

# **Analysis of DNA Recombination Proteins in DNA Damage Response**

De analyse van het gedrag van DNA recombinitie eiwitten  
veroorzaakt door DNA schade

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# ANALYSIS OF DNA RECOMBINATION PROTEINS IN DNA DAMAGE RESPONSE

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## SCOPE OF THE THESIS

The aim of the research described in this thesis is to investigate the response of the homologous recombination proteins Rad51 and Rad54 to DNA damage in mammalian cells. Homologous recombination is one of the DNA repair pathways involved in the repair of DNA double strand breaks (DSBs) and DNA interstrand cross-links (ICLs). These classes of DNA damage are extremely toxic and it is known that even a single DSB, if not repaired, can lead to cell death. If improperly repaired, DNA sequence could be altered, resulting in a mutation. Mutations in DNA can cause cancer, one of the most widespread diseases in our modern society. Basic research in the field of DNA repair not only enables us to understand more of the origins of cancer, but is also important when trying to eliminate tumors: radiotherapy and chemotherapy are common approaches to cure patients from cancer. These therapies are aimed to inflict a lethal amount of DNA damage to tumor cells. The types of DNA damage inflicted by these therapies include those that can be repaired by homologous recombination. Thus, basic research in the field of recombination repair is important for both understanding the origin of cancer and for the elimination of cancer cells.

**Chapter one** is the introductory chapter of this thesis. Section 1.1 explains the reasons to do basic research in the field of DNA repair and the different levels of research. Section 1.2 gives an overview of the different classes of DNA damage and the associated DNA repair pathways. Section 1.3 goes deeper into the repair pathways of DSBs and summarizes the different sub-pathways of homologous recombination. More concise information of the involved proteins is given in section 1.4, while involvement of the same proteins in another process, i.e. DNA replication, is reviewed in section 1.5. A brief summary of their involvement in meiotic processes is given in section 1.6.

In **chapter two**, the behavior of the recombination protein Rad51 is monitored. When DSBs or ICLs are induced in cells the Rad51 protein will redistribute into discrete nuclear foci. Using this feature of the protein, we analyzed different mutant cell lines known to be sensitive to ionizing radiation (IR), which induces DSBs, and agents that cause ICLs. Any altered Rad51 response indicates that the mutation affects proper functioning of Rad51-involved DSB repair. Thus, using Rad51 as an indicator we could detect which other proteins were involved in the same pathway and which were possibly not involved.

**Chapter three** describes the functions of the recombination protein Rad54. One way to investigate the functions of a protein is to eliminate the protein by a knock-out mutation. The result is that one can see what happens in the absence of the protein. In our case, we replaced the wild type protein by a (HA-) tagged version, using a knock-in construct. This gave us the opportunity to visualize the protein in the cell, to see how the protein replies to DNA damage and to monitor its interaction with Rad51. Different cell fixation techniques were used and compared. In addition, we used a biochemical approach, which revealed that Rad54 can alter the double-stranded DNA conformation, which could promote the formation of joint molecules.

In **chapter four**, our own results and recent findings of other research groups with respect to Rad54 are reviewed. The multiple roles and functions of Rad54 in repair of a DSB via homologous recombination are discussed.

**Chapter five** describes the response of Rad54 to hydroxy-urea (HU). HU is a replication inhibitor, not a direct DNA damaging agent. HU causes replication forks to

stall and one of the side effects is that DSBs can arise in the vicinity of the stalled forks. Absence of Rad54 indeed leads to an accumulation of DSBs. Importantly however, is that Rad54's behavior is different in the context of IR-treated cells compared to HU-treated cells (kinetic differences). Therefore we argue that the Rad54 activity in HU-treated cells reflects a role in processing stalled replication forks, rather than in DSB repair.

## LIST OF ABBREVIATIONS

BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
BER	base excision repair
BIR	break-induced replication
BLM	Bloom's syndrome
BRL	buffalo rat liver
Cq	chloroquine
DAPI	4',6-diamidino-2-phenylindole
dNTP	deoxy-ribonucleoside triphosphate
dsDNA	double-stranded DNA
DSB	double strand break
EM	electron microscopy
ES cell	embryonic stem cell
GFP	green fluorescent protein
HA	hemagglutinin
HJ	Holliday junction
HNPCC	hereditary non-polyposis colorectal cancer
HR	homologous recombination
HU	hydroxy-urea
HUIC	hydroxy-urea inducible complex
ICL	interstrand cross-link
IF	immunofluorescence
IR	ionizing radiation
JM	joint molecule
MMC	mitomycin C
MMR	mismatch repair
MMS	methylmethanesulfonate
MN	micronuclei
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
PBS	phosphate-buffered saline
PFGE	pulsed field gel electrophoresis
RPA	replication protein A
RTS	Rothmund-Thomson syndrome
SCE	sister chromatid exchange
SDSA	synthesis dependent strand annealing
SSA	single strand annealing
SSB	single strand break
SSB protein	single strand binding protein
ssDNA	single-stranded DNA
TLS	translesion synthesis
WRN	Werner's syndrome
XP	xeroderma pigmentosum
YFP	yellow fluorescent protein

## CHAPTER ONE

### **INTRODUCTION**

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## **1.1 Introducing DNA repair**

The information for a cell's survival, maintenance and function is stored in its DNA, thus it is essential to keep that information intact. However, our DNA is under continuous threat; we are exposed to UV-light, consume genotoxins present in food, or inhale these via cigarette smoke and fumes, and produce oxygen radicals during our normal cellular metabolism. Also, our DNA is subject to spontaneous hydrolysis and deamination (160). All these processes damage our DNA (and proteins) and in this way, each cell of our body suffers an estimated 10,000-1,000,000 DNA lesions every day (159, 163, 220). That most of us do not succumb to lethal DNA damage underlines the success and importance of our cells' DNA repair mechanisms.

Deficiencies in our DNA repair mechanisms can give rise to cancer. Cancer can originate from unrepaired (or unsuccessfully repaired) DNA lesions, which give rise to mutations. Mutations in the DNA cause the altered functions/behavior of cells, giving rise to tumor induction and outgrowth (49, 93, 260). Tumors in cancer patients can be removed mechanically (surgically), but also via radio, chemo and immuno-therapy (and in theory, gene therapy). Most of these methods are based on damaging the DNA causing cell death. Inflicting sufficient levels of DNA damage to kill tumor cells, but not to kill the adjacent healthy cells requires more insight in DNA repair.

Basic research not only elucidates why some chemotherapeutic agents are more successful to certain types of cancer than others, but also contributes to the design of new anticancer drugs. In addition, basic science led to the discovery that dysfunction of some DNA repair proteins is linked to specific types of cancer and to syndromes leading to cancer and/or premature ageing. Sometimes this dysfunction is inherited (e.g. XP, Xeroderma Pigmentosum, caused by deficiency in nucleotide excision repair) and then it is worthwhile to regularly screen persons at risk in order to prevent cancer.

The amount of DNA damage inflicted during chemotherapy depends on the state of the cell cycle (whether the cell is actively replicating, dividing or resting), on import/export of the drug, on the ubiquitination/protein degradation (since some proteins involved in repairing the damage are also actively involved or subject to protein degradation), on the state of chromatin and other DNA binding proteins (which could prevent access of the drug by covering the DNA). Finally, to understand the amount of damage caused and the way it could be repaired, a biochemical approach to study DNA and proteins at the molecular level is necessary.

## **1.2 Overview: different DNA repair pathways**

When a cell is confronted with an altered DNA metabolism due to DNA damage, it can decide to commit suicide (when there is too much damage to repair) or to induce cell cycle arrest in order to create time to repair the lesions. Repair is not only essential to avoid mutations, but also needed to avoid that the lesions interfere with transcription and replication processes. Cells have evolved different repair systems to counter virtually all different kinds of DNA lesions. Every system deals with a special kind of damage. An overview of different types of damage, caused by different sources and repaired via different systems is shown in table 1.

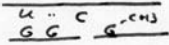
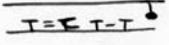


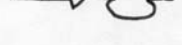





agent/source	X-rays oxygen radicals, alkylating agents, spontaneous hydro- lysis, deamination	UV light, polycyclic aromatic hydro- carbons	X-rays, $\gamma$ -rays	DNA crosslinking agents	Replication errors
					
examples of damage	C-->U, abasic sites, single strand breaks (SSBs), O <sup>6</sup> - meG	bulky adducts; cyclobutane pyri- midine dimers, (6-4) photopro- ducts	double strand breaks (DSBs)	interstrand cross- links, intrastrand crosslinks	base mismatches, insertion/deletion loops
					
pathway(s)	Base Excision Repair (BER)	Nucleotide Excision Repair (NER)	Homologous Re- combination (HR), nonhomologous end-joining (NHEJ)	HR, NER, MMR(?)	Mismatch Repair (MMR)

Table 1. Overview of different sources of DNA damage and examples of associated DNA lesions. The different classes of DNA lesions are repaired by specific DNA repair pathways, but in some cases lesions can be repaired via different pathways.

One repair system is **BER (base excision repair)**, see Fig. 1A), devoted to recognize minor damage to bases and sugars in the DNA. It is the safeguard against damage caused by oxygen radicals, methylation, spontaneous deamination, hydrolysis (which are all inherent to normal cellular metabolism) and SSB (single strand breaks). Briefly, the majority of BER repaired DNA lesions are recognized by highly specialized DNA glycosylases. They often recognize only one substrate and remove the damaged base from the DNA, leaving a sugar phosphate backbone (abasic site, which can also arise by spontaneous hydrolysis). The APE1 endonuclease now cleaves the abasic site (though some glycosylases have intrinsic endonuclease activity and do not need APE1 for cleavage). SSBs in the DNA strands are recognized and bound by PARP and this involves XRCC1 and PNK. After XRCC1-PNK binding or APE1 cleavage there are two possibilities to proceed. Short patch BER, which is the main mode, and which replaces the damaged base using XRCC1, DNA Polymerase  $\beta$  and DNA ligase III. Alternatively, a minor pathway called long patch BER is used. This pathway excises the base and mediates incorporation of a new nucleotide but also a small patch of few nucleotides adjacent to the excised base (2-10 bases) and uses PCNA, DNA polymerase  $\delta$  or  $\epsilon$  and DNA ligase I. The short stretch of replaced adjacent bases are removed by FEN-1.

A second pathway, **NER (nucleotide excision repair)**, see Fig. 1B), deals with all kinds of bulky adducts such as DNA intra-strand cross-links, 6-4 photoproducts and pyrimidine dimers. Briefly, there are two sub-pathways, which differ in the way they recognize the damage. During global genome NER, scanning for DNA damage is based on recognizing helix distortion, which is in humans performed by the XPC-HR23B (homologue of Rad23B) proteins. In the transcription coupled NER sub-pathway recognition is based on the ability of the DNA lesion to halt the DNA transcription machinery. The stalled RNA polymerase (II) complex must then be positioned in an efficient way by the combined actions of the CSA and CSB proteins

in order to recruit other NER proteins to remove the transcription blockage. The two sub-pathways converge at this step. Now 25 or more NER proteins come into action, and a dynamic protein complex is built up composed by the different individual components. One of the most important factors within the complex is the TFIIH transcription factor, which includes the helicases XPB and XPD, required to open the DNA structure around the lesion. Replication protein A (RPA) will then coat the DNA of the undamaged strand, while the NER endonucleases ERCC1-XPF and XPG will cut the damaged strand 5' and 3' of the damaged base respectively. This results in the removal of a stretch of 20-32 oligo-nucleotides containing the damage. The NER protein complex disassembles, and the regular DNA replication machinery is recruited to fill the gap.

Disorders linked to improper NER are, among others Cockayne's Syndrome, trichothiodystrophy and Xeroderma Pigmentosum, a disease associated with a high risk for UV-induced skin cancer.

A third pathway, long patch **MMR** (**mismatch repair**, see Fig. 1C), is devoted to correct replication errors such as base mismatches and misaligned bases or base loops. In the mammalian system, the MutS $\alpha$  (MSH2-MSH6) heterodimer recognizes base mismatches and loops containing 1-2 displaced bases, while the MutS $\beta$  (MSH2-MSH3) heterodimer has a preference for base loops of bigger size. After initial recognition follows, in most cases, binding by the MutL $\alpha$  complex (hMLH1-hPMS2). MutL $\alpha$  interconnects the MutS complex with the various replication factors, endo and exonucleases needed for strand discrimination (between the correct, parental strand and the newly synthesized daughter strand) and for repair. Strand discrimination may be based on localizing the nearest replicating DNA polymerase. Then follows excision of a stretch of DNA (~ 1kb) including the lesion and DNA resynthesis to fill the gap.

The best-known clinical symptom linked to a dysfunctional MMR pathway is HNPCC. Mutations in the *MSH2* and *MLH1* genes are responsible for half of the cases.

DNA double strand breaks (DSBs), induced by ionizing radiation and endogenously produced radicals, can be repaired via NHEJ (non-homologous end-joining) and HR (homologous recombination). The latter pathway is also involved in repairing inter-strand cross-links in the DNA, which can be induced by some chemotherapeutic agents. Both NHEJ and recombination are discussed in the section DSB repair below.

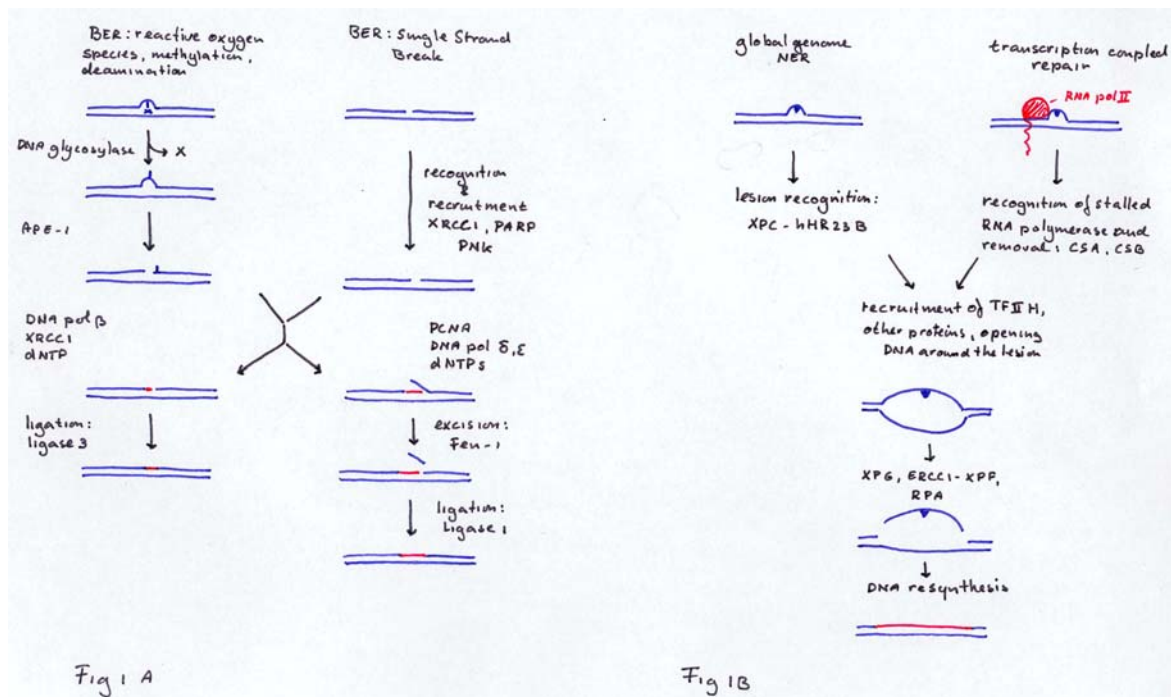


Figure 1. 1A, BER: differential recognition complexes (see text for more details). The majority of BER associated lesions are recognized by highly specialized DNA glycosylases, which remove the base and leave an abasic site. The latter is cleaved by APE1, creating a gap. Single strand breaks are recognized by PARP and equally involves XRCC1 and PNK. After primary recognition the repair can occur via filling of the 1 nucleotide sized gap with a nucleotide by DNA polymerase  $\beta$  and help of XRCC1 (in red), after which the nick is ligated by Ligase III. Alternatively, repair involves Polymerase  $\delta$  or Polymerase  $\epsilon$ , which resynthesize an adjacent stretch of (2-8) nucleotides (in red) after which the adjacent stretch of original nucleotides (sticking out) are excised by FEN1. Sealing of the nick occurs by Ligase I.

1B, also NER has two ways of recognizing lesions (see text for more details). During global genome repair lesions are recognized by XPC and hHR23B, during TCR the stalled RNA polymerase is recognized (and removed) by CSA and CSB. During the next step TFIIH and other proteins are recruited, leading to the opening of the DNA strands around the lesion and coating by RPA. The lesion and adjacent nucleotides are removed using the endonucleases ERCC1-XPF and XPG. The remaining gap (of 20-32 nucleotides) is filled (in red) by polymerases and sealing of the nick by ligases resulting in the fully restored end product.

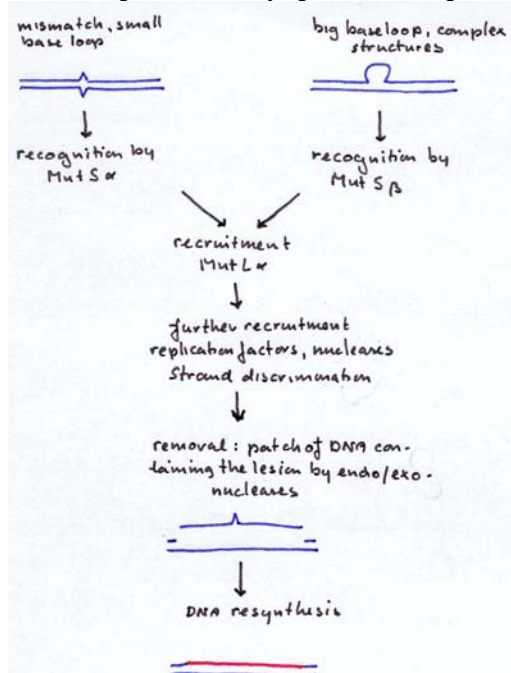


Figure 1C, MMR also two recognition complexes: MutS $\alpha$  recognizes mismatches and small base loops, MutS $\beta$  recognizes bigger base loops (containing more than 2 displaced bases). After primary recognition, MutL $\alpha$  binds

to MutS $\alpha$  or MutS $\beta$  and initiates the further recruitment of replication factors, nucleases. During this step the right strand has to be identified, only after that the lesion (and a stretch of adjacent nucleotides of ~1kb) is removed and the gap filled by the replication machinery and sealed by ligases. The newly synthesized DNA is shown in red. For more details see text.

### **1.3 Double Strand Break repair**

#### **DSB repair in prokaryotes**

As mentioned in section 1.2 there are two main pathways for repairing DSBs: homologous recombination and NHEJ. Though bacteria possess NHEJ activity (478), the predominant DSB repair pathway is recombination. The bacterium *Escherichia coli* (*E. coli*) largely repairs the DSBs via RecA (and at least 25 other proteins, of which the most important are mentioned below) mediated recombination (Fig. 2). Briefly, when a DSB is produced, it is in most cases processed by the multi-protein complex RecB-RecC-RecD, which has both helicase and nuclease functions. RecB-RecC-RecD enters at the DSB, unwinds the DNA strands and degrades one of the strands in a 3'→5' direction, resulting in a 5' single-stranded tail, until it encounters a Chi ( $\chi$ ) sequence. At the  $\chi$ -sequence the RecB-RecC-RecD enzyme is remodeled resulting in a reduction of translocation speed of the helicase and redirection of the nuclease activity to the other strand (these  $\chi$ -sequences are dispersed throughout the genome). Alternatively, the broken ends can be processed by the nuclease RecE, resulting in a 3' single stranded tail. Equally, in absence of RecB-RecC-RecD, a helicase (e.g. RecQ) could separate the strands, or work in concert with a 5' exonuclease (probably RecJ) to render single-stranded DNA overhangs.

RecA coats the ssDNA, and SSB proteins (single strand binding proteins) facilitate RecA binding by removing secondary DNA structures. Excessive DNA coating by RecA can be modified by the RecF-RecO-RecR complex, which is most important for gap repair as opposed to DSB repair. RecF-RecO-RecR is also shown to assist by overcoming the SSB protein-DNA interactions, which could prevent RecA from DNA binding. The RecA coated DNA filament can pair to a homologous DNA molecule and initiate strand pairing and strand exchange. The structure of a single strand paired to double-stranded DNA is called a D-loop. Now DNA (re)synthesis by DNA polymerase I starts, using the undamaged homologous DNA as a template. The junctions between the two molecules are called Holliday junctions (HJ) and can move along the length of the DNA molecules (called branch migration). Branch migration can be driven by RecA, but it is likely that it is assisted by several helicases. For example, the RuvA-RuvB complex stimulates RecA promoted migration by about a five-fold. On the other hand, other helicases such as RecG, have the ability to reverse the direction of RecA driven branch migration (see also section 1.5). This may provide an alternative mechanism for resolution of the HJs.

However, the majority of these recombination intermediates is resolved by nucleolytic resolution of the HJs. The HJ resolvase RuvC recognizes and specifically cleaves HJs by two symmetric nicks, one on each side of the HJ. Depending on the orientation of the nicks in the second HJ with respect to those in the first HJ the result will be gene conversion without (Fig. 2-a) or with (Fig. 2-b) cross-over. Ligation of the nicks will render the fully restored recombination products.

