutated in the *SNM1* gene led to classification of *SNM1* in a group of genes involved in ICL repair that includes a number of DNA repair genes involved in nucleotide excision repair, HR and postreplication/translesion repair (Dronkert *et al.*, 2000). The role of Snm1 in

ICL repair is unknown. It has been postulated that *SNM1* might be involved in a step after ICL formation either in modulation of chromosome structure or restoration of the continuity of DNA (Magana-Schwencke *et al.*, 1982, Wilborn *et al.*, 1989). Recently obtained data concerning the human *Snm1* homologue - *Snm1C/Artemis* involved in NHEJ, one of the two major DSB repair pathway, put *Snm1* in a different light (Ma *et al.*, 2002). Since mouse mutant lacking NHEJ genes are IR sensitive, we tested IR sensitivity of *Snm1*$^{-/-}$ mice (Bosma *et al.*, 1991, Taccioli *et al.*, 1998). Interestingly, *Snm1*$^{-/-}$ mice are mildly hypersensitive to IR. Previously we have shown that the IR hypersensitivity of NHEJ defective mice is enhanced by a deficiency in a second major DSB repair pathway, HR (Essers *et al.*, 2000). Consistent with an involvement of *Snm1* in NHEJ, the IR hypersensitivity of *Snm1*$^{-/-}$ mice is significantly increased in the absence of *Rad54*, which imparts HR deficiency (see Figure 1). The difference in survival between *Snm1*$^{-/-}$ and *Rad54*$^{-/-}$*Snm1*$^{-/-}$ mice is not caused by global radiation toxicity but by bone marrow failure (Figure 2). To gain more insight into the underlying defect that causes increased radiation sensitivity of *Snm1*$^{-/-}$ mice in the absence of *Rad54* we have analyzed the radiation response in the absence of both genes at the cellular level. For this purpose phosphorylation of histone variant H2AX is a convenient marker. Phosphorylation of H2AX, leading to γH2AX, occurs in chromosome domains containing a DBS and can be detected by immunofluorescence as nuclear foci (Rakagou *et al.*, 1998, Modest *et al.*, 2001). Longer persistence of γH2AX foci *Rad54*$^{-/-}$*Snm1*$^{-/-}$ in primary MEFs in comparison to wild type, *Rad54*$^{-/-}$ and *Snm1*$^{-/-}$ cells suggests that in *Rad54*$^{-/-}$*Snm1*$^{-/-}$ cells problems associated with processing of the IR-induced DSB is compounded compared to either single mutant (Figure 3). Radiation induces a large spectrum of DNA lesions, including DSBs that can differ in the exact chemical nature of the DNA end. Possibly a subset of DSB exist that can be processed through a pathway either involving *Rad54* or *Snm1*. The kinetics of repair are expected to be reduced in *Rad54*$^{-/-}$*Snm1*$^{-/-}$ cells.

To test whether *Snm1* is involved in DSB repair through the HR or NHEJ pathway we analyzed the effect of *Snm1*-deficiency on assays reporting on HR and NHEJ. *Snm1*, in contrast to *Rad54*, does not appear to play a role in HR, at least as measured by spontaneous and DNA damage-induced SCE and gene targeting (Figure 4). Furthermore *Snm1*, in contrast to other NHEJ factors, is also not required for the processing of DSBs during V(D)J recombination because no difference in the amount of premature and mature B and T cell was detected in *Snm1*$^{-/-}$ mice (Figure 5). However, we can not exclude the possibility that the nature of the coding joints in the rearranged genes is affected in absence of *Snm1*. Thus, *Snm1* and *Artemis* differ in their cellular functions. While Artemis is a nuclease involved in V(D)J

recombination (Ma *et al.*, 2002) and most likely in NHEJ, there is no direct evidence of Snm1 involvement in those processes.