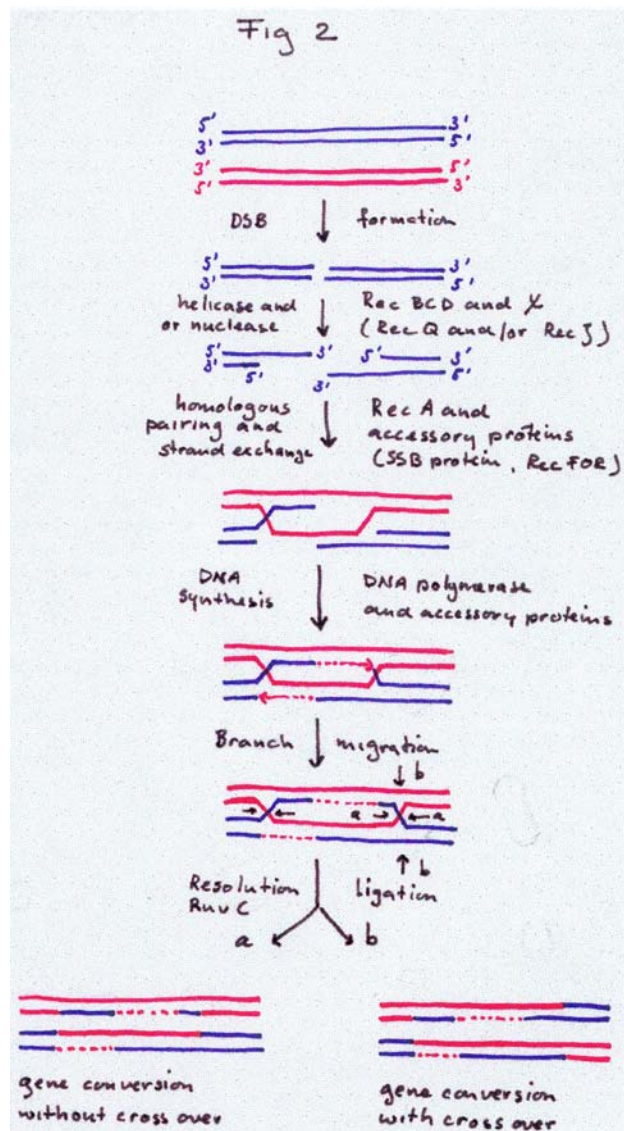


Figure 2. DSB repair via recombination in *E. coli*: After DSB formation the broken ends will be processed by the RecB-RecC-RecD complex resulting in single-stranded overhangs. For this also  $\chi$ -sequences are involved and strands can also be separated by helicases (e.g. RecQ) or degraded by nucleases (e.g. RecJ). During the next step of homologous pairing and strand exchange RecA is required, together with facilitators such as the SSB protein and the RecF-RecO-RecR complex (the latter only acts in absence of the RecB-RecC-RecD complex). Strand exchange is followed by DNA synthesis by DNA polymerase and accessory proteins and RecA driven branch migration occurs. HJs are resolved by RuvC and subsequent nicks sealed by ligases. Depending on the nicks in the second HJ relative to the nicks in the first HJ the recombination product will result in either gene conversion without cross-over (2-a) or with cross-over (2-b). See text for more information.

## DSB repair in eukaryotes: NHEJ vs HR

DSBs are induced by ionizing radiation, free oxygen radicals, chemotherapeutic agents, alkylating agents (e.g. MMS, methyl-methane-sulfonate), or originate from SSBs converted into DSBs. In addition, eukaryotic cells naturally produce DSBs e.g. during meiosis and VDJ recombination. Because as few as one DSB can be lethal for a cell, eukaryotes have developed two (main) pathways: NHEJ and HR. The critical difference is that NHEJ is quick, but error prone, while HR is slower, but accurate. HR relies on the principle of making a copy of undamaged homologous DNA analogous to RecA mediated recombination, while NHEJ simply sticks broken DNA



ends together and is accompanied by gain/loss of a few nucleotides around the break. Though HR is more accurate, cells not always have a homologous sister chromatid available and then NHEJ prevails. Another advantage of NHEJ above HR is that it does not need a reshuffling of other proteins bound to the DNA as this pathway only focuses on the broken DNA ends. In contrast, HR involves the DSB in context of the adjacent DNA and consequently has to deal with the proteins bound to this DNA.

However, in case it is important to avoid mutations, HR is the predominant pathway. Thus, in most (differentiated) cells of multi-cellular organisms NHEJ is the predominant pathway and HR is refined to a short period of cell life: during replication (S-phase of the cell cycle) and embryogenesis, in stem cells and gametocytes.

**NHEJ:** Each end of a broken DNA molecule is recognized and bound by the Ku70-Ku80 heterodimer (in yeast). In mammalian cells an additional unit, DNA PK<sub>CS</sub>, is targeted by the heterodimer to the DNA ends. Then, the ends are brought together and sealed by the DNA ligase IV-XRCC4 heterodimer. This mechanism of precise end joining (Fig. 3A) does not use any discrimination signal. When the broken ends of the DNA molecule are moved away and another DNA end from a second molecule is closer, the latter one may be joined to one end of the first molecule, resulting in a translocation. The system also loses efficiency when the ends cannot be joined directly. Another protein complex, consisting of the MRE11-RAD50-NBS1 (yeast Mre11-Rad50-Xrs2 complex (52, 466)), or Artemis (307, 375) may digest the breaks until they fit and can be joined. This however, is always accompanied by deletions of a few bases (Fig. 3B). A third sub-pathway involves joining based on small sequences of homology in the DNA (micro-homology; based on stretches of 2-6 complementary bases) and involves the aforementioned proteins. In this case the DNA is degraded until a sequence of micro-homology is detected and then the ends will be joined. This results in deletion of a few base pairs up till a few kb in length (Fig. 3C).

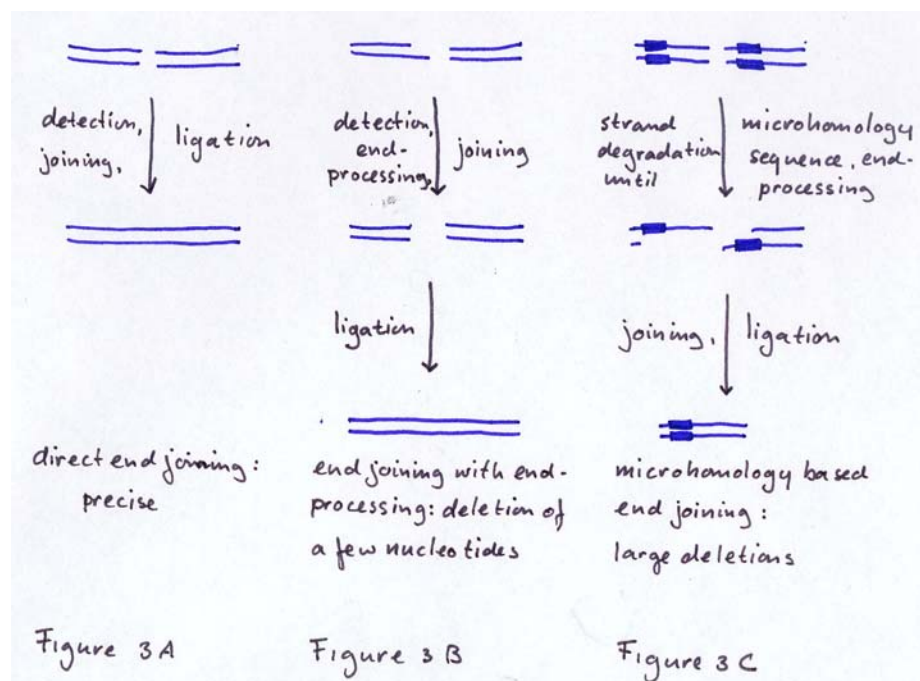


Figure 3. Three sub-pathways of NHEJ. 3A, direct end-joining: detection and joining of the broken ends by DNA-PK<sub>CS</sub> and Ku70-Ku80, followed by ligation. This is a precise process without inducing any mutations. 3B, end-

joining with end-processing. In this case the ends are not blunt or complementary in overhang and must be nucleolytically processed, presumably by MRE11-RAD50-NBS1, (among other nucleases), at the ends prior to be joined by DNA-PK<sub>CS</sub> and Ku70-Ku80. This process goes at the expense of a few nucleotides. 3C, end-joining based on micro-homology. Nucleases (e.g. MRE11-RAD50-NBS1) may degrade the broken ends until they meet small sequences of homology (2-6 nucleotides). Sticking these micro-homologous ends together reunites the two halves of the broken molecule, but goes at the expense of the DNA in between the two stretches of micro-homology. See text for more information.

Double strand break repair via **homologous recombination** (Fig. 4A) in eukaryotes (yeast) is likely similar to the *E. coli* model of DSB repair and both pathways and proteins involved are conserved. DSB ends are nucleolytically processed in a 5'→3' direction, resulting in 3' single stranded DNA ends of ~600nt long (50, 426), but can also exceed a 1kb in length (71). The proteins thought to be involved (at least indirectly) in this process form the MRE11-RAD50-NBS1 complex (304, 335, 452, 461). The ends are coated by the eukaryotic RecA homologue, RAD51, which is stimulated by RPA, the eukaryotic SSB protein homologue, and other recombination proteins as RAD52, RAD54 and RAD51 paralogues (for a detailed description see below). RAD51 initiates the search for a homologous partner and the strand exchange process, during which one (or both) tail(s) invade the homologous sister chromatid (or homologous chromosome). The information of this undamaged partner is copied by DNA synthesis performed by DNA polymerase  $\delta$ ,  $\epsilon$  and accessory factors (PCNA, RFC, see below). Subsequently HJs are formed, and molecules can be resolved by nicking in a process similar to that the described in the *E. coli* model (see **box 1**: Mus81) or eventually, without HJ resolution by HJ reversal (see **box 2**: BLM). Sealing of the nicks by DNA ligases completes the reaction. Depending on the orientation of the nicks during resolution, the resolution will result in gene conversion with or without crossover (Fig. 4A-a, 4A-b). Alternatively, the newly synthesized DNA of both ends re-anneal and nicks are ligated (144). This process is called synthesis dependent strand annealing (SDSA) and is also accompanied by gene conversion (Fig. 4A-c). Gene conversion (with or without crossover) is one way to repair DSBs via recombination but other types of recombination modes also exist (SSA and BIR, see below).

An alternative recombination pathway includes the RAD51 independent, but RAD52 dependent single strand annealing (SSA) as described for yeast (109, 173, 421) and mammalian cells (245, 246). Also SSA depends on 5'→3' degradation of the broken DNA ends (50, 71, 109, 174, 421, 426). As for gene conversion this probably depends on the MRE11-RAD50-NBS1 complex (123, 304, 335, 336, 452, 461). During strand annealing however, the cell uses a short homologous sequence (> 30nt in length) within the single stranded DNA to pair, rather than using the whole single stranded region to anneal to a homologous sequence. This repair mode can be applied to join broken DNA, which contains stretches of repeated sequences. The sequences on both sides of the broken DNA molecule will be paired, and the initial pairing is catalyzed by RAD52 (191, 224, 306, 360, 401, 424) (Fig. 4B). Non-complementary ends (the stretches of DNA in between the two repeated sequences) are recognized by the yeast MutS $\beta$  heterodimer and removed by yeast Rad1-Rad10 ((108, 172) the yeast homologue of ERCC1-XPF). Thus, this sub- pathway uses limited stretches of DNA homology to perform recombination and is always accompanied by deletion.

Another recombination repair pathway is called break-induced replication (BIR). It also needs DNA end processing identical to gene conversion and SSA and has been studied in yeast only. In contrast to gene conversion only one end of the broken



molecule invades another DNA molecule. Instead of copying the stretch of DNA, which was lost during breakage and processing of the broken molecule, now the whole template molecule is copied by replication until the end of the template (Fig. 4C). The other end of the broken molecule is lost.

BIR is likely to be important in repair of collapsed replication forks, may also be involved in repairing DSBs of other origin and can be used for replicating telomere regions in cells lacking telomerase (143, 294, 331). BIR is strongly dependent upon RAD52 and can occur in absence of RAD51 (270). Equally, mutations in yeast *RAD54*, *RAD57* and *RAD59* do not affect BIR, while gene conversion is abolished. Similarly, double mutants *RAD50-RAD51*, *RAD51-RAD59*, *RAD54-TID1* do severely impair BIR, but not as much as the yeast *RAD52* mutant (404). Based on experiments with centromeric plasmids (422) it had been suggested that requirement for Rad51 depends on the chromatin state of the DNA. When DNA is in a less constrained chromatin state Rad51 is not needed. Therefore, BIR may be more successful at special locations (e.g. telomeres) or periods where or when DNA is in a more open configuration (271).

Recent studies in yeast however, show that Rad51 may have a more prominent role than previously thought. Studies by Malkova and colleagues (271) showed that Rad51-independent BIR required a cis-acting element: the BIR facilitator. In absence of this facilitator and of Rad51, DSB induction leads to chromosome loss (271). Another study (89) using linearized plasmids containing one homologous end (to the tested chromosome) and a telomeric end, showed that the number of BIR events was 33-140 fold decreased in yeast *RAD51* mutants (with similar decrease for *RAD54*, *RAD55* and *RAD57* mutations as for the *RAD51* mutant). Though not as dramatic as in the *RAD52* mutant (no BIR products detected), it still shows a significant dependence on RAD51 (and other mentioned proteins). When looking for two independent BIR events the *RAD51* mutant showed frequencies similar to that of the *RAD52* mutant. These results suggest that Rad51 is needed for the majority of BIR events.

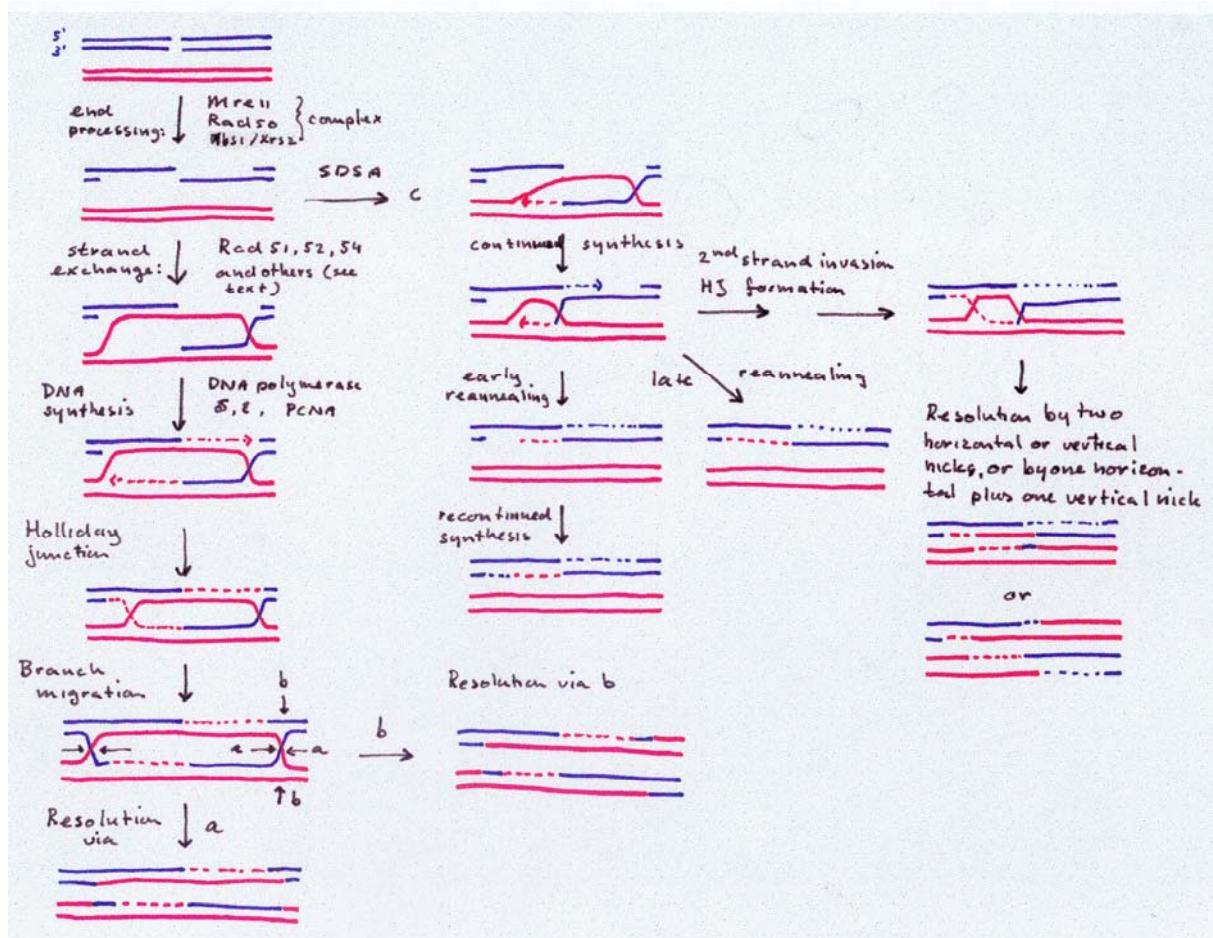


Figure 4. DSB repair via recombination in eukaryotes. 4A, gene conversion associated recombination: end-processing is the first step after the DSB formation and presumably involves MRE11-RAD50-NBS1. This is followed by coating of the single-stranded ends by RAD51, (assisted by accessory proteins such as RAD52, Rad54) leading to strand exchange of both strands of the broken molecule (in blue) with the template DNA (in red). The next step involves resynthesis of the broken DNA (by Polymerase  $\delta$  or  $\epsilon$ , PCNA) and branch migration may occur during or after resynthesis. Reannealing of the invading strand with the other half of the broken molecule leads to the formation of HJs. Resolution of the recombination intermediate by nicking results in gene conversion without cross-over (4A-a) or with cross-over (4A-b). Alternatively, only one strand anneals with the template and is dependent on DNA synthesis. This process is called SDSA (4A-c). After invasion, the synthesis continues on the template strand. It could then recontinue synthesis on the other half of the broken molecule, or it could perform the whole resynthesis of the gap on the template molecule and after that reanneal to the other half of the broken molecule. Alternatively the other broken half gets involved and its single-stranded tail anneals to the other strand of the template molecule. This results in the formation of a second HJ and this intermediate will be resolved by nicking, identical to 4A-a or 4A-b resulting in gene conversion without or with a cross-over. Resynthesized DNA is indicated by dotted lines, red indicates synthesis using the template strand, blue when sequences of the broken molecule were involved.

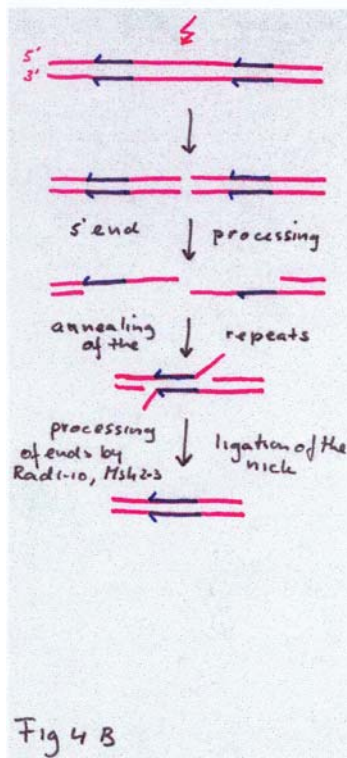


Figure 4B, SSA: in this case the DSB is in between repeated sequences (in black). Extensive end processing leads to single-stranded overhangs. The overhangs will anneal with each other at the complementary sequences of the repeats. The non-complementary ends of the intervening sequences will stick out and are clipped off by the Rad1-Rad10 endonuclease after recognition by Msh2-Msh3.

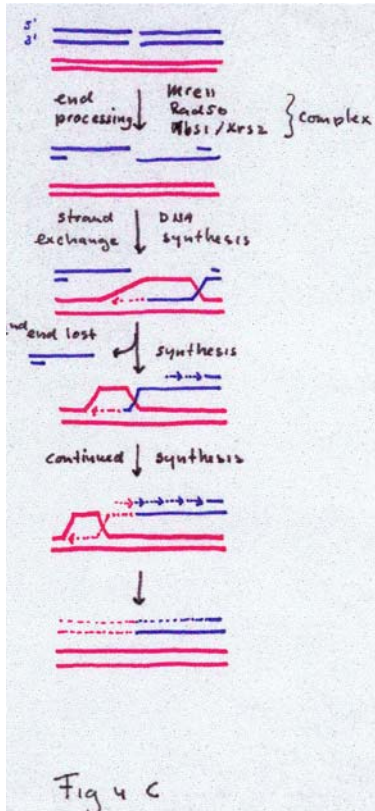


Figure 4C, BIR: also in this case end processing of the DSB is required to create single-stranded tails. As with SDSA one end invades the template strand (in red). In contrast to SDSA however, synthesis on the template

continues until the end of the chromosome, the other end is lost. Branch migration occurs during synthesis on the template strand, so that synthesis on the complementary strand of the broken molecule can occur using the newly synthesized DNA as a template. For a more extensive explanation see text.

#### **1.4 Proteins involved in the recombination repair core reaction: prokaryotic RecA and the eukaryotic RAD52 group of proteins**

Making use of the homology of Rad51 to RecA, extensive biochemical and genetic studies revealed the similarity between the RecA-mediated and Rad51-mediated recombination pathways. A whole set of yeast mutants showed deficiencies in recombination and replication processes, and exhibited sensitivity to DSB inducing agents. This group is usually referred to as the *RAD52* epistasis group and initially included *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57* and *RAD59*. Together with *RAD50*, *MRE11* and *XRS2* these genes are required for DSB repair via HR in yeast (13, 127, 207, 343, 361, 397, 398, 402). The yeast Rad51, Rad55 and Rad57 form a separate group of Rad51 and Rad51-like proteins, as are the mammalian homologues RAD51, RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3. Moreover, both Rad52 and Rad54 have their homologous proteins (Rad59 and Tid1/Rdh54 respectively).

**RecA** is involved in replication, recombination, mutagenic bypass via Pol V and the SOS induction (79, 216). *RecA* mutations abolish both conjugational recombination and damage provoked recombination repair (original references see (68, 164), reviewed in (370)). The deficiency causes 50-70% of the *recA* mutant population to die (82, 214, 371), which is due to replication problems. The protein is 49-100% conserved within all prokaryotes (267). It forms RecA nucleoprotein filaments by co-operative binding to ssDNA in a 5'→3' direction (193, 395, 480). Bound DNA can be 1.5 times extended in length (96). After binding to ssDNA it initiates strand exchange (138): aligning homologous sequences, formation of hybrid DNA of three or four stranded DNA molecules. Furthermore, it initiates and catalyses unidirectional branch migration (213, 244). The ATPase function of the protein is needed for the exchange and branch migration functions, but is also thought to be required for dissociation of the RecA filaments from the DNA (23, 31, 80, 268, 291, 319). In vitro experiments have shown no preference for 3' or 5' end mediated strand exchange concerning long tails, but in vivo there are probably only 3' end tails available due to end processing by RecB-RecC-RecD. The invaded 3' end can serve directly as a primer for an extending DNA polymerase (290).

**RAD51** is a 37kD sized protein, 40% homologous (17, 138, 427) to RecA, and biochemically characterized as an ATPase, responsible for homologous pairing (427) and strand exchange (430), forming RAD51 nucleoprotein filaments (for reviews on RAD51, repair and recombination see (20, 31, 331, 473)). Despite the homology, both yeast and human RAD51 have an extra N-terminal domain, which is conserved in eukaryotes (4, 223). RecA on the other hand, has a larger C-terminal domain, which is projected outside the nucleoprotein filament as shown by crystallization studies (419). Electron microscopy (EM) studies (26, 324) on nucleoprotein filaments of hRad51 show that the N-terminal appendage is flexible and contains a six-barrel domain (446), similar to Exo III, needed for DNA binding (5). Other domains within the N-terminal part are those required for binding of RAD51 to other RAD51 monomers (94), RAD52 (94), BRCA2 (392), RPA (417), p53 (248) and for phosphorylation by c-Abl (61, 497) and for binding ATP (61). In contrast to RecA

which can only bind ssDNA (215, 285, 355), Rad51 is able to bind ss and dsDNA in a pH independent manner, but still needs ATP and  $Mg^{2+}$  as co-factors (499). Salt (at high concentrations) stimulates the RAD51 ATPase function, weakens the RAD51-dsDNA binding, but stimulates aggregation of RAD51-ssDNA-dsDNA. Salt also converts the nucleoprotein filament from the condensed form into the extended form, which is a better substrate for efficient strand exchange (258). In a filament Rad51 binds the ssDNA at 4-9 nucleotides per monomer (315, 499), while RecA binds 3 nucleotides per monomer (415).

The RAD51 DNA dependent ATPase has a lower activity (17, 427) than that of RecA (267). Since unwinding of dsDNA by RecA is ATP dependent (326) RAD51's weak ATPase activity may explain why it promotes less strand exchange and cannot form extensive heteroduplex molecules alone (17, 139). It interacts with the DNA dependent ATPase RAD54 (this thesis and (69, 134, 180)), RAD52 (154, 298, 393) and RPA (107, 333) and is also stimulated by these proteins (RPA (17, 19, 140, 425, 427, 431), RAD52 (25, 323, 393, 400, 428, 463), RAD54 (134, 344)). RPA not only removes secondary structures in the ssDNA, but also stabilizes the joint molecule (JM) during strand transfer and prevents re-initiation of strand transfer and JM formation (19). As RPA has a higher affinity than RAD51 for ssDNA, the latter needs its accessory proteins (e.g. RAD52, RAD54) for efficient filament formation (interactions with RAD52, RAD54 described below). During strand exchange, strand invasion has been reported to occur in a 3'→5' direction (18, 19, 430), 5'→3' direction (140) or in both directions (289, 316). In vivo however, most widely available ends are 3' ends (due to end processing).

The yeast *RAD51* deletion mutant (398) is sensitive to ionizing radiation (IR) and MMS, showing the importance of the protein in DSB repair via recombination in yeast. That RAD51 is the key enzyme of recombination is most dramatically illustrated by the mammalian homologues: the *Rad51KO* mutation is embryonic lethal in mice (244, 457). Conditional mutants in chicken DT40 cells die due to accumulation of DNA damage and chromosomal instability as soon as Rad51 expression is switched off (413). Moreover, addition of Rad51 anti-sense oligonucleotides to cells inhibits cell growth and increases IR sensitivity (439). Moderate over-expression of Rad51 (1.6-3x) increases IR resistance (492), increases recombination repair relative to single strand annealing (11, 231) and also increases spontaneous (illegitimate) recombination (11, 231, 472, 487), leading to increased translocations, aneuploidy and chromosomal rearrangements (365). High over-expression levels (4-10x endogenous level) have adverse effects as reduced DSB induced recombination (202), reduced cell growth and increased apoptosis (113).

The behavior of Rad51 can be followed at the cellular level using immuno-fluorescent techniques. Rad51 forms nuclear foci as a response to DNA damaging treatments (this thesis and (141, 276, 320)), which can be observed in both yeast and mammalian cells within 30'-60' after induction (this thesis, (301, 423)). They (partially) co-localize with other Rad52 group foci (this thesis) and other proteins involved in DSB processing or replication, such as BLM,  $\gamma$ -H2AX, BRCA1, 2 (see [box 2](#), [3](#) and [4](#) respectively). Foci also form during S-phase (240, 442, 443) and during meiotic recombination processes. Though the steady state level of Rad51 protein is higher during S-phase than during other stages of the cell cycle (435, 490), it is not increased after IR (this thesis). In addition to RAD51 foci, the protein can also be found as sequestered in micronuclei (MN) (142). MN originate from chromosomal material that is not incorporated in daughter nuclei during cell division. Different types



of DNA damage give rise to different types of MN (275, 418, 470), thus it may be involved in disposing (different types of) damaged DNA into MN. Co-localization of RAD51 with other proteins will be discussed elsewhere.

Eukaryotic cells have different **RAD51 paralogues**. The first discovered paralogue is DMC1, which is a meiosis specific RAD51 homologue and has no obvious role in DSB repair outside the meiotic context. Other RAD51 like proteins are mammalian XRCC2, XRCC3, RAD51B, RAD51C, RAD51D (6, 54, 95, 153, 198, 253, 349, 351, 364, 444) and yeast Rad55 and Rad57 (196, 262, 263), which are all 20-30% homologous to RAD51 and to each other. They are considered to be accessory factors in the recombination process (40, 277, 328, 437, 438) (for a review see (194, 445, 448, 449)).

Yeast Rad51, Rad55 and Rad57 are involved in DSB repair (138), but have functional differences (182). Thus it is better to call them paralogues than homologues. Rad55 and Rad57 form a stable heterodimer and stimulate Rad51 nucleoprotein assembly (428). Therefore they are the functional homologues of RecO, RecR, which enhance RecA loading on ssDNA, and of (bacteriophage T7) UvsY protein, which loads UvsX on ssDNA (150, 179, 435, 495).

As mentioned previously, Rad51 mediated strand exchange can be stimulated by Rpa. However, when Rpa binds first to the DNA it inhibits Rad51 binding. The function of the Rad55-Rad57 heterodimer is to overcome this inhibition and to stimulate Rad51 mediated recombination in presence of Rpa (429). This finding is also supported in vivo: the cancer associated *RAD51* mutation (I345T), which is RPA displacement proficient by itself (in contrast to the wild type protein), can rescue the IR sensitivity of the *RAD55* and *RAD57* mutants (115). Other studies on *RAD55* and *RAD57* mutants show that the proteins are required for efficient mating type switching (383). Also, the mutants exhibit stimulated deletion formation (presumably via SSA), which is a phenotype similar to *RAD51* and *RAD54* mutants, showing the dependence of accurate HR on these proteins (3, 104, 243, 283) (review in (206)). The groups of Haber and Shinohara (301, 423) showed that Rad51 foci formation in the yeast *RAD55* mutant is not absent, but delayed. Both the delayed Rad51 and Rad52 foci disappear without repairing the break. Rad51 binds the broken strand, but fails to associate with the template (donor) strand in the mutant.

Though yeast mutant studies show the importance of Rad51 paralogues, mammalian mutant analysis shows a more dramatic outcome. Rad55 is closest to XRCC2 in homology and Rad57 to both XRCC3 and RAD51D (445, 456). However, the hamster *Xrcc2* and *Xrcc3* mutant cell lines, *irs-1* and *irs-1SF*, are unable to form Rad51 foci (this thesis, (33, 328)). Moreover, a *Xrcc2*KO mutation is embryonic lethal (92), thus *Xrcc2* is essential for cell survival. Also, *Rad51*BKO and *Rad51*DKO mice are embryonic lethal (350, 403). *Xrcc2* and *Xrcc3* mutants are moderately sensitive to IR, and UV light, but extremely sensitive to DNA inter-strand cross-linking agents (55, 122, 181, 190, 253, 349) and exhibit chromosomal instability (*ibid*, (42, 84, 458)). Identically, all five paralogue mutants in chicken DT40 are IR sensitive and show chromosomal instability due to defects in mitotic recombination (437, 438). It has also been suggested that *XRCC2*, *XRCC3* and *RAD51C* mutations may increase the risk of developing cancer (227, 484, 491).

RAD51B forms a complex with RAD51C, that is an ssDNA dependent ATPase and alleviates the RPA-RAD51 competition for ssDNA binding and thus has a function similar to RAD55-RAD57 (405, 432). RAD51B, RAD51C, RAD51D and XRCC2 are part of the RAD51B-RAD51C-RAD51D-XRCC2 complex, which

preferentially binds Y-shaped and HJ structures (494), and also binds ssDNA, single stranded gaps and nicks in dsDNA (278). Within the complex, RAD51C is the central protein and its C-terminus and linker region interact with RAD51B ('s N-terminus) and RAD51D ('s C-terminus). XRCC2 is bound by the N-terminal part of RAD51D (296). The RAD51C mutant is sensitive to IR and required for gene conversion (117). Interestingly, RAD51C also forms a complex with XRCC3 (225, 254, 277, 297, 482). The latter complex is also a DNA dependent ATPase, but it promotes DNA-DNA interactions, resulting in strand annealing (226, 277). Consistent with this biochemical property is the observation (41) of increased numbers of long, discontinuous tracks of DNA during heteroduplex formation in *Xrcc3* mutants, suggesting unstable or incomplete annealing. This may also have its consequences for the re-initiation of replication (see section 1.4).

Recently, it has been suggested that RAD51C is required for HJ resolution (257). Extracts of both *Rad51C* and *Xrcc3* mutants show low HJ resolvase activity similar to wild type extracts depleted of Rad51C. Addition of the RAD51C-XRCC3 complex and the RAD51B-RAD51C-RAD51D-XRCC2 complex restored this activity. Moreover, the RAD51C depleted extracts also lost the ability to branch migrate, an ability that could be restored by adding the RAD51B-RAD51C-RAD51D-XRCC2 complex. Therefore RAD51C could be considered as one of the accessory proteins of the putative mammalian HJ resolvase.

The **RAD52** is an almost exclusively nuclear protein (255) that interacts directly with RAD51 (154, 298, 393), is expressed during S/G2-phase (416) and facilitates RAD51 loading on the ssDNA (25, 323, 393, 400, 428, 463). However, it also functions in initial pairing in RAD51 independent recombination (191, 224, 265, 270, 359, 503). It is the key protein for pairing of repeated sequences in single strand annealing (173, 306, 360, 401, 424). Disabled in RAD51 dependent and independent recombination, DSB repair is virtually absent in the yeast *RAD52* mutant (74). RAD52 can bind all three different subunits of RPA and is thought to recruit RPA to the DSB to assist the strand exchange process and/ or recruit the DNA polymerases needed for repair synthesis (155, 401, 424). Interaction with RPA (and RAD51) is mediated via the C-terminus (155, 292, 333, 393). RAD52 itself clusters DNA and has a preference for binding ssDNA (and DNA ends with a single stranded overhang) over dsDNA (249, 367). It also binds the WRN helicase, stimulating the unwinding of DNA ends, but preventing the unwinding of HJ, the reversal of chicken foot structures (see [box 2](#) and section 1.4) and the exonuclease activity of WRN. WRN on its turn stimulates the Rad52 dependent annealing (22). Binding of ERCC1-XPF (the mammalian Rad1-Rad10 homologue involved in single strand annealing) to RAD52 will increase the endonuclease activity of the former and attenuate the annealing property of the latter (309).

Yeast and human RAD52 display 30% identity (24, 313, 399), with weak homology at the C-terminus and strong homology (70%) at the N-terminus and share similar biochemical properties (305, 432). Most conservation between yeast's Rad52 and its homologue Rad59 is found at the N-terminal part (13, 345, 462). The human RAD59 homologue has not been found yet, but there are two alternative splicing forms of RAD52, which both contain the conserved N-terminus (205) and may be they partially compensate for the putative homologue's functions. The long form (46kD) forms a heptameric ring (416), the short form (24kD) binds ss and dsDNA in an undecameric form (192, 358). The C-terminus of the full-length protein may be located near the DNA binding sites positioning RAD51 and RPA to transfer DNA.

Binding of ssDNA prior to dsDNA to the short form is important, otherwise the ssDNA will be sterically hindered by the already bound dsDNA (192). RAD59 may form rings similar to RAD52 and even heteromeric rings with RAD52 (90).

The RAD52 homo-oligomerization domain can be phosphorylated by c-Abl (204, 394). The latter protein has a role in DNA damage signaling (199) and normally shuttles between cytoplasm and nucleus (436), but can be activated upon DNA damage and during S-phase (259). Following DNA damage RAD52 (and exogenous RAD52GFP) form nuclear foci and co-localize with similarly induced RAD51 and RAD54 foci (this thesis, (102, 255, 256). Uncompromized RAD52 foci formation depends on c-Abl (204). Mammalian Rad52 however, is not essential for Rad51 foci formation, as *Rad52* mutants still form these foci (this thesis, (131)), while yeast mutants are unable to form RAD51 foci and nucleoprotein filaments (301, 423). Yeast *RAD52* deletion mutants are deficient in DSB repair, mitotic recombination, mating type switching, form very low levels of viable spores in meiotic cells, are sensitive to DNA damage (74, 127, 383). In contrast, the vertebrate *RAD52* mutants have a weak phenotype (366, 489). These mice have no abnormalities concerning fertility or viability, and mouse and chicken cells are not sensitive to IR and MMS, and only show a reduced recombination frequency. Though the highest expression level is at the lymphoid organs (and testes) (313), there are no abnormalities concerning the immune system of *Rad52KO* mice (366). The absence of a more severe phenotype could be explained by a redundancy function of RAD52 in vertebrates (366). A possibility is the existence of a(n unknown) homologue, as yeasts have Rad59. Another possibility is that RAD51 paralogues can partially compensate. For example, chicken DT40 cells deficient in RAD52 or XRCC3 have a mild, similar phenotype, but depletion of both proteins (using conditional mutants) causes extensive chromosome breaks and leads to cell death (121). Also, other RAD51 paralogues may have non-reciprocal, but overlapping roles in DSB repair or in other RAD52 associated functions. For example, though both yeast's Rad52 and Rad55-Rad57 are essential for meiotic RAD51 dependent recombination, only RAD52 or the RAD55-RAD57 complex is required for RAD51 dependent mitotic recombination, not both (130).

**RAD54** is another protein, which is conserved from yeast to humans but has no prokaryotic homologue. As such, it contrasts RAD51, which is conserved from prokaryotes to eukaryotes, but it needs much more accessory proteins to perform in a similar manner as prokaryotic RecA. The reason is that the eukaryotic system is more complicated. The size of the *E. coli* genome is  $4 \cdot 10^6$  nucleotides on one (circular) chromosome, mammalian cells contain  $\sim 6 \cdot 10^9$  nucleotides, spread over several (linear) chromosomes (114). At metaphase the DNA in human chromosomes is at least a 10,000 fold compressed (162). To achieve this, the DNA is wrapped by histone octamers into nucleosome fibers of 11nm, which are packed in series of 30nm fibers. These are folded into loops of fibers of different sizes, which are, on their turn, folded into bigger loops by a protein scaffold, and subsequently wound to form a chromosome (51, 53, 106, 230, 327, 339). Prokaryotic DNA is not organized in such a way, and consequently RecA does not deal with such a degree of complexity around the broken DNA. Theoretically, there is some space of dsDNA within the chromatin structure from where Rad51 could start, but it still needs to remove the nucleosomal proteins (282), which hinder the strand exchange (357). Rad54, which stimulates Rad51 mediated strand exchange (69, 134, 180, 344), could facilitate access to these chromatin structures (422), as it belongs to the SWI/SNF2 (super)family (II) of chromatin remodeling factors (97, 146, 340). These



proteins contain the seven characteristic motifs, including Walker A/B type nucleotide binding motifs (99, 303). They remodel chromatin by loosening histone-DNA contacts, which allows the nucleosomes to move (9, 453, 465), or completely remove the histone octamer from the DNA (261, 317, 348). Some of the members are helicases, others are remodeling factors associated with DNA methylation and linked to replication via PCNA (29, 66), and can be more generally described as DNA translocases (479). However, most members function in a multi-protein complex (110, 203). Many superfamily II members, e.g. RAD54 (344, 434), have no classic helicase function, but may induce superhelical twists (152). This raises the possibility to alter the state of DNA curvature (167), to promote triplex DNA formation and to unwind DNA (460).

Biochemical studies on RAD54 show that the protein has no classic helicase activity (282, 344, 346, 347, 434, 464), but it can alter the DNA topology by inducing superhelical stress (this thesis, (282, 368, 464)). RAD54 proteins are monomeric in solution (347), but exist as multimers when bound to DNA (di or higher order multimer (347), tri or hexamer (368)). RAD54 is a dsDNA dependent ATPase (344, 434) and could be targeted to the homologous chromatid/chromosome by RAD51ssDNA (282, 464). The DNA translocase function of RAD54 creates positive supercoils ahead and negative supercoils behind the translocating protein, which could displace histones and other DNA binding proteins. Superhelicity could also promote unwinding of the dsDNA, thereby facilitating hybridization with incoming RAD51 bound ssDNA to form a D-loop (282, 368, 409, 411, 464). Rad54 binding to the Rad51 nucleoprotein filament renders the filament more robust against nucleolytic degradation, salt destabilization and against Rad51 dissociation (281). These Rad51-Rad54 interactions are quite species specific as the yeast Rad54 protein has reduced ATPase activity in presence of human RAD51 filaments compared with yeast Rad51 filaments (and vice versa) (410). Experiments using nucleosomal DNA show that RAD54 must have some chromatin remodeling activity as the nucleosomal arrays are more accessible to restriction enzymes. This activity could be stimulated by Rad51, however it is significantly weaker than the activity of established chromatin remodeling factors such as ACF or SWI/SNF. In addition, no significant nucleosomal mobilization has been detected. On the other hand, Rad54 stimulated D-loop formation is more efficient on nucleosomal DNA than on naked DNA substrates (7, 8, 177). In vitro, Rad54 also increases the rate of heteroduplex extension and is able to remove Rad51 from dsDNA (200). Both chromatin remodeling and heteroduplex extension during recombination are dependent on Rad54's ATPase and translocase functions.

There are about twice as many Rad51 proteins ( $4.7 \cdot 10^5$  molecules) as Rad54 proteins ( $2.4 \cdot 10^5$  molecules) in the nucleus (of mouse ES cells) (101). Both are increased in S/G2-phase in mammalian cells and yeast (101, 490) and mRNA levels are up-regulated during late G1 (70, 112, 188, 195, 490). In yeast, Rad54 can be 10-fold increased after induction with MMS, but DNA damage (by IR) does not increase the Rad54 steady state levels in mammalian cells. IR, MMS and DNA inter-strand cross-linking agents can induce nuclear Rad54 foci formation (this thesis). These foci partially co-localize with Rad51, Rad52 foci under similar conditions and with PCNA during S-phase. The foci are highly dynamic structures, as in vivo studies on DSB induced foci (102) show that Rad51 is a stable core component and that Rad54 (and Rad52) has a more transient role as it has a shorter residence time in the foci. Rad51 foci can form in *Rad54KO* mouse cells. However, these are probably not as stable as

in wild type cells, as visualization of these foci depends on the fixation method (this thesis). This would be consistent with the biochemical observations, which suggest a promoting and stabilizing role for Rad54 in heteroduplex formation. Rad51 foci also form in the yeast *RAD54* mutant, but there is no extension of the invading strand, neither is the break repaired (301, 423) consistent with the biochemical observations. Mutant analysis in yeast shows that Rad54 is important, but not essential for mating type switching (383), and the mutant displays increased deletion mutations (3, 104, 206, 243, 283), and is sensitive to DSB inducing agents (e.g. MMS). However, MMS sensitivity in the yeast mutant can be rescued to some extent by human RAD54 (195), in contrast to observations from in vitro experiments. These demonstrated the inability of the human RAD54 to stimulate yeast Rad51 mediated recombination and vice versa (282).

Chicken and mouse Rad54 deficient cells (and mice) show decreased mitotic recombination and mild sensitivity to DSB and cross-link inducing agents (IR, Mitomycin C (MMC), MMS) (30, 100, 103). In addition, *Rad54KO* mice display telomere shortening and end-to-end fusions (175) similar to the Rad51D mutant (441). Interestingly, when the tumor associated Rad54G484R mutation is introduced in the wild type alleles in yeast, the yeast cells become sensitive to IR and show reduced recombination frequencies (407).

Yeast Rad54 also has a homologue, Rdh54B or Tid1. Similar to Dmc1 however, this protein's main function is in meiotic recombination processes. The mutant cells have no hypersensitivity to DNA damaging agents (300), supporting the idea that the protein has no major function in recombination associated DSB repair (in otherwise wild type genetic backgrounds).

## **1.5 Recombination and replication**

Recombination and replication processes are linked as indicated by the following observations (81). Firstly, replication is part of the recombination process that accompanies bacterial conjugation or transduction. Secondly, in certain viruses (e.g. bacteriophage T4) recombination is essential for replication initiation after a few rounds of viral replication. Thirdly, recombination proteins are involved in the restart of stalled replication forks (see below). Also gives this association of recombination and replication the possibility to repair miscellaneous DSBs occurring in mitotic cells (as discussed in 1.3). Fourthly, in some cases break induced replication (BIR, reviewed in (331)) can be used to replicate major parts of a chromosome (270) and maintain telomere replication (37, 217, 234, 264, 265, 284). Lastly, many types of recombination need extensive replication (210).

During normal replication processes, replication forks are frequently paused or halted, leading to fork collapse, fork regression or fork reloading and restart (83, 111, 143, 211, 214, 229, 272, 273, 376). This is due to secondary structures in the DNA, nucleosomes or other DNA or protein barriers on the way of the progressing fork (a classic example being the Tus protein, blocking the replication fork by binding to the Ter site on the DNA (294)). Also, collisions with the transcriptosome are inevitable, since replicative polymerases are faster than transcriptional polymerases (43, 286). Forks retain the capacity to resume replication for more than a five minutes after collision (251). When the replication machinery is somehow deficient or when nucleotide pools drop, replication forks can also be slowed down or halted. Another source of halting forks is DNA lesions. *E. coli* cells suffer from 3,000-5,000 lesions/generation under normal conditions (81). Some of these lesions cause

mispairing (leading to replication errors), but do not block replication, while other lesions induce distortion of the DNA helix, which stop replication. Sometimes this lesion is a SSB. Replication of the broken strand results in fork collapse and a DSB (228). These can be repaired via recombination as discussed in section 1.3.

Due to the nature of replication, lesions on leading and lagging strands lead to different possibilities during DNA synthesis. Normally, equal leading and lagging strand synthesis is coordinated by coupling the polymerases responsible for leading and lagging strand synthesis. Since lagging strand synthesis starts at RNA primers distributed at regular intervals (30-400bp in eukaryotes, 1-2kb in prokaryotes), a block on the lagging strand may cause the involved polymerase to stop and to resume synthesis at the next primer (433). Blocks at the leading strand may uncouple the coordinated synthesis (75, 433): the leading polymerase is halted and the lagging polymerase continues alone, causing a gap of more than 1 kb (330) (roughly the size of an Okazaki fragment) on the leading strand template. In some cases lagging strand synthesis stops a few bases after the blocked leading strand synthesis (e.g. at rRNA clusters) or stops synthesis a 50-70bp before the blocked leading strand synthesis (e.g. at the Ter site).