The role of *Snm1* in repair of IR-induced DNA damage remains a challenging puzzle, similarly to its role in ICL repair. Possibly, both ICL agents and IR introduce a common subset of damages that require *Snm1* for repair or processing. It will be important to determine the subset of lesions that is acted upon by *Snm1*. Taking under consideration the homology between *Artemis* and *Snm1* one could postulate that *Snm1* is a nuclease, similarly to *Artemis* and takes part in processing of DNA repair intermediates that require cleavage of the phosphodiester backbone of the DNA.

Acknowledgements

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Summary

This thesis focuses on characterization of genes involved in DNA repair. I concentrate on homologous recombination (HR) and non-homologous end joining (NHEJ), mechanisms involved in repair of double-strand breaks (DSBs), cross-link (ICL) repair, meiotic recombination and processes ensuring genetic diversity of immunoglobulins, which are thought to be linked to the DSB repair mechanisms. General information about these processes and genes involved in the reactions can be found in **Chapter 1**. The thesis describes the characterization of two genes *Rad54* and *Rad54B* and their function in HR, meiosis and immune system development. Additionally, a possible function of *Snm1*, ICL gene, in ionizing irradiation (IR) induced DNA damage is investigated.

    Chapter 2 describes in detail chromosomal localization and intron/exon borders of *Rad54*. The expression pattern of the protein is analyzed and linked to the potential function of *Rad54* in HR. Ratios between Rad54 and the key protein of HR - Rad51 are estimated and possible explanations for the difference in expression level are discussed.

    In **Chapter 3** we test if absence of *Rad54* influences the split-dose recovery in embryonic stem cells. We propose that the change in the recovery pattern in absence of *Rad54* could be due to a selective increase of the sensitivity of the normally relatively resistant S-phase of the cell cycle.

    In **Chapter 4** experimental work concerning the role of *Rad54B* in HR is presented. *Rad54B* is a paralog of *Rad54* and its function in mammalian HR has not been defined. Chromosomal localization, generation of *Rad54B*$^{-/-}$ mice and phenotypic characterization of the *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mice and cells is described. Mice and cells are tested for their sensitivity to genotoxic agents like IR inducing DSBs and mytomycin C (MMC), an agent inducing ICL. Levels of HR in *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ embryonic stem (ES) cells are measured and the course of meiosis is analyzed in spermatocytes derived from these mice.

    In **Chapter 5** a role of *Rad54* and *Rad54B* in somatic hypermutation (SHM) is investigated. SHM is secondary diversification process of immunoglobulin (Ig) genes. It occurs in B cells and presence of DSB in the hypermutation domain of Ig genes has been associated with this reaction. Here we analyse mutation load, mutation frequency, the base exchange pattern and the distribution of somatic mutations along the rearranged Vλ light chain genes.

Chapter 6 describes experiments with $Rad54^{-/-}Snm1^{-/-}$ mice and cells. Here we try to identify the cause of the sensitivity of $Snm1^{-/-}$ mice to IR. We test the cells for responses to IR induced DNA damage and defects in HR and NHEJ. The possible functions of $Snm1$ in these damage responses are discussed.

Samenvatting

Dit proefschrift beschrijft de karakterisatie van een aantal genen die een belangrijke rol spelen bij het herstellen van schade aan DNA. Hierbij richt ik me voornamelijk op een tweetal breukherstel-mechanismen: 1) homologe recombinatie (HR), en 2) niet-homologe eind-koppeling (NHEK), die betrokken zijn bij herstel van dubbelstrengs breuken (DSB), cross-links (kruisverbindingen) en processen die ten grondslag liggen aan meiose en de genetische diversiteit van immunoglobulines . Verder beschrijft dit proefschrift de karakterisatie van de genen *Rad54* en *Rad54B*, en hun mogelijke functies in het proces van HR en tijdens de meiose en ontwikkeling van het immuunsysteem. Daarnaast werd de eventuele rol van het *Snm1* gen bestudeerd bij het herstel van DNA breuken veroorzaakt door Röntgen straling.

Hoofdstuk 1 geeft een algemene inleiding en uiteenzetting van deze mechanismen en processen, en een beschrijving van de genen waarvan is aangetoond dat ze hierbij een cruciale rol spelen, is uiteengezet in.