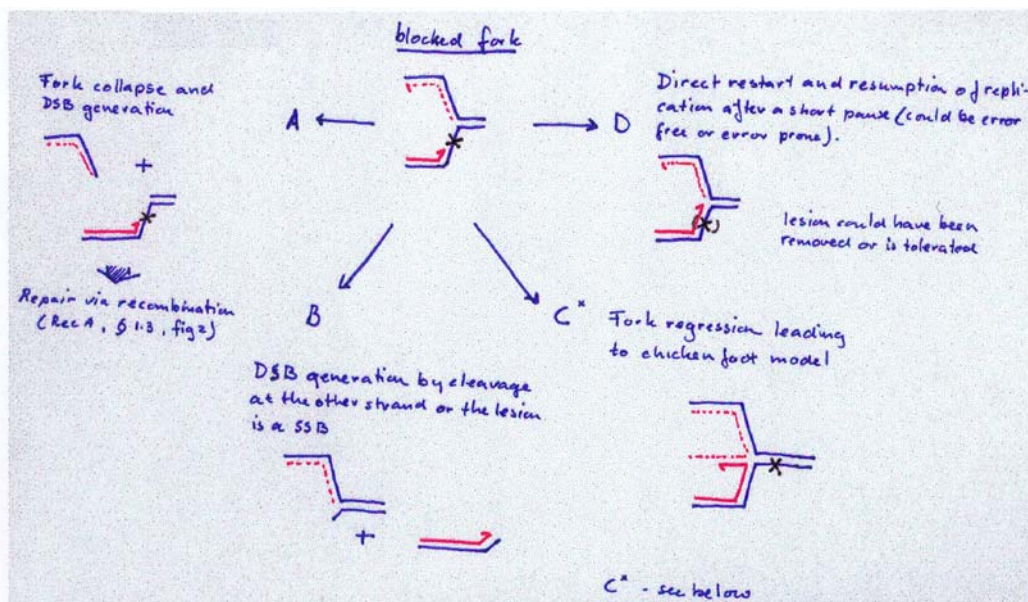
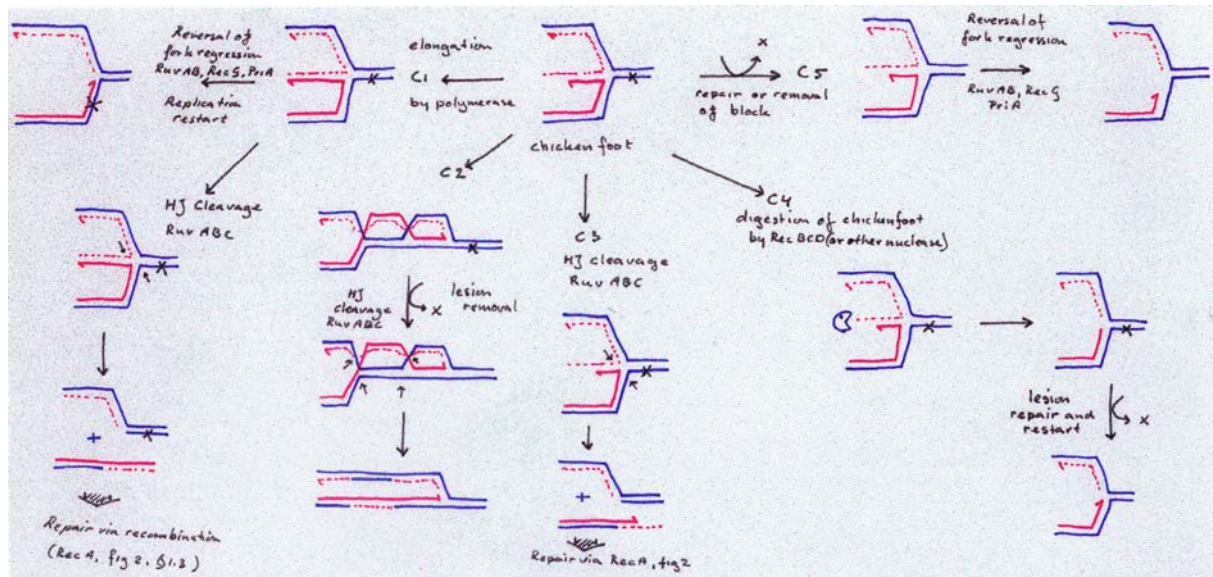


Figure 5. Resolution of stalled replication forks in *E. coli*: after the lesion (the black X) is encountered the replication fork can collapse followed by a break in one of the parental strands (in blue). This could happen in the strand on which lagging strand synthesis (dotted red line) or leading strand synthesis (continuous red line) occurs, but in both cases RecA dependent DSB (5A and 5B respectively) repair as shown in figure 2 is needed. Stalled replication forks can also lead to fork reversal, during which the newly synthesized strands anneal to each other (5C) or direct restart of replication takes place after a short pause (5D). The pause could be used for repair of the lesion or for translesion synthesis. The reversed fork (5C) is called a chicken-foot for which RecA is needed as well as helicases and translocases as RecG, DnaB, RuvA-RuvB or occurs spontaneously (see text). The chicken-foot can be solved in several ways. The first (5C1) is by continued synthesis. In this case the polymerase associated reading from the undamaged parental strand continues replication past the lesion of the damaged parental strand. So its newly synthesized daughter strand contains the same information as the damaged parental strand. Annealing of the two daughter strands, subsequent synthesis followed by reannealing to the parental strands bypasses the lesion and DNA synthesis using the parental strands can continue. For reversal of the reversed fork RuvA-RuvB or RecG, and PriA are needed. Another possibility is that the reversed fork after the elongation on the daughter strand is recognized and processed by RuvA-RuvB-RuvC. This leads to a DSB, which is repaired as shown in figure 2. 5C2, part of the annealed daughter strands reanneal to their complementary parental strands, while a part remains annealed to each other, causing two HJs. While a second reaction removes the DNA lesion, the HJs are cleaved by RuvA-RuvB-RuvC. After exchange of a part of the strands the synthesis can recontinue. Another possibility (5C3) is that the chicken-foot itself is directly recognized as a four-way junction and processed by RuvA-RuvB-RuvC, resulting in a DSB (and repaired as shown in Fig. 2).

Alternatively (5C4), the annealed daughter strands are degraded by RecB-RecC-RecD or another nuclease, while the lesion is repaired. The fifth possibility starting from the chicken-foot (5C5) is that the foot remains in this configuration until the lesion is removed, after which reversal of the foot (by RuvA-RuvB, RecG, PriA, see text) restores the original fork and replication can resume.



Most **prokaryotic** replication studies have been performed using *E. coli*. As shown in in Figure 5, nearly all ways to deal with a stalled replication fork in *E. coli* need RecA at some step. RecA binds the single-stranded regions at/near the fork (with or without SSB protein), but can also induce fork regression or is needed for recombination processes (for a review on RecA functions see (79, 216)). Since there are numerous spontaneous DNA lesions, causing stalled replication forks, it is not surprising that the mortality of RecA mutants is already high (50-70%) under normal conditions (82, 214, 371). The inability to repair naturally occurring stalled forks may suggest that the main function of RecA is to repair stalled forks and that recombination repair is a by-product, not the main function (78). When a stalled fork collapses (Fig. 5A, B) it will lead to DSB repair via RecA mediated recombination, which is explained in section 1.3. Regression of the stalled fork leads to the so-called chicken foot structure (Fig. 5C). Fork regression is the first step of stalled fork resolution when it does not collapse or restart (as shown in Fig. 5A, B, D) and can be visualized using EM (158, 471). Positive supercoils generated ahead of replicating polymerases (252) can lead to spontaneous fork regression to produce the chicken foot (81, 354). Regression may also be caused by the RecG helicase, via coordinated unwinding of both newly synthesized daughter strands and re-annealing of the parental strands (286, 287). Daughter-strand-annealing can be stimulated by RecA. Enzymatic regression could possibly be performed by the helicase and translocase DnaB. DnaB conditional mutants exhibit stalled forks and die as soon as DnaB is switched off. DnaB could remove the stalled replication machinery, thereby enabling access for RecG and RecA to the stalled fork (197). Eventually, regression could be stimulated by the RuvA-RuvB helicase (294, 389), but at least needs RecA (369, 390). RecA binds the ssDNA regions near the fork to protect it against degradation, but can also cause annealing of the bound single strands (78, 158, 286, 295). The resulting intermediates after chicken foot generation can be processed as indicated in the figure to function as substrates for replication restart (Fig. 5C1-C5). Nicks in the chicken foot (and recombination intermediates) are made by the RuvA-RuvB-RuvC



resolvase/endonuclease complex, since the foot is not more than a HJ. Replication restart needs loading of the primosome, which consists of PriA-PriB-PriC complex, primase DnaG, helicase DnaB and DnaC and T. PriA will load DnaB on both recombination intermediates generated after HR mediated DSB repair (Fig. 5A, B), direct fork restart (Fig. 5D) and the chicken foot end products (Fig. 5C)]. In some of the latter cases (Fig. 5C-5), the chicken foot needs re-regression to restore the original fork. RecG will re-regress the regressed fork before PriA loads DnaB and the rest of the primosome is assembled (137). RuvAB could, like RecG, reverse the polarity of RecA mediated strand exchange, so RecA could, with assistance of RuvA-RuvB or RecG, be responsible for fork restoration (81). Not only DnaB, DnaC (269), but also RecF deficient cells have problems with fork reloading (77, 209), indicating that also RecF(RuvO-RuvR) is needed for replication restart.

Another option is that a replication fork pauses when it meets a lesion (Fig. 5D). In this case, the original DNA polymerase (III) just resumes replication or is changed for the error prone DNA polymerase IV or V. This DNA polymerase is less stringent in inserting the right nucleotide opposite the lesion and could lead to mutations. After this DNA polymerase has inserted the (wrong?) nucleotide it is changed for a high fidelity DNA polymerase and the replication is resumed. This process is called TLS, translesion synthesis. However, stalled forks are preferentially repaired via accurate recombination dependent replication (as in Fig. 5A, B, C) and not via error prone TLS (27, 288).

**Eukaryotes**, which use similar systems to repair stalled forks have evolved numerous different DNA polymerases. There are the high fidelity DNA polymerases  $\delta$  and  $\epsilon$ , and those with specialized tasks for mitochondria (DNA polymerase  $\gamma$ ) and primer synthesis (DNA polymerase  $\alpha$ ) (118), but there is a group which is often referred to as error prone DNA polymerases (i.e. polymerases for TLS).

Some are for specialized repair processes (e.g. DNA polymerase  $\beta$  for BER (280, 311, 406), REV1 for abasic sites (322)), or involved in spermatogenesis or somatic hyper-mutation. However, most of them recognize special substrates and incorporate the right nucleotide opposite that lesion, but are error prone in inserting nucleotides opposite other DNA than their preferred lesion (for reviews see (21, 119, 135, 168, 169, 221)). Crystallization studies on one of these, DNA polymerase  $\eta$ , shows that the active site is more open than those of high fidelity DNA polymerases. This is the reason why it can replicate through distortive DNA lesions, where the narrow cavities of the fidelity DNA polymerases are stuck (451). DNA polymerase  $\eta$  (147, 149, 184, 185, 279, 477) is considered to be quite efficient during TLS compared to for example DNA polymerase  $\zeta$  (132, 133, 148, 183, 186, 232, 233, 321, 500), which has an extremely low efficiency. Therefore, even within TLS there is a hierarchy, with a general preference for DNA polymerase  $\eta$  above the other error prone TLS polymerases (21), though it also depends on the type of lesion.

It is evident that recombination dependent replication (Fig. 5 A, B, resolved via DSB repair as in Fig. 4) and fork regression generating chicken foot structures also occur in eukaryotic cells (11). However, most available data on replication and recombination in eukaryotic cells originate from cell biology and genetic studies, while there is very little information available on biochemical characterizations. Replication in eukaryotic cells takes place in replication factories attached to the nuclear matrix (28, 166, 332, 382, 414, 474, 488). Therefore, it requires the DNA to move to and from established sites on the nuclear matrix, rather than that the replication machinery moves to and along the DNA (165, 420). Replication foci can

be visualized using immuno-fluorescence techniques and have been shown to include replication proteins as DNA polymerase  $\alpha$ , and DNA polymerase  $\epsilon$ , PCNA and RPA (125, 165, 166, 239, 314).

Biochemical interactions between RAD51, RAD52 and RPA have been reported (see 1.3) and co-localizations of the proteins can be visualized using IF. RAD51 and RPA foci co-localize at sites of ssDNA (356), conform their biochemical function. RPA foci assemble before RAD51 foci and disappear faster (130). PCNA was the first protein seen in replication foci (39, 59), but foci show different patterns during S-phase progression (166, 201). This could be explained by the fact that easily accessible, transcriptionally active DNA is replicated in early S-phase (242) and that the constitution of the replication machinery changes during S-phase (362). DNA polymerase  $\epsilon$  is only active during late S-phase and associated with replication of inactive heterochromatin coated DNA. Therefore it only (transiently) co-localizes with PCNA during late S-phase (98, 125). PCNA (219, 476, 498) diffuses onto DNA ends of linear DNA (47), but requires RFC (105, 352, 454) to be loaded as a homo-trimeric ring (sliding clamp) on DNA without ends. RFC not only loads the trimer onto the DNA, but also determines the length of the RNA primers made by DNA polymerase  $\alpha$  (44, 85, 86, 308, 329, 408, 455). When loaded onto the DNA, PCNA can interact with both DNA polymerase  $\delta$ , and  $\epsilon$  (46, 98, 125, 236, 237, 318, 353) and FEN1 which is required for Okazaki fragment processing (171, 241, 459, 486). The prokaryotic sliding clamp is left behind during lagging strand synthesis (222) and it could be that PCNA is left behind as well, serving as docking platform and strand polarity marker for other proteins. During DNA damage, RPA and DNA polymerase  $\alpha$  (45, 312) can be phosphorylated, which could lead to modifications on the replication machinery. This leads to slow down of the S-phase progression (rather than arrest (247, 338, 363, 377)) allowing the cell to replicate without inducing further damage or to repair or reinitiate stalled replication forks. *Xrcc3* mutants however, are deficient in slowing down the progression of the replication fork after DNA damage. Addition of the RAD51C-XRCC3 complex or of RAD51 to the *Xrcc3* mutant cell extracts restore the slow down of the fork progression similar to wild type levels (156).

Analysis of *RPA* mutants (107, 154, 155) (no deletion mutant, that is lethal (44)) on genome stability reveal that the mutants exhibit decreased recombination and spore viability. Recombination levels can be restored by over-expression of RAD52 (155). *Xrcc3* mutants on the other hand show, besides the previously described recombination deficiencies, aberrant replication: it has elevated levels of endo-reduplication. Both XRCC3 and RAD52 interact with RPA and deregulation of RPA in the XRCC3 mutant causes the faulty replication initiation. Over-expression of RPA in wild type cells also increases endo-reduplication and over-expression of RAD52 in both the XRCC3 mutant and RPA over-expressing cells suppresses endo-reduplication (496). Both *RFC* and *PCNA* mutants are MMS sensitive like recombination mutants and show accumulation of ssDNA gaps and nicks (10, 12). Moreover, the FEN1, PCNA and RFC mutants all show synthetic lethality with mutations affecting the RAD52 group of recombination proteins (171, 187, 189, 293, 302, 450, 459).

That eukaryotic recombination proteins are linked to the replication process is also indicated by another set of IF experiments. Human MRE11-RAD50-NBS1 and RAD51 are in different repair complexes (141, 276) as the MRE11-RAD50-NBS1 related end processing of DSBs is required before NHEJ or HR can take place.

Placing a grid over the cells before IR results in DSBs in the exposed nuclear areas only. Consequently, MRE11 foci were found in the exposed areas and localized to the breaks. RAD51 foci were also induced, but were also found in unexposed areas, not co-localizing to the breaks (320). This suggests IR-induced RAD51 proteins migrate to the immobile replication factories attached to the nuclear matrix, rather than that they migrate to the DSB to initiate repair on the spot.

The presence of recombination protein foci in normal, healthy yeast cells using exogenously expressing Rad52YFP indicate the role of recombination in replication. During S-phase cells frequently generate just one, short-living RAD52 focus. This is caused by replication fork stalling which frequently happens during replication. Stalled or pausing forks may resume after a while, which could explain the disappearance of the focus. The collapsing forks give rise to a DSB, which activates Rad52 (249, 250). In this case the focus persists for more than an hour and results in cell cycle arrest. In addition, fast growing cell populations show a high percentage (~10% in ES cells) of Rad54 (and Rad51) positive cells (this thesis) and Rad51 foci have been reported to appear in S-phase previously. These cells (this thesis, (240, 442, 443)) show more than just one focus, but identical to prokaryotic RecA, the proteins could be involved in more than just repairing collapsed forks during replication or in more (or other) replication processes than Rad52.

Direct involvement in replication processes for the latter proteins have been shown using yeast *Schizosaccharomyces pombe* *rhp51Δ* and *rhp54Δ* deletion mutants (*S. pombe* homologues of Rad51 and Rad54 respectively). These not only show abnormal replication structures, but also activate replication origins very inefficiently. Studying these cells Segurado et al. (388) found that the activation of replication origins is associated with JM formation. This suggests that recombination proteins are as important for replication initiation in eukaryotes as for prokaryotes. In support with this is the observation (379) that mouse p53 mutants can show increased recombination, when challenged with replication elongation inhibitors, which cause stalled forks. However, normal cells do not show increased recombination levels when treated with replication initiation inhibitors. It has been suggested that the RAD51B-RAD51C-RAD51D-XRCC2 complex targets RAD51 to ssDNA regions near halted replication forks but direct evidence is lacking (208). *Xrcc3* mutants however, are sensitive to hydroxyurea (HU), while HU can also induce Rad51 and Rad54 foci in wild type cells (this thesis, (266, 378)) and the MRE11-RAD50-NBS1 complex (299). HU blocks the ribonucleotide reductase resulting in depletion of dNTPs (447) Nucleotide depletion causes replication fork stalling (38, 381). Both the BLM and WRN helicases (box 2) have also been shown to migrate to sites of ongoing replication in HU treated cells, form HU induced foci and are required for recovery of HU induced replication arrest (73, 391).

## **1.6 Recombination proteins and meiotic processes**

To discuss the role of the recombination repair proteins in meiosis is outside the scope of this thesis. Briefly, the reason that recombination proteins are often associated with meiosis (502) is because meiosis needs pairing of the chromosomes before separation into daughter cells. During this process meiotic recombination occurs, hence the need for the recombination repair proteins (e.g. RAD51) and some meiosis specific proteins (e.g. RAD51 homologue DMC1 or RAD54 homologue RDH54/TID1). Recombination is a 500-1000 fold increased as compared to mitotic

cells (372). Recombination is initiated by breaks caused by meiosis specific endonucleases, rather than by breaks caused by DNA damaging agents. A second essential difference is that mitotic recombination (preferentially) uses the sister chromatid (the exact copy of the broken DNA molecule) while during meiotic recombination the homologous chromosome is used. Mutations resulting from this process are given to the offspring and therefore recombination is considered to be responsible for creating genetic diversity during evolution.



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## CHAPTER TWO

### **THE USE OF RAD51 AS AN INDICATOR FOR DEFECTS IN DNA DAMAGE RESPONSE**

Based on the article by M. Dronkert et al. in *Mol. Cell. Biol.* 20(13): 4553-61, 2000 and on the article by M. Kraakman-van der Zwet et al. in *Mol. Cell. Biol.* 22(2): 669-79, 2002



## ABSTRACT

The Rad51 protein has a central role in repairing both double strand breaks (DSBs) and interstrand cross-links (ICLs) via homologous recombination. We developed an immunofluorescence assay, which visualized DSB or ICL-induced Rad51 foci in cells. We applied this assay when studying cell lines that contained mutations in other homologous recombination genes (*Rad52*, *Rad54*, *Xrcc2* and *Xrcc3*), to see the effect of the respective mutation on DNA damage-induced Rad51 foci formation. *Xrcc2* and *Xrcc3* are essential for Rad51 foci formation, *Rad54* is important, and *Rad52* is dispensable for Rad51 foci formation. Secondly, we used the assay to screen another mutant (*Snm1KO*) to see whether the corresponding gene is involved in the same pathway as Rad51. The *Snm1KO* cells are sensitive to ICLs, but the protein is probably not involved in the same pathway, as the mutant was still able to induce Rad51 foci. Thirdly, the assay can be applied to screen mutant cell lines with unknown mutations but are known to be sensitive to DSB or ICL inducing agents (such as V-H4 and VC-8). Applied this way, the assay was an essential tool in the discovery of the *BRCA2* mutation in the VC-8 cell line.

Thus, this assay can be used for studying the effects of known mutations on the Rad51 behavior, but can also be an essential help for the discovery of gene mutations of mutants whose mutation is unknown.

## INTRODUCTION

Double-strand breaks (DSBs) can be induced in the DNA by ionizing radiation (IR), as well as chemically and endogenously produced radicals. DNA interstrand cross-links (ICLs), can be caused by several anti-cancer drugs used for chemotherapy (see chapter one). Both types of DNA lesions can be repaired via homologous recombination (2, 13, 22, 24, 38). Homologous recombination differs from other DNA repair pathways in the sense that cells make a copy of a piece of undamaged, identical DNA to repair the damaged DNA. As a consequence, DNA lesions are repaired with high fidelity, in contrast to some other, error prone ways of repairing DSBs (e.g. non-homologous end-joining, see chapter one).

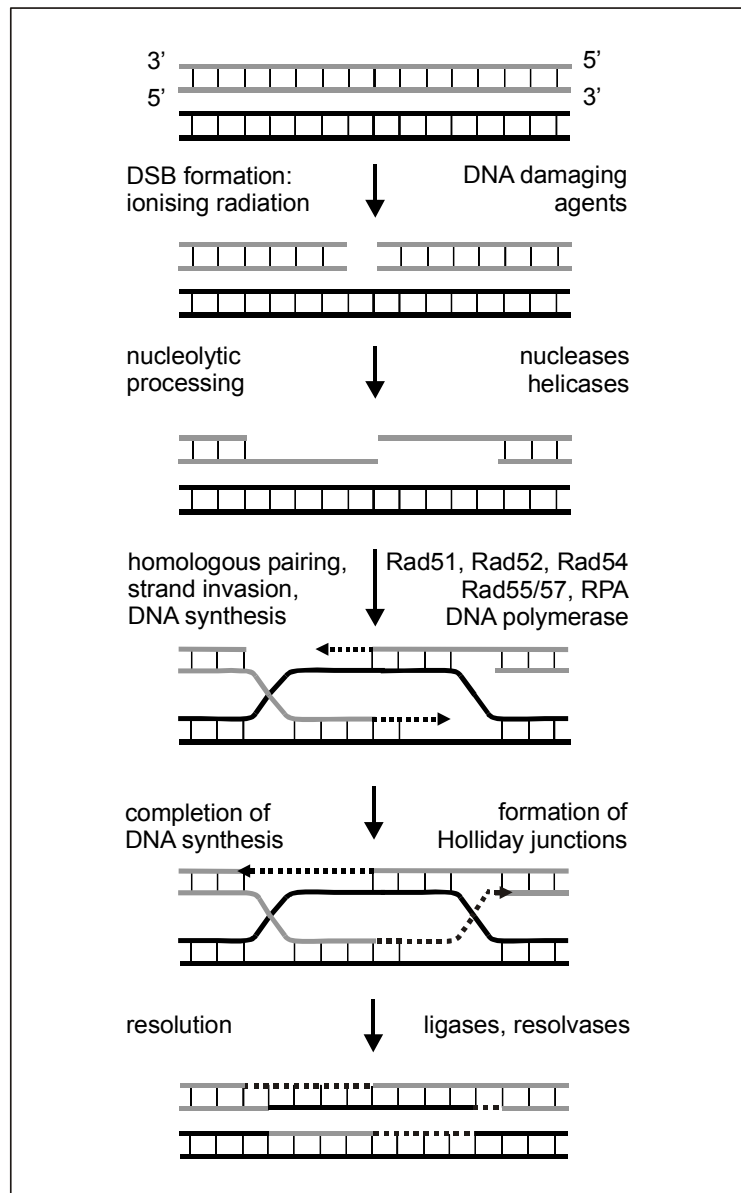


Figure 1. A model of DSB repair via homologous recombination. After the grey DNA molecule is broken by IR or DNA damaging agents (step2), the strands are processed resulting in single stranded DNA tails (step3). In order to restore the broken sequence the grey molecule will pair with the homologous sequence of a partner (black molecule) and strand invasion takes place (step4). The partner molecule is used as a template by the DNA replication machinery and after completion the structure has to be resolved (step5). After ligation the grey DNA molecule is restored, containing a copy of the black molecule at the side of damage. See text for a more detailed explanation.

As shown in the yeast *Saccharomyces cerevisiae*, DSB repair via homologous recombination repair (Figure 1) starts with locating the damage, after which both broken ends are nucleolytically processed. This consists of trimming the DNA ends in a 5'-3' direction and results in 3' single-stranded DNA overhangs. The processing nuclease(s) have not been unambiguously identified. Among candidate proteins that could be involved are the Mre11-Rad50-Xrs2(NBS1) multi-protein complex and Exo I. Then follows the search for the homologous partner, which may be on either the homologous chromosome or sister chromatid. Once the partner has been found, strand invasion of the 3' single-stranded overhang takes place. Thus, an undamaged piece of DNA can be used as a template by the replication machinery. Rad51, Rad52, Rad54, Rad55, Rad57 and RPA are the main proteins involved in the homologous pairing and strand invasion processes. The consequence of using a homologous partner for DNA replication is that each DNA molecule has one strand partially paired with its partner. To resolve the resulting structure (the example shown in Figure 1 shows a joint molecule with two Holliday junctions, one at each side), resolvases cut the Holliday junctions. Now the two DNA molecules are separated and the nicks are sealed by ligases.

ICLs are supposed to be repaired via homologous recombination in a way analogous to DSBs. However, the ICL is likely to be converted into a DSB before recombination repair starts.

Most of the knowledge on the recombination repair pathway is obtained using the yeast *S. cerevisiae* as a model organism. The mammalian system is much more complex, also due to the existence of multiple proteins homologous (or paralogous) to the original yeast proteins. To understand more about the function of these proteins in mammalian cells, and to see which other proteins are also involved in DSB or ICL involved recombination repair, we followed the behavior of the key enzyme Rad51 in repair deficient cells using immunofluorescence techniques (IF).

In mammalian homologous recombination, the 37kD sized DNA dependent ATPase Rad51 is a centrally important protein. Firstly, it binds to the 3'-single stranded DNA tails, then forms a nucleoprotein filament and thereafter performs the search for homologous DNA and mediates strand invasion (1, 3). Higher eukaryotes developed at least five paralogues (XRCC2, XRCC3, Rad51B, Rad51C, Rad51D), while yeast has two paralogues (Rad55, Rad57), whereas prokaryotes have only one Rad51 homologue (RecA) (37). The importance of Rad51 is underscored by the fact that *Rad51* knockout (KO) mutations are embryonic lethal (in contrast to yeast *RAD51KO* mutations (29)) (17, 39) and by the observation that when the *Rad51* gene is switched off in chicken cells, the cells accumulate chromosome breaks and die (32). As described in chapter three, the protein can be visualized by IF and has a particular feature: it redistributes into nuclear foci as a response to IR (which induces DSBs) or to interstrand DNA cross-link inducing agents as 1,3-bis(2-Chloroethyl)-1-nitrosourea (BCNU) and Mitomycin C (MMC).

Tashiro and colleagues found that Rad51 proteins also form foci at sites of replication after UV-C irradiation (36). In untreated cells Rad51 is also found in foci in late S and G2-phase of the cell cycle (16, 35). These findings may suggest that Rad51 is also involved in recombination repair of stalled replication forks.

Using the IF assay, we can study the influence of mutations in known genes on DNA damage-induced Rad51 foci formation. Firstly, disruption of Rad51 foci formation in

the mutant shows the importance of the gene product for Rad51 foci formation or stabilization. In contrast, the mutant's ability to form Rad51 foci suggests that the gene product acts later than Rad51, is not essential to Rad51 function, or acts in a Rad51 unrelated pathway. Therefore, we can also use the IF assay to screen mutant cell lines for defects in genes involved in Rad51-associated recombination repair. Any abnormal Rad51 foci formation shows involvement of the respective mutant gene in DNA damage induced Rad51 foci formation. Secondly, co-localization of a protein with Rad51 shows that the two proteins act in near proximity to each other and it could indicate a close interaction between the proteins themselves. Thirdly, by treating the mutant cell line with different DNA damaging agents, one can discriminate between the mutant's induced Rad51 response to the different treatments, e.g. a mutant could respond differently to IR, inducing DSBs, than to MMC, inducing ICLs.

## RESULTS AND DISCUSSION

We studied the influence of the Rad52 and Rad54 proteins, which are known to act with Rad51 in recombination repair on Rad51 foci formation by using the mouse *Rad52KO* and *Rad54KO* mutant embryonic stem cells and applying IR and ICL inducing agents. Rad52 forms heptameric ring structures (33) and protects the single-stranded DNA ends against degradation by binding to it. It stimulates Rad51 mediated strand exchange and binds (1, 3, 30, 40), like Rad54 (25), to the Rad51 protein. Rad54 is 85kD sized, (strong) dsDNA dependent ATPase (25, 26, 34) that facilitates partial unwinding of the DNA (see chapter three, Figure 5), which could make DNA structures and sequences more accessible for the Rad51 nucleoprotein filament. Absence of Rad54 protein affects the stability of Rad51 foci in mouse embryonic (ES) stem cells (see chapter three). In contrast, knocking out the *Rad52* gene had no effect on Rad51 foci induction after DNA damage, as the Rad51 foci still formed in the *Rad52KO* cells (Figure 2). However, the Rad52 protein is known to interact with Rad51 based on biochemical studies. To see whether we could visualize Rad51-Rad52 interactions in the cell, we studied an ES cell line expressing the Rad52 protein with a fluorescent tag (Green Fluorescent Protein, GFP, see Figure 3). Interestingly, upon DNA damage induction Rad52-GFP also redistributed into nuclear foci that partially co-localized with Rad51. Thus Rad52 does not influence the Rad51 foci formation or the stability, but it does co-localize. Rad54 also co-localizes with Rad51 foci after IR, but absence affects the stability of the induced Rad51 foci (see chapter three). The difference in importance for repair between the two proteins can also be seen from the sensitivity of the respective KO ES cell lines; the *Rad54KO* is sensitive to IR and cross-linking agents (9, 10), while the *Rad52KO* is not (28). Since yeast has a Rad52 homologue (Rad59, see chapter one), we suggest that it is likely that a protein with a similar function will (at least partially) take over Rad52's (repair) functions in the mouse *Rad52KO* ES cells.

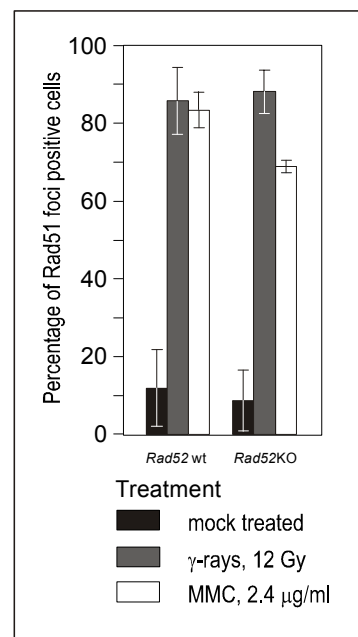


Figure 2. Rad51 foci formation in mouse *Rad52KO* embryonic stem cells two hours after induction of DSBs (IR) or ICLs (by MMC). Cells were fixed, stained for Rad51 and counted. The percentage of cells positive for Rad51 is displayed. Error bars show the standard of the mean of 3 independent experiments. IB10 is the parental cell line of the *Rad52KO* cell line and used as *Rad52wt* (wild type).

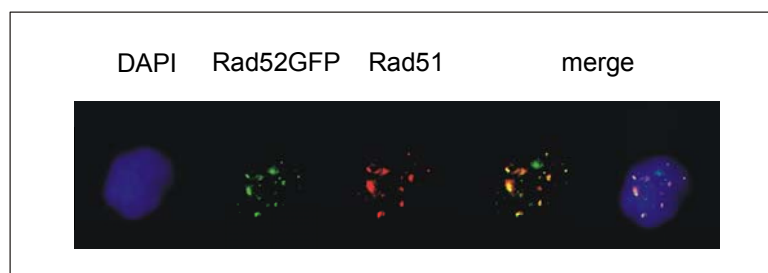


Figure 3. IF picture of exogenously expressed Rad52GFP foci (green) co-localizing with Rad51 foci (red) two hours after foci induction by  $\gamma$ -irradiation (12 Gy). The nucleus is counterstained by 4', 6-diamidino-2-phenylindole (DAPI).

Another separate group of proteins, involved in homologous recombination, are the Rad51 paralogues (XRCC2, XRCC3, Rad51B, Rad51C and Rad51D) (37). Attempts to make KO mutations in mice resulted in embryonic lethal phenotypes (7, 27, 31), just as is the case for KO mutations in *Rad51* (17, 39). These observations suggest that these closely related proteins cannot substitute for each others' essential functions. Two hamster cell lines exist, *irs-1* and *irs-1SF* (11, 12), which have mutations (not KO mutations) in the *Xrcc2* and *Xrcc3* gene, respectively (18). The cell lines are, like the *Rad54KO* ES cells, sensitive to IR and are even more (extremely) sensitive to cross-linking agents. We found that these cell lines were unable to form Rad51 foci after treatment with IR or cross-linking agents (Figure 4). Thus, they are involved at the same moment (or before) as Rad51 and are essential for DNA damage induced Rad51 foci. Given their similarity to Rad51, we would suggest that these proteins have a function in promoting or stabilizing the Rad51 nucleoprotein filament. Similar results have been reported (5, 20).

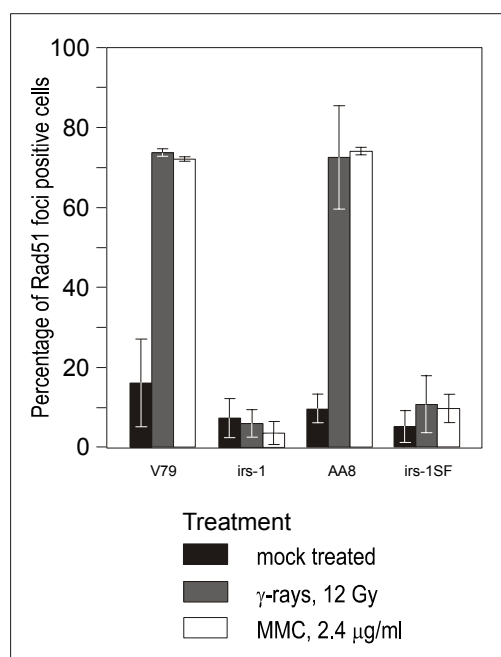


Figure 4. IF data of *irs-1* (*Xrcc2* mutant) and *irs-1SF* (*Xrcc3* mutant) cell lines presented as bar graphs, showing the percentage of Rad51 foci positive cells. V79 and AA8 are the respective parental cell lines. Cells were treated with agents at indicated doses, fixed two hours after treatment and counted for foci. Error bars show the standard of the mean of 3 independent experiments.

To determine what other proteins are involved in the pathway leading to DNA damage induced Rad51 foci formation, we screened mutant cell lines deficient in DSB or ICL repair or both. The mouse *Snm1KO* ES cell line is hypersensitive to MMC, which induces ICLs. This is a phenotype similar to the mutant cells belonging to the RAD52 group, and makes *Snm1* a possible candidate gene to fall within this group of recombination repair genes. Therefore the *Snm1KO* cell line was treated with IR, and with two different cross-linking agents; MMC and BCNU. The results presented in Figure 5 and Figure 6 show that the absence of Snm1 did not affect the DNA damage response of Rad51 as measured by foci formation. This indicates that Snm1 is either not essential to the Rad51 associated recombination repair pathway, or works downstream from Rad51 or is involved in a completely different pathway. The latter is the most likely, since additional survival experiments showed that the *Snm1KO* ES cells are hypersensitive to MMC, but not to other cross-linking agents or IR (for a complete list of the tested agents see Dronkert et al. table 2 (8)), in contrast to other, recombination repair related cell lines, as *irs-1*, *irs-1SF* and the *Rad54KO* (9, 18).

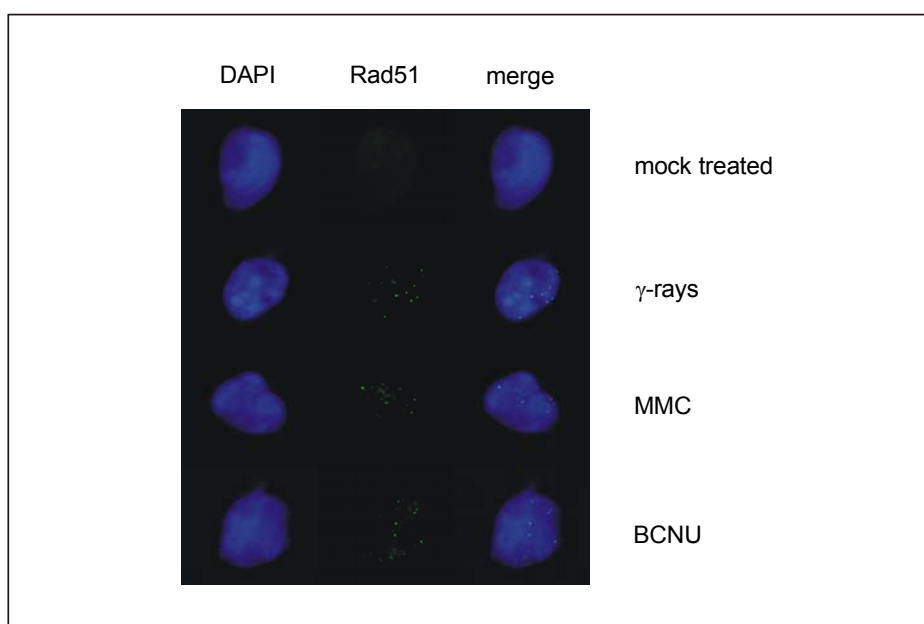


Figure 5. IF pictures of the *Snm1KO* ES cell line. Absence of Snm1 does not affect the IR, MMC or BCNU induced Rad51 foci formation. Pictures were taken two hours after treatment with  $\gamma$ -rays (12 Gy), MMC (2.4  $\mu$ g/ml) or BCNU (0.3 mM). Cells were stained with Rad51 and counterstained with DAPI.



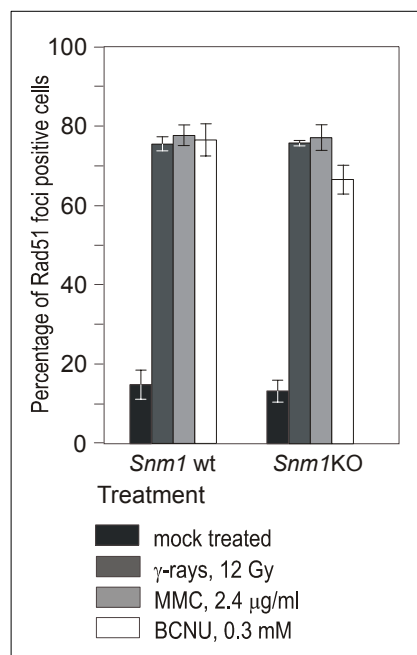


Figure 6. IF data of the *Snm1*KO ES line presented as bar graph, showing the percentage of Rad51 foci positive cells. Two hours after treatment cells were fixed, stained for Rad51 and counted for foci. Error bars show the standard of the mean of 3 independent experiments. IB10 was used as wild type control and is the parental cell line.

Cell line V-C8 is a hamster mutant cell line whose gene mutation was unknown when the IF assay was performed. It is sensitive to IR and extremely sensitive to cross-linking agents (21, 47, 48). However, it is also sensitive to other DNA damaging agents such as adriamycin, methyl methanesulfonate and UV light. In addition, it shows radioresistant DNA synthesis after IR and increased spontaneous and induced genetic instability, suggesting that it has a defect in some type of DNA recognition, DNA repair or cell cycle checkpoint regulation (21). Based on its complex and rather unique phenotype it was put in a separate X-ray sensitive mutant complementation group: the XRCC11 complementation group (21, 42, 47). Due to its sensitivity to a broad spectrum of DNA damaging agents it was impossible to discover the mutated gene as the group of candidate genes was too big. Complementation studies with *DNA-PKCS*, *Ku80*, *Xrcc2*, *Xrcc3* and *Xrcc4* had been performed in an attempt to localize the mutation, but without positive result (42).

Hamster cell line V-H4 (47, 48) shows instability and radio-resistant DNA synthesis similar to VC-8 however, it features normal DSB rejoining as measured by DNA elution (42, 47).

Both cell lines were treated with IR and MMC to see whether the mutation affected the Rad51 response. V-H4 cells showed no difference to the parental cell line regarding the Rad51 foci induction (see Figure 7, Figure 8). In contrast to V-H4, the Rad51 response is clearly affected in V-C8, so the gene product is essential for DNA damage-induced Rad51 foci formation and acts at the same stage as Rad51 or upstream within the same pathway (Figure 7, Figure 8). This observation implied that the mutation in V-C8 must be in a gene involved in recombination repair. Previous complementation studies show that V-C8 cells are not deficient in *Xrcc2* or *Xrcc3* (42, 47). Therefore, V-C8 should be deficient in another gene involved in Rad51-associated repair. V-C8 cells were transfected with *RAD51*, *RAD51B*, *RAD51C* and *RAD51D* cDNA (data not shown), but the cDNA constructs did not rescue the mutant.

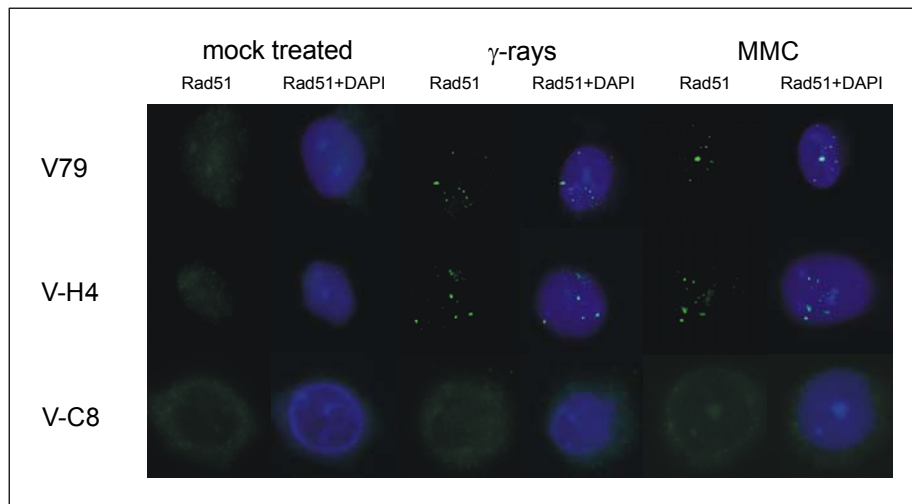


Figure 7. IF pictures of V-H4, V-C8 and V79 cells. V79 is the parental cell line of both V-H4 and V-C8. Cells were fixed two hours after treatment with  $\gamma$ -rays (12 Gy) or MMC (2.4  $\mu$ g/ml). Cells were stained for Rad51 and counterstained with DAPI.

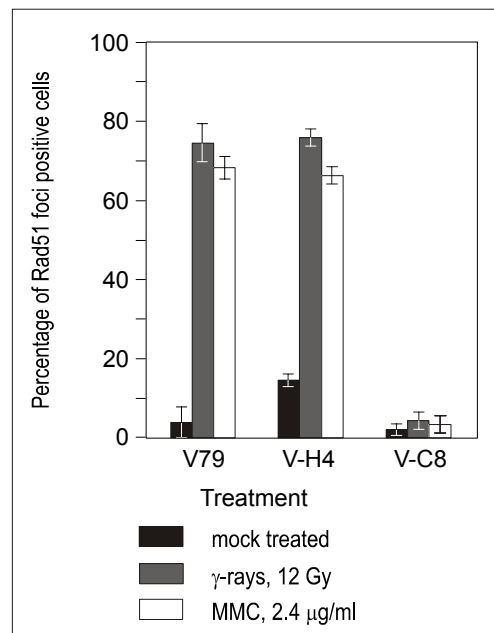


Figure 8. IF data of V79 (parental line), V-C8 and V-H4 cell lines presented as bar graphs. The percentage of Rad51 foci positive cells is displayed. Error bars show the standard of the mean of 3 independent experiments. Cells were fixed, stained for Rad51 and counted for foci two hours after treatment. The mutation of the V-H4 cell line does not influence the Rad51 foci formation, the mutation of the V-C8 cell line abolishes the induced Rad51 foci formation (compare with parental line V79).