In **Hoofdstuk 2** wordt uitvoerig ingegaan op een aantal eigenschappen van het *Rad54* gen en het gecodeerde eiwit. Zo wordt in detail de chromosomale localisatie en de intron/exon overgangen van *Rad54* gen beschreven. Verder wordt ingegaan op de subcellulaire localisatie van het Rad54 eiwit, en de mogelijke rol die het eiwit vervult bij het breukherstel-mechanisme via HR. De hoeveelheid Rad54 eiwit ten opzichte van de hoeveelheid van Rad51, een eiwit dat een centrale rol speelt in het HR proces, werd zo nauwkeurig mogelijk bepaald, en op basis hiervan word een model gepresenteerd met dynamische aspecten (zowel spatieel als temporeel) van deze twee eiwitten tijdens het proces van HR.

Hoofdstuk 3 beschrijft experimenten waarin het effect van uitschakeling van het *Rad54* gen in embryonale muizencellen op het herstel/ overleving na "split-dose" blootstelling aan röntgenstraling werd getest. Wij stellen voor dat het verschil in herstelpatroon door afwezigheid van *Rad54* mogelijk het gevolg is van een selectief gevoeliger worden van de fase van de celcyclus waarin het genoom gedupliceerd wordt, de S-fase, die onder normale omstandigheden relatief ongevoelig is.

In **Hoofdstuk 4** wordt experimenteel werk behandeld betreffende de rol van *Rad54B* tijdens HR. *Rad54B* is een paraloog van het *Rad54* gen, maar de functie van het gecodeerde eiwit is nog onbekend. De chromosoomlocalisatie van *Rad54B*, genereren van *Rad54B$^{-/-}$* muizen en de fenotypische karakterisering van deze muizen, tevens ook *Rad54$^{-/-}$Rad54B$^{-/-}$* muizen staat in dit hoofdstuk beschreven. Behalve de muizen, werden ook

primaire cellen afkomstig van deze muizen onderzocht, tijdens herstel van DNA schade. Zowel muizen als cellen werden getest op hun gevoeligheid voor genotoxische agentia, zoals Röntgenstraling en mitomycine C, een stof die onder andere DNA kruisverbindingen veroorzaakt. De eficientie van HR in *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ embryonale muizencellen werd gemeten, net als het beloop van het proces van meiose in spermatocyten afkomstig van deze muizen.

In **Hoofdstuk 5** is de rol van *Rad54* en *Rad54B* onderzocht in het proces van somatische hypermutatie. Dit is een mechanisme in B cellen dat een rol speelt bij de secundaire diversificatie van immunoglobuline genen. Verder is de aanwezigheid van DSBen in het hypermutatie-domein van Ig genen, kenmerkend voor dit proces. In dit hoofdstuk analyseren we het aantal mutaties, de mutatie-frequentie, het patroon van base-veranderingen en de distributie van somatische mutaties over de gere-organiseerde V λ light chain genen.

Hoofdstuk 6 beschrijft experimenten waarin gebruik gemaakt werd van *Snm1*$^{-/-}$ en *Rad54*$^{-/-}$*Snm1*$^{-/-}$ muizen en hiervan afkomstige cellen om de gevoeligheid van *Snm1*-deficiente muizen voor Röntgenstraling beter te kunnen begrijpen. De *Snm1*$^{-/-}$ en *Rad54*$^{-/-}$*Snm1*$^{-/-}$ cellen werden getest om de reactie op door Rontgenstraling veroorzaakte DNA schade en de efficiëntie van HR en NHEK vast te stellen. De mogelijke functie van *Snm1* in deze schade herstelprocessen wordt verder uitgediept.

List of publications

Essers J., Hendriks R.W., Wesoly J., Beerens C.E.M.T., Smit B., Hoeijmakers J.H.J., Wyman C., Dronkert M.L.G., Kanaar R. (2002). Analysis of mouse *Rad54* expression and its implications for homologous recombination. DNA Repair 1, 779-793.

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