BRCA1 and BRCA2 proteins are also reported to interact with RAD51 (4, 6, 23, 44-46) and therefore complementation studies were performed. Both human chromosome 13 (containing *BRCA2*) and mouse *Brca2* cDNA rescued the hamster V-C8 cells in terms of cross sensitivity to IR and MMC, and complemented the radio-resistant DNA synthesis and spontaneous and MMC-induced chromosomal instability (for a more extensive report see Kraakman-van der Zwet et al. (15). More evidence for the fact that the mutation involved the *Brca2* gene was the finding that V-C8 cells lack the *Brca2* protein, as assayed by Western blot analysis (15). The IR and MMC induced Rad51 foci formation was also restored in m*Brca2* transfected V-C8 cells

(see Figure 9). Importantly, when RAD51-GFP was transfected in V-C8 and V79 cells, only the latter (containing Brca2) was able to form IR-induced RAD51GFP foci (15). Many cancer-associated *BRCA2* mutations are in the RAD51-interaction domain (14, 19) affecting RAD51's sub-cellular location, and additional experiments revealed that V-C8 cells have reduced Rad51 protein levels in the nucleus compared to V79 cells (15). Thus, Brca2 is essential for the formation of DNA damage-induced Rad51 foci as the mutation affects Rad51 localization to the nucleus. However, this may not be the only reason for deficient foci formation, as it has also been shown that Brca2 stimulates Rad51-mediated DNA pairing and strand exchange in presence of RPA (43). It has also been proposed that Brca2 has a shuttle function in bringing Rad51 to the single-stranded DNA, releases Rad51 at the spot and return later to remove it from the DNA (41). Therefore, it is not surprising that V-C8, missing such a for Rad51 important protein, is unable to form IR-induced Rad51 foci.

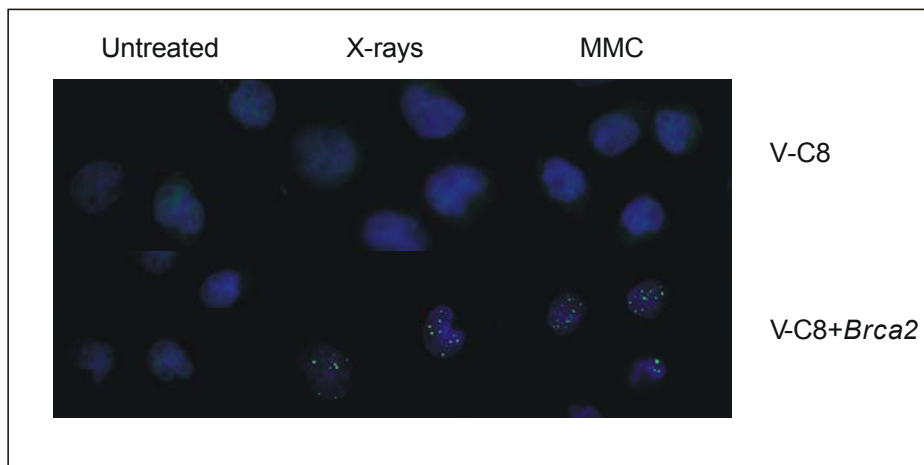


Figure 9. IF picture of V-C8 cell line vs. V-C8 with *mBrca2* gene, showing the absence of Rad51 foci in the mutant (V-C8) and presence of the Rad51 foci in the rescued cell line (V-C8+*Brca2*) after induction with IR (12 Gy) or MMC (2.4  $\mu$ g/ml). Cells were stained for Rad51 and counterstained with DAPI.

Thus, the Rad51-immunofluorescence assay is an important tool for analyzing mutant cell lines. It is a quick method, as absence of induced-Rad51 foci would indicate that the mutant falls in the group of recombination repair deficient cell lines. Used this way, it showed its value in identifying the mutation in the V-C8 cell line.

This assay could even have a function when Rad51 foci formation is not absent: the relative importance to Rad51 foci formation could be discovered when it is known that the protein interacts with Rad51 (e.g. in case of the *Rad52KO*).

## MATERIALS AND METHODS

### Cell culture

Mouse embryonic stem cells were cultured on a 1:1 mix of DMEM and BRL (buffalo rat liver) cell cultured medium, enriched with 10% foetal calf serum, 0.1 M non-essential amino acids, 50  $\mu$ M  $\beta$ -mercapto-ethanol and 500 U/ml leukaemia inhibitor factor. Hamster cells were grown on F10/DMEM (1:1 mix) or in DMEM, supplemented with 10% foetal calf serum. All cell cultures were maintained at 37° C in a 5% CO<sub>2</sub> atmosphere humidified to 95-100%.

### Transfection of exogenous DNA

Transfections (of the bacterial artificial chromosome containing murine *Brca2*) were performed using the GenePORTER™ transfection reagent according to the manufacturer's protocol (BIOzym).

### Immunofluorescence

Exponentially growing cells were seeded on gelatinized glass slides 24 hours before the experiments. Cells were mock treated or  $\gamma$ -irradiated (12 Gy, <sup>137</sup>Cs source), washed in phosphate buffered saline (PBS) and incubated for two hours at 37° C in fresh medium. In case of MMC treatment, cells were incubated with 2.4  $\mu$ g/ml MMC in fresh medium for one hour, washed twice in PBS, and also incubated for two hours in fresh medium. After the last incubation step (all treatments), cells were washed 3x in PBS and fixed in ice-cold methanol for 20 minutes at -20° C and 10 seconds in acetone (at -20° C). Cells were blocked in PBS+ (20 mM glycine, 0.5% BSA in PBS) and incubated with rabbit antiserum against hRad51 (FBE2, 2307) for 90 minutes at 20° C, washed 3x in PBS+, and incubated with Alexa 488/594 conjugated goat anti-rabbit immunoglobulin G (Molecular Probes). Cells were counterstained with DAPI (4',6'-diamidino-2-phenylindole) in vectashield. Experiments were performed 3x and at least 100 cells per experiment were analyzed, error bars in the figures show the standard error of the mean. Cells were considered to be Rad51 foci positive if the nucleus contained 2 or more Rad51 foci. For pictures shown in Figures 3 and 9 (Rad52GFP and VC-8+ *Brca2*, respectively) a 2% paraformaldehyde fixation (5) at 20° C was used (two hours and eight hours after treatment, respectively).

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## CHAPTER THREE

### THE MOUSE RAD54 RECOMBINATIONAL DNA REPAIR PROTEIN AFFECTS DNA CONFORMATION AND DNA DAMAGE-INDUCED RAD51 FOCI FORMATION

Extended version of the article by Tan et al. in Current Biology 9(6):325-328, 1999

## ABSTRACT

Error-free repair of ionizing radiation-induced DNA double-strand breaks by homologous recombination requires the Rad51 and Rad54 proteins in the yeast *Saccharomyces cerevisiae*. Key steps in recombination, homologous DNA pairing and strand exchange, are mediated by Rad51, while Rad54 stimulates the recombination activities of Rad51. Mammalian homologues of Rad51 and Rad54 have been identified. Here we demonstrate that mouse Rad54 forms ionizing radiation-induced nuclear foci that colocalize with Rad51. Interaction between mouse Rad51 and Rad54 is induced by genotoxic stress, but only due to lesions whose repair requires Rad54. mRad54 is shown to be important for the stability of IR-induced mRad51 foci. Rad54 belongs to the SWI2/SNF2 protein family whose members modulate protein-DNA interactions in an ATP-driven manner. We show that in the presence of a ligase, purified human Rad54 protein introduces negative and positive supercoils in nicked DNA at the expense of ATP hydrolysis. This finding suggests that this feature may provide stability to Rad51 mediated joint molecule formation and is consistent with our observation that Rad54 is important for the stability of induced Rad51 foci.

## INTRODUCTION

Among the most genotoxic DNA lesions generated by ionizing radiation are DNA double-strand breaks (DSBs). Their accurate repair is essential to prevent chromosomal fragmentation, translocations, and deletions. The persistence of chromosomal aberrations, resulting from incorrect DSB repair, can lead to carcinogenesis through activation of oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity. Probably due to the extreme genotoxicity of DSBs, several mechanistically distinct DSB repair pathways have evolved, including homologous recombination, DNA end-joining, and single-strand annealing. Homologous recombination requires extensive regions of homology and repairs DSBs accurately using information on the undamaged sister-chromatid or homologous chromosome (3, 18, 21, 32). DNA end-joining uses no or extremely limited sequence homology to rejoin broken ends in a manner that need not to be error-free (10, 19, 42). Single-strand annealing can operate in a specialized case when the DSB occurs between or within directly repeated DNA sequences. DSB repair through this pathway results in the loss of one of the repeats as well as the DNA segment between the repeats (23).

Extensive genetic analyses established that DSBs are efficiently repaired through homologous recombination in the budding yeast *Saccharomyces cerevisiae* (13, 31). Mutants in the *RAD52* epistasis group are defective in DSB repair through homologous recombination and display sensitivity to ionizing radiation and the alkylating agent methyl methanesulfonate (MMS). Key genes within the *RAD52* group include *RAD51* and *RAD54* because mutations in these genes result in extreme cellular sensitivity to ionizing radiation. Rad51 shows limited amino acid sequence similarity to the *Escherichia coli* RecA strand exchange protein and displays similar biochemical properties (24, 36, 39). Both proteins assemble a presynaptic nucleoprotein filament composed of single-stranded DNA, coated with one protomer per three nucleotides. Nucleoprotein filament formation is followed by homologous DNA pairing and DNA strand exchange. The additional *RAD52* group members Rad52, Rad55, and Rad57 have been implicated in stimulating the recombination activities of Rad51 (29, 37, 40). Rad54 is a member of the SNF2/SWI2 family of proteins that have been implicated in many aspects of DNA metabolism such as transcription, repair, and recombination (22). The protein family is characterized by conserved sequence motifs found in DNA helicases (11). However, none of the SNF2/SWI2 family proteins have been shown to possess helicase activity (30). It has been suggested that the conserved motifs that confer helicase activity to some proteins provide a more general function of which helicase activity represents a subset (17). Recent biochemical experiments have revealed that the *S. cerevisiae* Rad54 protein promotes Rad51-mediated D-loop formation between linear single-stranded DNA and homologous supercoiled DNA (33).

The importance of the Rad52 homologous recombination pathway is underscored by its conservation from fungi to humans. Human genes with sequence similarity to *S. cerevisiae* *RAD51*, *RAD52* and *RAD54* have been identified (21, 32). The human Rad51 and Rad52 proteins display similar biochemical activities compared to their yeast counter parts (2, 4, 15, 28, 35). In addition, disruption of the mouse and chicken *Rad54* genes results in ionizing radiation sensitive cells with a reduced level of homologous recombination (5, 12). Here, we report on the cellular behavior of the mouse Rad54 protein (mRad54) in response to DNA damage and on the interaction of the human Rad54 protein (hRad54) with DNA.

## RESULTS AND DISCUSSION

To study the behavior of mRad54 after IR treatment of cells, a knock-in construct was generated in which exons 4-18 were replaced by the corresponding cDNA supplemented with a carboxy-terminal histidine-hexamer hemagglutinin (His<sub>6</sub>HA) tag. Homologous integration of the construct within the *mRAD54* locus of embryonic stem (ES) cells ensured expression of His<sub>6</sub>HA-tagged mRad54 from the endogenous *mRAD54* promoter (Figure 1A). The protein (88 kDa) was detected using  $\alpha$ -hRad54 and  $\alpha$ -HA antibodies on immunoblots from cell lines *mRAD54*<sup>-/-HA</sup> and *mRAD54*<sup>+/-HA</sup>. These ES cells contain knock-out and wild-type *mRAD54* alleles, respectively, in addition to the His<sub>6</sub>HA-tagged allele (Figure 1B). The HA-tag did not interfere with the biological activity of mRad54 since cells expressing only the tagged protein displayed the same IR resistance as cells expressing untagged mRad54 (Figure 1C).

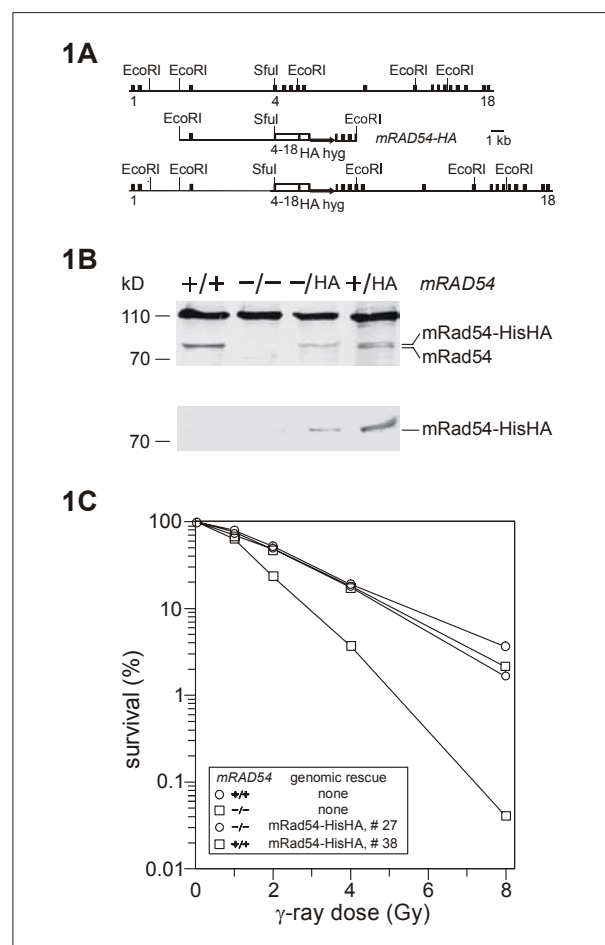


Figure 1. Generation and characterization of ES cell lines containing endogenously expressed HA-tagged mRad54. A, Structure of the genomic *mRAD54* locus (top), the HA-tagged *mRAD54* knockin construct (middle), and the targeted locus (bottom). Black boxes represent the 18 exons encoding mRad54. HA, hemagglutinin tag; hyg, hygromycin resistance gene. B, Immunoblot of extracts from *mRAD54* wild type (+/+), KO (-/-), and knock-in (-/HA and +/-HA) ES cells, probed with  $\alpha$ -hRad54 and  $\alpha$ -HA antibodies (upper and lower panel, respectively). The nature of the 110-kDa protein that cross reacts with the  $\alpha$ -hRad54 antibodies is unknown. C, Clonogenic survival assays of the indicated ES cell lines after ionizing radiation treatment.

When the steady-state level of mRad54 was measured after treatment of cell line *mRAD54*<sup>-HA</sup> with IR, no dose- or time-dependent difference in mRad54 levels was detected (Figure 2A). A similar result was obtained for mRad51. However, although the steady-state level of mRad54 did not change, the protein was redistributed after IR treatment, as detected by immunofluorescence microscopy (Figure 2B). After IR treatment, mRad54 was detected as bright foci in the nuclei of the majority of the cells. This staining pattern is similar to that reported for Rad51 (16, 25, 26). Therefore, cells were also stained for mRad51, which revealed co-localization of some mRad54 and mRad51 foci (Figure 2B). DAPI staining of irradiated cells showed that foci-containing cells did not show signs of apoptosis (i.e. apoptotic blebbing) or were otherwise aberrant.

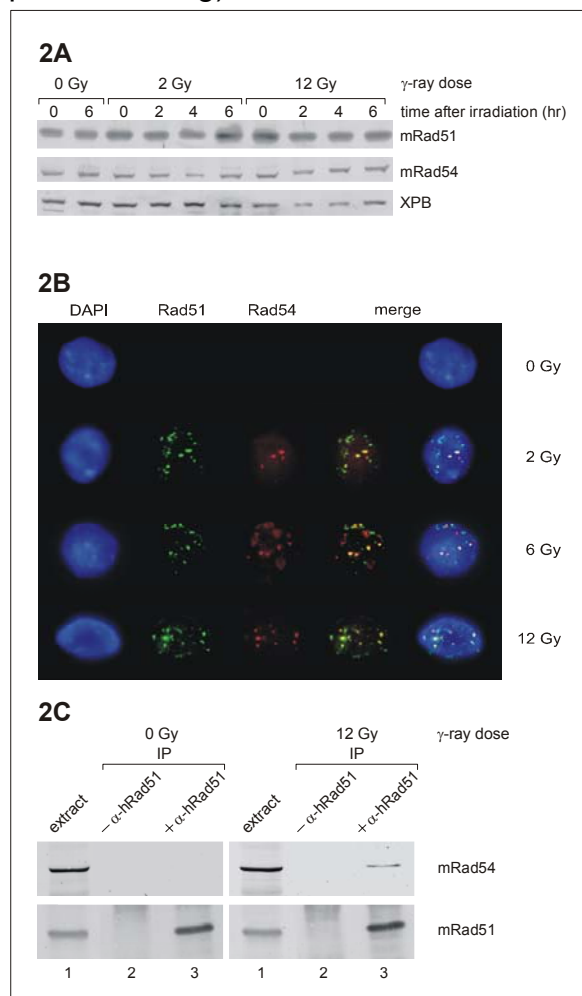


Figure 2. IR-induced interaction between mRad51 and mRad54. A, Steady-state levels of mRad51 (top) and mRad54 (middle panel) proteins: No dose or time dependent change after  $\gamma$ -irradiation. The XPB protein (lower panel) serves as loading control. B, Ionizing radiation-induced co-localization of mRad51 and mRad54 foci as detected by immunofluorescence within cells fixed six hours after treatment. The first three columns show separate nuclear (DAPI), mRad51 and mRad54 staining, respectively, while the latter two show merged combinations. C, Co-immunoprecipitation of mRad54 with immobilized  $\alpha$ -hRad51 antibodies requires prior ionising radiation treatment of the cells. Lane 1, protein extracts; lane 2, immunoprecipitation (IP) using pre-immune serum; lane 3, IP using  $\alpha$ -hRad51 antibodies. The immunoblots in the upper and lower panels were probed  $\alpha$ -HA and  $\alpha$ -hRad51 antibodies, respectively.

To determine whether the IR-induced mRad51 and mRad54 foci co-localized because of an association of the two proteins, immunoprecipitation experiments were performed. Immobilized  $\alpha$ -hRad51 antibodies were used to precipitate

mRad51 from *mRAD54*<sup>-HA</sup> ES cell protein extracts, and the precipitate was analyzed for the presence of mRad51 and mRad54. While mRad51 was detected in the precipitate from extracts of unirradiated and irradiated cells, mRad54 was co-immunoprecipitated only from the extract of irradiated cells (Figure 2C). Similarly, IR-dependent co-immunoprecipitation of mRad51 and mRad54 was observed using immobilized  $\alpha$ -HA antibodies. To ensure that co-precipitation was not mediated via the DNA, we repeated the immunoprecipitations in the presence of the DNA intercalating agent ethidium bromide and obtained similar results (data not shown).

The kinetics of IR-induced mRad51 and mRad54 foci formation was examined to determine whether the redistribution of these proteins upon induction of genotoxic stress was a dynamic process. The percentage of cells showing both mRad51 and mRad54 foci, *i.e.* double positive cells, increased from 8.5 to 76% over a two hour period after IR treatment with doses of 2 to 12 Gy (Figure 3A). No significant

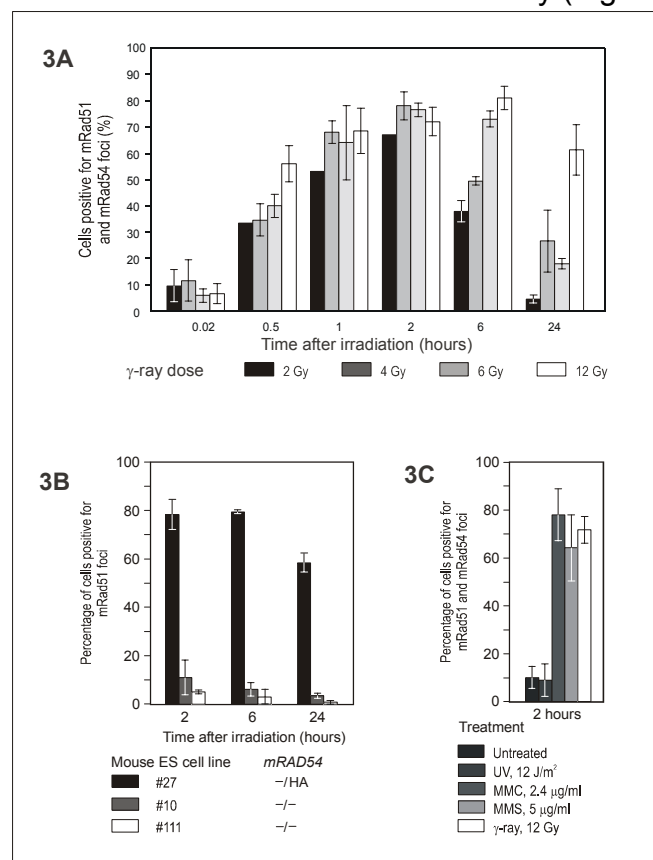


Figure 3. Kinetics of mRad51 and mRad54 foci formation. A, ES cells (*mRAD54*<sup>-HA</sup>) were treated with the indicated doses of IR, fixed at given time points, and stained for mRad51 and mRad54. The percentage of cells containing both mRad51 and mRad54 foci is displayed. Cells with two or more nuclear foci of one type were considered as positive for that type. Each data point, obtained in three independent experiments, was based on the analysis of at least 100 nuclei. B, As in A, except *mRAD54*<sup>-/-</sup> cells were used and the percentage of cells containing mRad51 foci is plotted. C, As in A, except that the cells were fixed two hours after treatment with the indicated agents.

dose dependent difference in the induction rate of mRad51 and mRad54 foci was observed. However, the decrease in the percentage of double positive cells over time was dependent on IR dose. Twenty-four hours after treatment with 2 Gy, the percentage of double positive cells was reduced to levels found in untreated cells. Treatment with the lethal dose of 12 Gy did not result in a substantial reduction

(Figure 3A), suggesting that foci remaining after 24 hours in these lethally irradiated cells reflect unrepaired DNA damage.

To test whether the IR-induction interaction between mRad54 and mRad51 affects mRad51 foci formation, mRad51 foci formation was examined in *mRAD54*<sup>-/-</sup> ES cells. IR did not induce mRad51 foci formation in *mRAD54*<sup>-/-</sup> cells (Figure 3B) under methanol/acetone fixation conditions. To determine whether the lack of IR-induced mRad51 foci in the *mRAD54*<sup>-/-</sup> ES cells was dependent on the fixation method applied to the cells, mRad51 was also examined by IF using an alternative fixation method. Instead of fixing the cells with methanol and acetone, they were fixed with para-formaldehyde. In contrast to the results obtained with the methanol/acetone fixation method, IR induced mRad51 foci were detected in *mRAD54*<sup>-/-</sup> ES cells fixed with para-formaldehyde (Figure 4, right). This effect was observed in two independently obtained *mRAD54*<sup>-/-</sup> cell lines. The *mRAD54* proficient and deficient cell lines were isogenic and differed solely in their *mRAD54* alleles. We suggest that in the absence of mRad54 the stability of mRad51 foci is affected. They can be observed when the proteins in the cells are cross-linked with para-formaldehyde at 20° C, but in the absence of mRad54 they are not resistant to methanol/acetone treatment at -20° C (Figure 4, left).

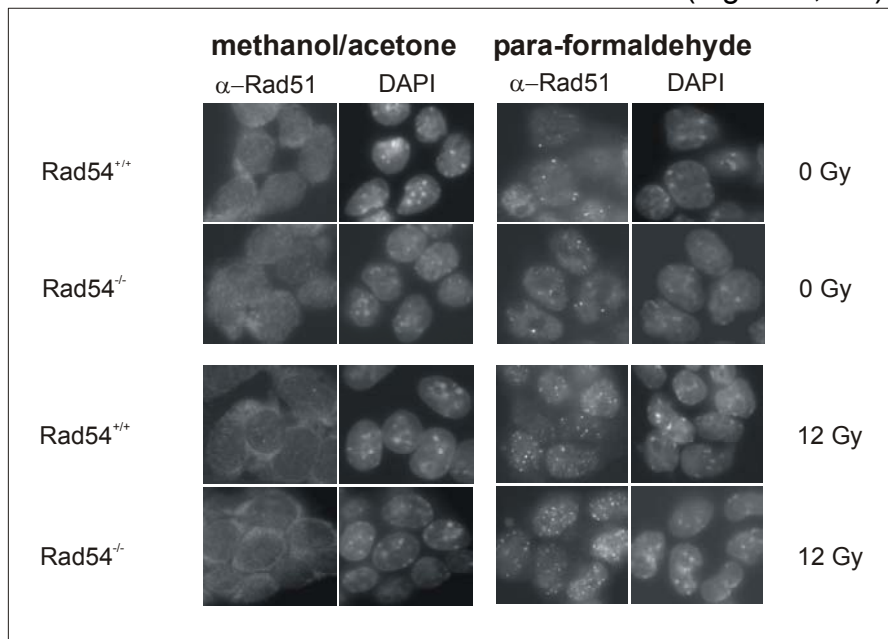


Figure 4. Rad51 irradiation induced foci formation in Rad54 proficient and deficient ES cells depends on the fixation method.

*Rad54*<sup>+/+</sup> and *Rad54*<sup>-/-</sup> mouse ES cells were irradiated with 12 Gy and fixed after 2 hours with either methanol/acetone (first panel) or 2% para-formaldehyde (second panel) as described in the materials and methods. Using an antibody against Rad51, foci formation was compared in untreated cells (first two rows) and irradiated cells (3<sup>rd</sup> and 4<sup>th</sup> row). To discriminate between the different cells the nuclei are visualized by DAPI staining and shown next to the Rad51 staining. Before irradiation hardly any Rad51 foci can be discriminated after methanol/acetone fixation in both cell lines, while after fixation with para-formaldehyde most nuclei of both *Rad54*<sup>+/+</sup> and *Rad54*<sup>-/-</sup> ES cells show several foci. After ionizing radiation the *Rad54*<sup>+/+</sup> ES cells fixed with methanol/acetone show some Rad51 foci where they cannot be discriminated in *Rad54*<sup>-/-</sup> ES cells. After fixation with para-formaldehyde irradiated cells demonstrate numerous Rad51 foci in both *Rad54*<sup>+/+</sup> and *Rad54*<sup>-/-</sup> ES cells.

This observation is consistent with observations reported by Mazin and colleagues (27). Their biochemical data reveal that Rad51 nucleoprotein filaments can form in the absence of Rad54. However, Rad54 protected the Rad51-

nucleoprotein filament against restriction enzymes, increased the salt resistance of the filament and prevented Rad51 protein dissociation from the nucleoprotein filament. Thus, we observed the cellular consequence of the biochemical Rad54 function: Rad51 foci can form in the *mRad54KO*, but are less stable.

Brca1 and the Rad51 paralogues Xrcc2 and Xrcc3 are required for Rad51 foci formation (see chapter two and references therein). Absence or mutations in any of these genes lead to disrupted Rad51 foci formation. In case of the *Xrcc3* mutant hamster cell line *irs-1SF*, the Rad51 foci formation was studied under methanol/acetone (chapter 2) and paraformaldehyde (7) fixation conditions and Rad51 foci were absent using both types of fixation. Thus, whereas Rad54 is important for the stability of the Rad51 foci, Xrcc3 is essential for the foci formation. The relative importance of Rad54 and Xrcc3 in foci formation is reflected by the biological importance of the proteins: the *mXrcc3KO* is embryonic lethal, while the *mRAD54KO* is viable.

To determine whether other types of DNA damage elicit mRad54 foci formation, cells were treated with mitomycin C (MMC), methyl methanesulfonate (MMS) or ultraviolet (UV) light. Foci containing both mRad51 and mRad54 were induced by MMC and MMS, but not by UV light (Figure 3C), which correlates with the DNA damage sensitivities of *mRAD54<sup>-/-</sup>* ES cells (12). This result is consistent with the observation that MMS treatment induces Rad51 foci in both primary and transformed mammalian cells (16, 25). In contrast, no redistribution takes place in response to lesions whose repair does not depend on mRad54. We conclude that mRad54 redistributes in the cell and associates with mRad51 upon induction of genotoxic stress generated by DNA damage that requires mRad54 for its repair. Our observations are consistent with experiments showing that Rad51 and Rad54 from *S. cerevisiae* and human can interact *in vitro* (8, 14, 20). However, in contrast to the *S. cerevisiae* proteins, which interact under normal physiological conditions, significant interaction between the mouse proteins requires DNA damage.

In addition to the physical interaction, Rad51 and Rad54 interact functionally. During recombinational repair Rad51 mediates joint molecule formation between a single-stranded region on the damaged DNA and an intact homologous duplex DNA. Rad51 initiates homologous pairing by forming a nucleoprotein filament on the single-stranded DNA (3, 6). The *S. cerevisiae* Rad54 protein has been shown to stimulate the pairing activity of Rad51 (33). However, the molecular basis of this stimulation is not understood. *S. cerevisiae* and human Rad54 are double-stranded DNA-dependent ATPases that belong to the SNF2/SWI2 protein family (33, 41). Although members of this family contain seven conserved motifs characteristic of helicases (11, 30), helicase activity of these proteins using oligonucleotide displacements assays has not been detected (30, 33, 41). One interpretation of these results is that although proteins of this family do not disrupt base pairing, they might still be able to locally unwind the DNA double helix. To further investigate this possibility, the interaction of hRad54 with double-stranded DNA was examined using a topological assay. Single-nicked plasmid DNA was incubated with purified hRad54 protein and the reaction mixture was then supplemented with DNA ligase. Any protein-induced change in linking number ( $\Delta Lk$ ) will be detected as a change in the electrophoretic mobility of the DNA. In the presence of ATP, hRad54 generated topoisomers that migrated with native



superhelix density DNA (Figure 5A), indicating that the protein induced an extensive  $\Delta Lk$ . The amount of converted DNA increased with increasing hRad54 concentration (Figure 5B). The hRad54-induced  $\Delta Lk$  required ATP hydrolysis since it was not observed in the absence of ATP, in the presence of the non-hydrolyzable analog ATP $\gamma$ S (Figure 5B), or with hRad54<sup>K189R</sup> (Figure 5A), which carries a single amino acid substitution that blocks ATP hydrolysis (41).

The direction and extent of the hRad54-induced  $\Delta Lk$  was determined by two-dimensional gel electrophoresis. In the presence of ATP, hRad54 introduced negative supercoils in the plasmid DNA (Figure 5C). Topoisomers with a  $\Delta Lk$  of up to  $-23$  were resolved, indicating that hRad54 binding can induce a specific linking difference ( $\sigma = \Delta Lk/Lk_0$ ) of at least  $-0.08$  which is even lower than that of native superhelix density DNA ( $\sigma = -0.06$ ). Although a widening of the topoisomer distribution was observed in the absence of ATP, the center of the distribution was unchanged. The negative supercoils introduced by hRad54 could either result from a change in twist due to unwinding of the DNA double helix, or from a change in writhe due to DNA wrapping around the protein surface. Given that the *E. coli* recombination protein RuvB which contains similar conserved motifs to those found in Rad54 and the SNF2/SWI2-containing protein complex change twist (1, 34), we favor the possibility that the negative supercoils induced by hRad54 are due to DNA unwinding.

We suggest that the stimulation of Rad51-mediated homologous DNA pairing by Rad54 could be due to unwinding of the double-stranded DNA recombination partner (33). Unwinding will facilitate pairing because the DNA in the Rad51 nucleoprotein filament is in an extended conformation (3, 6). In comparison with *E. coli* RecA protein, hRad51 makes only short heteroduplex joints (3). Through its association with hRad51 and its ATP-dependent DNA unwinding activity, hRad54 might provide stability to the hRad51-mediated joint molecule thereby allowing extension of the hRad51 filament and of heteroduplex DNA. This role is consistent with our demonstration that mRad54 affects the stability of mRad51 foci induced by genotoxic stress.

In addition to a role in promoting Rad51-mediated joint molecule formation, Rad54 could influence homologous pairing indirectly by affecting chromatin structure. Rad54-mediated DNA unwinding might result in displacement of histones that could be inhibitory to homologous pairing. Such a role is in agreement with the functions of other SNF2/SWI2 family members that have been implicated in chromatin remodeling and removal of proteins from DNA (30). Dual roles of these proteins, a general role in affecting chromatin and a specialized role in DNA metabolism, provide an explanation for why a number of DNA repair pathways, such as transcription-coupled nucleotide excision repair, genome overall nucleotide excision repair, post-replication repair, and homologous recombination, all require a specific SNF2/SWI2 family member (11). Although the effect on chromatin could have been provided by a single protein, their additional function within each DNA repair pathway demand specialization.

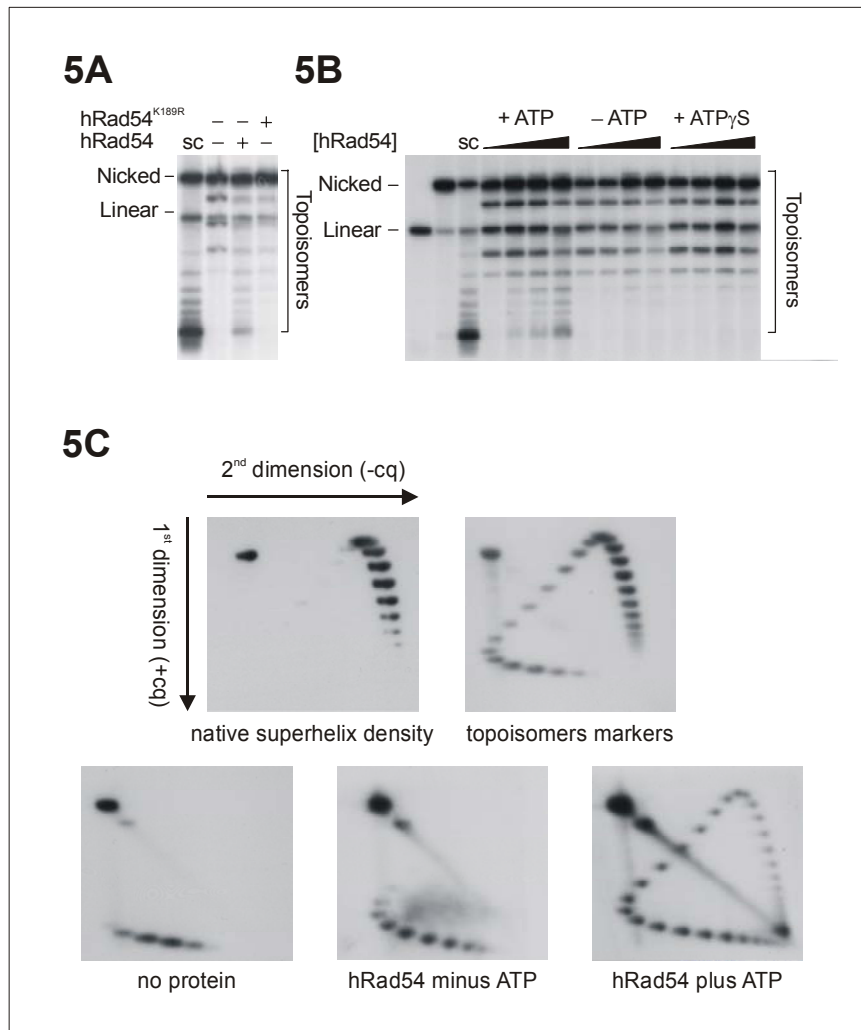


Figure 5. Binding of hRad54 to DNA induces negative supercoiling. A, Equal amounts of hRad54 and hRad54<sup>K189R</sup> proteins were incubated with singly-nicked plasmid DNA in the presence of ATP. After ligation of the nick the resulting distribution of topoisomers was analyzed by agarose gel electrophoresis. sc, native superhelix density DNA. B, The induction of an extensive  $\Delta Lk$  by hRad54 is dependent on protein concentration and requires ATP hydrolysis. The amount of hRad54 used was 0, 15, 30 and 40 ng. C, Binding of hRad54 induces a negative  $\Delta Lk$ . Series of two-dimensional gels containing the indicated DNAs. Marker topoisomers of the plasmid are displayed in the upper two panels. The lower three panels show gels containing plasmid DNA ligated in the absence of hRad54 (no protein) and in the presence of hRad54, either without (hRad54 minus ATP) or with (hRad54 plus ATP) ATP. cq, chloroquine.

## MATERIALS AND METHODS

### Cell culture and survivals

ES cell culture and cell survival assays were carried out as described (12). Measurements were performed in triplicate. Standard errors of the mean were within 4-16%, except for the dose of 8 Gy for the *mRAD54*<sup>-/-</sup> line, which showed an error of 28%.

### Immunoprecipitation

For immunoprecipitations, cells were lysed by resuspension in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, and 0.5 % Triton X-100 (NETT buffer). All manipulations were carried out at 4° C. The extract was clarified by centrifugation followed by the sequential addition of  $\alpha$ -hRad51 antibodies and protein G sepharose beads to the supernatant. The beads were washed three times with NETT buffer. The immunoprecipitate was fractionated by SDS-PAGE and gels were analyzed by immunoblotting using rabbit  $\alpha$ -hRad51 and rat  $\alpha$ -HA antibodies.

### Immunofluorescence

Cells were grown on gelatinized glass slides, treated with DNA damaging agents, fixed at different time points with methanol/acetone and processed as described (12, 26). Slides were incubated for 1.5 hours at 20° C with  $\alpha$ -hRad51 and  $\alpha$ -HA antibodies, followed by a 1.5 hours incubation with Alexa 488-conjugated goat  $\alpha$ -rabbit and Alexa 594-conjugated goat  $\alpha$ -rat secondary antibodies obtained from Molecular Probes. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Cells with two or more nuclear foci of one type were considered as positive for that type. Each data point, obtained in three independent experiments, was based on the analysis of at least 100 nuclei. Standard deviations are indicated in Figure 3. Image acquisition and processing were done as described (26).

### Topological analysis

The hRad54 and hRad54<sup>K189R</sup> proteins were purified as described (41). Singly-nicked plasmid DNA (pBluescript II KS) was prepared as described (9). Reaction mixtures (60  $\mu$ l) contained 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2 mM ATP, 26 mM NAD, 50  $\mu$ g/ml BSA, and 50 ng DNA unless stated otherwise. After 10 min at 30° C, one unit *E. coli* DNA ligase was added and incubation was continued for 50 min. Purification of DNA and two-dimensional gel electrophoresis were performed as described (9, 38). Marker topoisomers were generated by topoisomerase I in the presence of different concentrations of ethidium bromide.

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## CHAPTER FOUR

### **RAD54, A JACK OF ALL TRADES IN HOMOLOGOUS RECOMBINATION**

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## ABSTRACT

Homologous recombination mediates the transfer or exchange of genetic information between homologous DNA molecules. It plays important roles in central processes in the cell such as genome duplication and DNA damage repair. Recent experiments reveal the surprising versatility of one of its central actors, the Rad54 protein.

## INTRODUCTION

Homologous recombination is essential for the accurate transmission of the genome because it corrects errors during chromosomal replication and mediates DNA damage repair. Discontinuities in double stranded DNA, particularly DNA double-strand breaks (DSBs), are the major initiators of homologous recombination. Homologous recombination restores the continuity of a broken DNA molecule by using an intact and homologous DNA molecule as a template, usually the sister chromatid (25). A model for the accurate healing of a DSB by homologous recombination is schematically depicted in Figure 1.

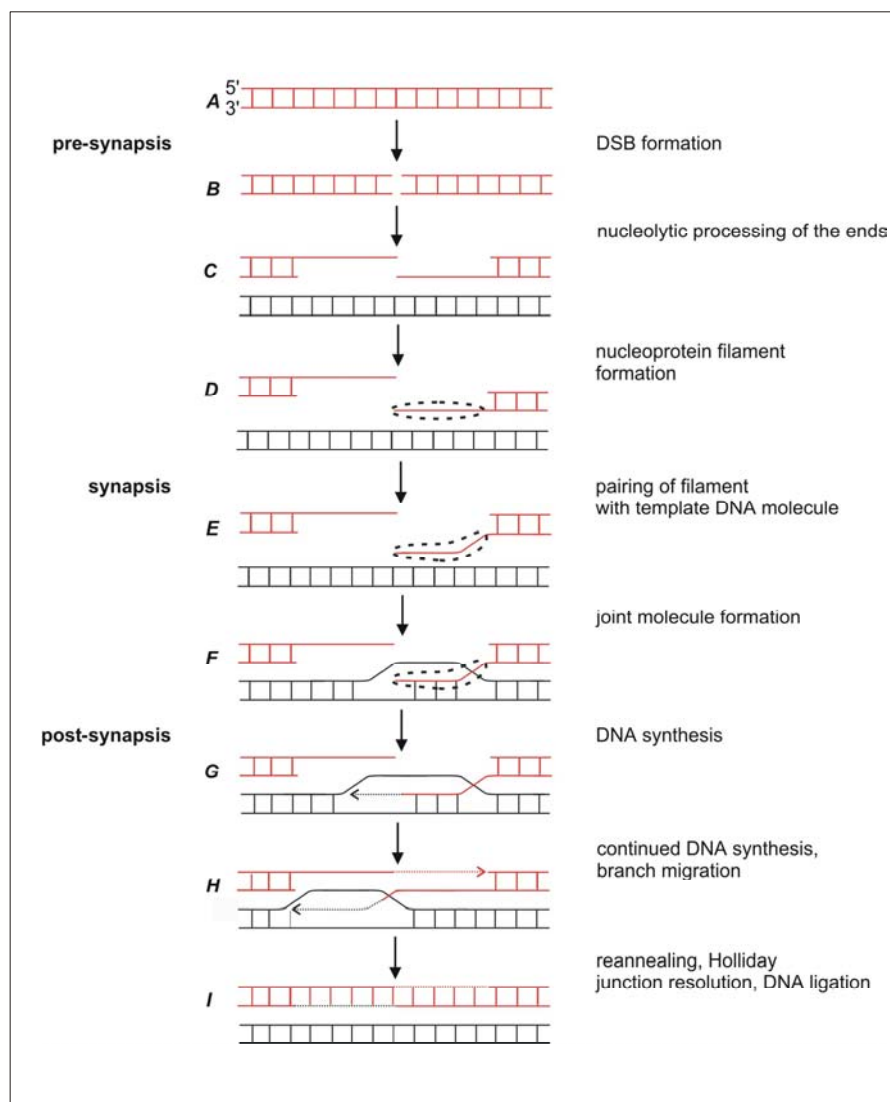


Figure 1. Schematic diagram of DNA double strand break repair by homologous recombination. The pathway is divided into three steps, pre-synapsis (A-D), synapsis (E and F), and post-synapsis (G-I). As depicted, intact DNA (A) suffers a double strand break (B). The broken DNA ends are processed to produce single stranded 3' overhangs (C). A Rad51 nucleoprotein filament is formed on the single-stranded DNA tails with the help of accessory proteins (D). The filament is indicated at one of the DNA ends only by the dashed oval. During synapsis the Rad51 nucleoprotein filament can pair with homologous sequences in the template DNA (E). This results in joint molecule formation where the single stranded end of the broken DNA is base paired to one strand of the template (F). During post-synapsis the broken single stranded end can prime DNA synthesis to recover any DNA sequences lost (G). Branch migration, Holliday junction resolution and ligation restore two complete copies of the DNA spanning the original break (H and I).



For convenience a division into three mechanistically distinct steps can be made, although these steps are likely to occur in a coordinated fashion. Step one, the stage of homologous recombination before physical contact between the recombining partner DNAs has been established, is referred to as pre-synapsis (Figure 1, A-D). During this step the DNA ends at the break site are processed into single stranded DNA tails with 3' extensions. The identity of the exonuclease involved in this step has not yet been firmly established. Alternatively, single-stranded DNA could be generated by the combined action of a helicase and an endonuclease. Subsequently, the single-stranded DNA tail is converted into a nucleoprotein complex that can recognize homologous double-stranded DNA. The assembly of this complex, referred to as a nucleoprotein filament, requires the single stranded DNA binding protein RPA and the homologous recombination proteins Rad52 and Rad51. RPA is required to reduce the secondary structure in the single stranded DNA, while the Rad52 protein mediates the exchange of RPA for Rad51. The mature nucleoprotein complex is a helical filament containing one Rad51 monomer per three nucleotides of single stranded DNA. In step two, synapsis, the Rad51 nucleoprotein filament executes a central feature of homologous recombination, the recognition of a homologous double stranded template DNA and, once recognized, the formation of a joint molecule between the single-stranded tail of the broken DNA and undamaged template DNA (Figure 1, E-F). The joint molecule intermediate provides the substrate for DNA synthesis, requiring a DNA polymerase(s) and its accessory factors that restore the missing information during step three, post-synapsis (Figure 1, G-I). During this step, branch migration of the crossed DNA strands (Holliday junctions) allows the generation of heteroduplex DNA between the joined DNA and the structure-specific endonucleases separate the recombined molecules into duplex DNAs. Finally, the continuity of the strands is restored by a DNA ligase.

#### RAD54

The *RAD54* gene was discovered in a genetic screen for ionizing radiation hypersensitive *Saccharomyces cerevisiae* mutants (25). The gene is part of an epistasis group that also includes *RAD51* and *RAD52*. Mutants in this group are defective in homologous recombination and DSB repair. The genes are conserved in mammals although their relative contribution to recombination and repair must differ as evident by severity of the respective knockout phenotypes. Disruption of *Rad51* causes embryonic lethality, disruption of *Rad54* results in mild recombination and DNA repair defects, while no obvious phenotype is associated with *Rad52* disruption (6, 9, 14, 20, 26).

The amino acid sequence of Rad54 places the protein in the SWI2/SNF2 family of DNA-dependent ATPases that are best known for their nucleosome remodeling activity. The ATPase activity of Rad54 is specifically triggered by double-stranded DNA (18, 24). This contrasts with the ATPase activity of Rad51, which is stimulated most efficiently by single-stranded DNA (3). Thus, Rad51 and Rad54 appear to act on the different DNA partners during homologous recombination (Figure 1). Rad51 and Rad54 interact directly at the protein level (5, 10, 12, 18), although *in vivo* in mammalian cells this interaction can only be demonstrated after the induction of DNA damage (this thesis). Recently, Rad54 has been highlighted in several studies that describe mechanistic functions indicating it could play a role at many stages of homologous recombination (Figure 1).

## PRE-SYNAPTIC ROLE OF RAD54

As mentioned above, the Rad52 protein plays a critical role at the pre-synaptic stage of homologous recombination, which is not surprising given that it interacts with the major players involved in establishing the Rad51 nucleoprotein filament; Rad51, RPA and single-stranded DNA (25). A role for Rad54 at this stage did not seem obvious, because the focus of nucleoprotein filament formation is on single stranded DNA and not double stranded DNA, which triggers Rad54's ATPase activity. Rad54 does however interact with Rad51. Given that this interaction in mammalian cells requires DNA damage (this thesis), it is possible that Rad54 interacts with Rad51 in the context of the nucleoprotein filament. An obvious advantage for this interaction is to target Rad54 activity to a useful site, the homologous double stranded DNA template that will be recognized by the filament (17). However, this is not strictly speaking a role of Rad54 in pre-synapsis.

Recent experiments suggest that Rad54 actually plays an active role in pre-synapsis by stabilizing the Rad51-single stranded DNA interaction (Figure 2) (16). Rad51 single-stranded DNA filaments were less sensitive to disassociation by salt in the presence of Rad54, Rad54 increased the protection of DNA in a Rad51 double-stranded DNA filament from restriction enzyme digestion and Rad54 inhibited transfer of Rad51 from one DNA substrate to another. In addition, Rad54 increased the salt resistance of the Rad51 strand transfer reaction. It was argued that these activities require a 1:1 molar ratio of Rad54 to Rad51. This was demonstrated only for reactions with short DNA substrates. For filament stabilization on longer substrates and for stimulation of the important strand exchange reaction substoichiometric amounts of Rad54 sufficed. Given the likely possibility that Rad54 forms oligomers on DNA of up to six protomers (21), a short DNA with a few such oligomers, for instance at the ends, would appear to have stoichiometric association of Rad54 relative to Rad51 bound at one monomer per three nucleotides or base pairs. Thus, the functionally important stoichiometry of the complex remains an intriguing unanswered question.

These pre-synaptic functions of Rad54 did not require its ATPase activity as the K341R mutant, defective in ATP hydrolysis, functioned as well as wild type protein in filament stabilization (16). This is perhaps fortuitous and also raises further interesting questions. The ATPase activity of human Rad54 is very salt sensitive (24) and based on the standard ATPase conditions this seems to be true for yeast Rad54 also (18, 22). Therefore, the ATPase activity would be unable to play a role in salt stabilization of Rad51 nucleoprotein filaments at the concentrations tested. However, this salt sensitivity of Rad54 ATPase and the standard *in vitro* conditions for strand exchange are not compatible with likely *in vivo* conditions. Although the *in vitro* conditions allow for measurable reactions, their deviation from expected *in vivo* salt and temperature conditions may indicate that stabilizing elements are missing. The point was made that Rad54 increased the salt stability of the strand exchange reaction. It would be very interesting to know if the converse is also true, does the Rad51 nucleoprotein filament make the Rad54 ATPase activity more salt resistant?

## ROLE OF RAD54 DURING SYNAPSIS

During synapsis, the nucleoprotein filament forms a joint molecule between the invading single-stranded DNA and the double stranded DNA template molecule. This occurs at locations on the template homologous to the invading sequence through a rather mysterious step deterministically known as the search for homology (Figure 2). It was demonstrated several years ago that Rad54 stimulates Rad51 dependent joint molecule formation *in vitro* (18). One event that has to occur to allow joint molecule formation is a disruption of base pairing in the double stranded DNA template partner. The biochemical activities that have been described for Rad54 suggest an obvious mechanistic role at this stage of homologous recombination.

The double stranded DNA dependent ATPase activity of Rad54 is not required for the pre-synaptic function of Rad51 nucleoprotein filament stabilization. However, other activities of Rad54, such as its ability to change DNA topology, do require ATPase function (this thesis, (19, 21)). Both the yeast and human protein can introduce supercoils into DNA (this thesis, (17, 27)). The observations that these supercoils are not protein constrained, that they are both positive and negative, combined with direct imaging of Rad54 anchored supercoiled domains in plasmids suggest that Rad54 uses the energy of ATP hydrolysis to translocate along (this thesis, (17, 21, 27)). A translocating protein tracking the DNA helix will introduce supercoils if there is sufficient frictional torque to prevent it from freely rotating (15). Additional biochemical tests such as the ability to displace a triplex forming oligonucleotide and oligonucleotide length dependent ATP hydrolysis rate are consistent with Rad54 being a DNA translocating motor (11). The ATPase activity of Rad54 and its ability to induce supercoils into DNA are both stimulated by interaction with a Rad51 nucleoprotein filament (17, 27). This would have the advantage of stimulating Rad54 activity where it is needed, namely at regions of homology recognized by the Rad51 nucleoprotein filament.

A DNA translocating motor could have direct or indirect mechanistic roles in joint molecule formation. In association with a Rad51 nucleoprotein filament this could simply provide a means to move along a double-stranded DNA target in search of homologous regions. Though without further guidance, movement alone is just as likely to be away as toward a region of homology. More plausibly, a Rad54 translocating motor attached to the Rad51 filament end of a broken chromosome would induce supercoils into the template (17, 21, 27). Negative supercoiling favors unpairing of double stranded DNA, and thus joint molecule formation. Indirectly, changing DNA structure by negative or positive supercoiling will alter the affinity of DNA binding proteins, possibly resulting in the displacement of proteins that would otherwise block joint molecule formation. Alternatively, Rad54 moving along the double-stranded DNA template could displace proteins directly. Indeed these are the types of activities associated with SWI/SNF2 family members that define their mechanistic role in many processes requiring protein access to genomic DNA (4). The Rad54-Rad51 interactions that stimulate joint molecule formation require proteins from the same species indicating specific structural interactions (17). It will be interesting to determine the arrangement of these two proteins and their DNA substrates in functional complexes to see if interaction with Rad51 orients Rad54 to translocate along template DNA with a given polarity and to determine their functional stoichiometry.

## RAD54 AND CHROMATIN

In eukaryotes, all DNA transactions actually occur in the context of DNA packaged into chromatin. Since Rad54 belongs to the SWI2/SNF2 family of chromatin remodeling proteins it seemed obvious to propose that the role of Rad54 in synapsis is to alter accessibility of template DNA via chromatin remodeling. This function has recently been tested directly in a variety of *in vitro* situations. The simplest such situation involved the action of Rad54 on single positioned nucleosomes on a short piece of DNA. Rad54 was able to displace these nucleosomes from their original position and sometimes remove them from the DNA, all in an ATP dependent fashion and stimulated by interaction with a Rad51 single-stranded DNA filament (1). On nucleosomal arrays both yeast and *Drosophila* Rad54 exhibited ATPase dependent remodeling activity as evident by increased accessibility to restriction enzyme digestion. However, where it was tested, no significant nucleosomal mobilization was observed (11). The nucleosomal remodeling observed on arrays was greatly stimulated by Rad51, but this did not require homologous single-stranded DNA. Thus, nucleosomal remodeling need not be coupled to synapsis. Though these results all point to a role for Rad54 in chromatin remodeling it should be noted that Rad54 was much less efficient and effective. More protein was needed and less dramatic effects, in nucleosome remodeling, were observed than with the established chromatin remodeling factors (ACF, SWI/SNF) used as controls. The functional significance of nucleosomal remodeling by Rad54 should be clarified when the structural consequences of Rad54 action on nucleosomal arrays are determined in more detail.

However it is clear that Rad54 enables synapsis in a nucleosomal context. Though bacterial RecA, not surprisingly, was unable to perform strand exchange and joint molecule formation with a nucleosomal template DNA, eukaryotic Rad51 could do so efficiently in the presence of Rad54 (2, 11). Alexiadis and Kodanaga (2) note that chromatin is the natural substrate for DNA transactions in the nucleus and they then show that the *Drosophila* proteins worked better in a D-loop formation assay with nucleosomal DNA compared to naked DNA. This stimulation required Rad54's ATPase activity, but did not depend on superhelical tension in the target double-stranded DNA. As noted earlier, the enzymatic activity of Rad54 is optimal in conditions not expected to reflect the *in vivo* situation perhaps due to missing factors. With this in mind, it is interesting to note that the ATPase activity of yeast Rad54 was specifically heat-stabilized by the presence of nucleosomal arrays (11).

## POST-SYNAPTIC ROLE OF RAD54

All events after initial strand exchange are referred to as post-synaptic processes. These events include extension of the heteroduplex, branch migration and replication, all processes that could utilize a DNA translocating motor. Indeed, Rad54 can increase the rate and extent of heteroduplex extension *in vitro*, in an ATPase dependent manner (22). Other evidence for a post-synaptic role is the increased ATPase activity of Rad54, viewed as an indicator of translocating potential, in the presence of double stranded DNA partially coated with Rad51 (13). Rad51 bound to double strand DNA would exist as an intermediate in strand exchange after synapsis. One, often overlooked, aspect of dynamic molecular reactions is the need to disassemble protein complexes after they have done their job. Such a role has been suggested for Rad54 in post-synapsis. Presumably, Rad54 tracking along DNA would displace no longer needed recombination proteins in its path. Biochemical

evidence for this is provided by the observation that Rad54 can disassemble a Rad51 filament of double stranded DNA in an ATPase dependent manner (23). This is in contrast to the salt stabilization of Rad51 filaments on double stranded DNA demonstrated by Mazin and colleagues (16). However, it is difficult to directly compare these results as the *in vitro* assays were very different. For instance, stabilization was tested for short times (5 minutes) and the role of Rad54 ATPase functions was not directly addressed. Because Rad51 removal was more effective with a species matched Rad54 and occurred on both circular and linear DNA, this activity is not simply an indirect effect of Rad54's ability to alter DNA topology. Significant displacement of Rad51 by Rad54 did require a time course of hours, in contrast to the standard strand exchange reaction time course of a half hour or less. However, this may be explained by differences in the *in vitro* reaction conditions such as the length of homologous DNA available for strand exchange in the various *in vitro* reactions, which varied from a few tens to several thousand nucleotides.

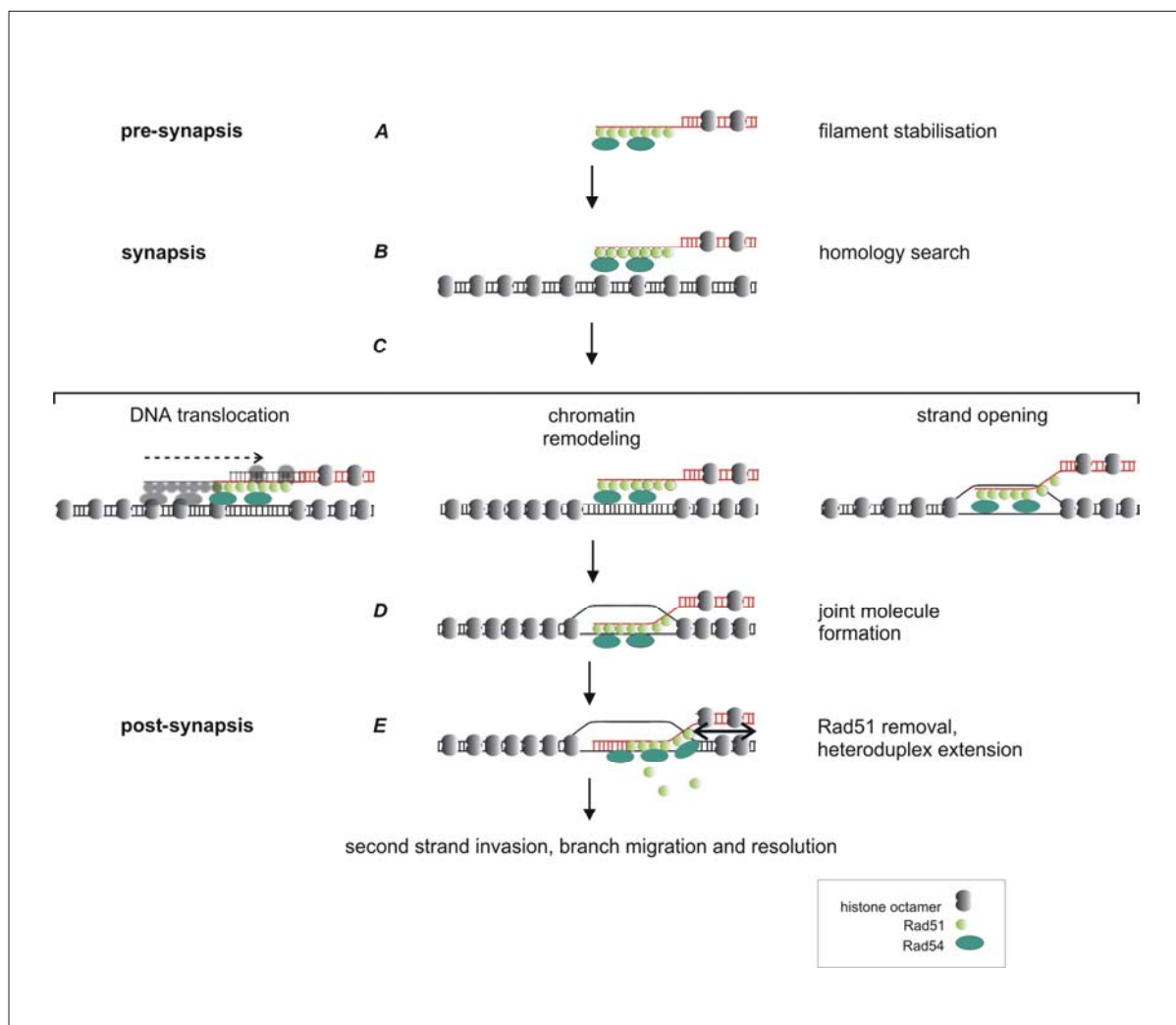


Figure 2. Rad54 functions throughout homologous recombination.

For simplicity the steps of homologous recombination described in Figure 1 are shown for one end of the broken DNA (red), and only the participation of Rad51 (light green circles), Rad54 (dark green ovals) and histone octamers (grey) is indicated. (A) In pre-synapsis Rad54 interacts with Rad51 and the Rad51 nucleoprotein

filament, where a role for stabilizing the filament has been suggested. During synapsis there are several possible mechanistic roles for Rad54. (B) Rad54 in association with the Rad51 nucleoprotein filament contacts template DNA to search for homology. (C and D) For illustrative purposes three mechanistic advantages of Rad54 translocating along template DNA are depicted separately, though they are related and likely coordinated activities. To the left, Rad54 translocating along template DNA could facilitate homology localization. Translocation is indicated by the dashed arrow. The change in position of the Rad51/Rad54/histone/broken DNA complex along the template DNA is indicated by showing its current position in color and its previous position in grey. In the center, Rad54 induced chromatin remodeling would remove histone octamers or other chromatin bound proteins and allow access to template DNA. To the right, superhelical tension induced by Rad54 would favor unpairing of the double stranded template and joint molecule formation. (E) Finally, in post-synapsis, Rad54 could help to displace Rad51 from the double stranded intermediate and thereby favor the forward reaction and eventual completion of recombinational repair.

## PERSPECTIVE

The data reviewed here identify mechanistic steps at all stages of homologous recombination that can be effected by Rad54. The well-defined *in vitro* reactions, in which Rad54 was tested, were designed to isolate one step in the recombination process. The division of homologous recombination into stages is a convenient tool for analysis, but in reality the reaction is likely to occur in a concerted and coordinated manner without regard to the cartoon models we draw to help us to understand this process. Nonetheless all of the data summarized here can be synthesized into a coherent pathway of Rad54 function throughout recombination (Figure 2). Rad54 was shown to stabilize Rad51 filaments on single stranded DNA, a pre-synaptic function. The mechanistic advantages of this would be to assemble a complex needed for further steps and to commit a single stranded DNA end to homologous recombination in favor of other fates. There is a lot of data to support a role for Rad54 in synapsis. Rad54, associated with a Rad51 single-stranded DNA filament, encounters a double-stranded DNA template and is then stimulated to hydrolyze ATP and translocate along the double-stranded DNA. This may serve one or more mechanistic functions in recombination. First, it could facilitate the localization of homology. Second, it could favor unpairing of the template double-stranded DNA and joint molecule formation. Third, it could remove inhibitory proteins bound to the template DNA. Reassuringly, given the nuclear environment in which Rad54 has evolved, Rad54 is also apparently best suited in these functions if the template DNA is packaged into nucleosomes. Finally, some activities of Rad54 would be most useful in post-synapsis. Assuming Rad54 does not disassociate from the Rad51 single-stranded DNA filament upon joint molecule formation it would then be poised to aid in post-synaptic functions, such as enhancing heteroduplex extension and possibly removing Rad51 from double stranded DNA heteroduplex intermediate. The ability to both stabilize and destabilize Rad51-DNA filaments described for different steps is not necessarily contradictory. The stabilization of filaments on single stranded DNA did not require ATP hydrolysis, whereas the destabilization of filaments on double stranded DNA did require ATP hydrolysis. The different activities identified for Rad54 can be accounted for in a progression of different molecular assemblies with specific functions needed throughout homologous recombination (Figure 2).

From the synthesis of these accumulated data interesting ideas of the coordinated mechanisms of eukaryotic recombination are rapidly emerging. Fortunately, these interesting ideas also suggest experimental approaches to test them. There is still a controversy in the literature over the relative stoichiometry of Rad51 and Rad54 needed for function (7, 16). One way to address this would be to observe functional recombination intermediates using a variety of different combinations of DNA substrates and quantify the position and amount of Rad54 in the complexes. This can be addressed by methods such as electron microscopy and scanning force microscopy imaging but will require efficient methods to prepare, identify, and analyze functional recombination intermediates. Such direct imaging can also be used to define the structural changes in nucleosomal arrays induced by Rad54 in order to understand how they would favor synapsis. Similarly, any information on the arrangement of the different proteins in joint molecules would likely provide new insight into the mechanism of this complex process. However, this is still *in vitro* analysis and the suggested mechanistic importance of Rad54 activities should also

be tested *in vivo*. It would for instance be nice to create separation of function mutants that are defective in one or a few of the defined *in vitro* activities and then see what effect they would have when introduced into cells. Progress in live cell imaging and single molecule analysis are also likely to contribute greatly to our understanding of homologous recombination (8).



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## CHAPTER FIVE

### **RAD54 COUNTERACTS DNA DOUBLE-STRAND BREAK FORMATION BY THE DNA REPLICATION INHIBITOR HYDROXY-UREA**

## ABSTRACT

We have studied the response of the mammalian homologous recombination (HR) protein Rad54 to stalled DNA replication. Previously, we have demonstrated the involvement of Rad54 in DNA double-strand break (DSB) repair and shown that the protein accumulates into nuclear foci upon treatment with DSB-inducing agents, including ionizing radiation (IR). Here we show that the mouse Rad54 protein also forms nuclear foci upon treatment with the DNA replication inhibitor hydroxy-urea (HU), which causes stalled DNA replication forks. Lack of Rad54 causes hypersensitivity of the cells to both IR and HU. Interestingly, the percentage of cells positive for Rad54 foci returns much faster the initial level in case of induction by HU compared to induction by IR, suggesting a fundamental difference in the lesion with which Rad54 is associated. We show that the absence of Rad54 leads to accumulation of DSBs following HU treatment. Although HU-induced stalled replication forks can be converted into DSBs that require repair by HR, we argue that the accumulation of HU-induced DSBs in the absence of Rad54 is not due to a defect in HR-mediated DSB repair because this process is likely to be very inefficient in the presence of HU, even in DSB-proficient cells. Possibly, a function of Rad54 is to regress stalled replication forks, thereby preventing collapse, such that they can be processed and restarted without the need for double-strand DNA breakage.

## INTRODUCTION

DNA double-strand breaks (DSBs) are a lethal type of DNA damage in cells. Even a single DSB, if left unrepaired, can cause cell death. Therefore it is extremely important for a cell to repair this type of DNA damage. Cells developed two DNA repair systems to counter the lethal threat of DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR) (see chapter one). The main difference is that NHEJ sticks the break ends together in an untemplated manner, while the latter uses the undamaged, homologous sister chromatid (or chromosome) as a template to repair the broken DNA molecule (16, 21). Most DSB repair via HR takes place in S/G2-phase, during the rest of the cell cycle NHEJ is the predominant pathway. During S-phase however, also other cellular processes take place, the most important being DNA replication. During replication, the replication fork may slow down and pause for short periods, which could result in a stalled replication fork. This can occur due to DNA lesions, nicks, and also due to collision with other proteins or protein complexes (e.g. RNA polymerases), which could block the replication fork's progress (2). Stalled forks could collapse, leading to a gap or a DSB. Stalled replication forks occur in virtually every cell during every cell cycle and recombination proteins play an important role in promoting their progression. Studies on bacteria show that recombination proteins are required to generate a structure on which replication can resume and to allow bypass of the lesion (13). The recombination proteins could also come into action when fork collapse would lead to the generation of a DSB that requires HR for its repair.

Hydroxy-urea (HU) is an agent affecting the replication processes; it inhibits ribonucleotide reductase, causing a depletion of the cell's deoxy-ribonucleoside triphosphates (dNTPs) (20). Depletion of, or even a drop (below 80%) in, a yeast cell's dNTP pools will result in replication arrest and in stalled replication forks (8). DSBs may arise in the close vicinity of the stalled forks by breaks in the leading or lagging strand templates. The replication inhibition is reversible and simply removing HU from the cells will result in re-synthesis of the dNTPs and restart of DNA replication. Studies by Lundin and colleagues (10) show that mammalian recombination proteins are important following HU-induced replication arrest. They suggest that HR is involved in repair of DSBs following stalled replication forks, using the HR-defective and *Xrcc3*-deficient hamster cell line *irs-1SF*.

Here, we investigated the importance of the recombination protein Rad54 during HU-induced replication arrest. Similar to DSB-inducing ionizing radiation (IR) treatment, treatment with the replication inhibitor HU induces the redistribution of Rad54 into discrete nuclear foci. This foci induction is of biological relevance as we, using mouse *Rad54KO* cells, show that absence of the protein leads to increased sensitivity to HU. We also show that the kinetics of Rad54 foci disappearance depends on the agent that induced them. HU-induced Rad54 foci disappear much faster than Rad54 foci formed at IR-induced DSBs. Furthermore, our data implicate Rad54 involvement in processing HU-induced abnormal replication intermediates because its absence causes the accumulation of DSBs.

## RESULTS

### HU induces nuclear Rad54 foci in mouse ES cells

Our previous studies (chapter three; (18)) showed that Rad54 foci can be induced in ES cells by treatment with IR, agents that cause DSBs (such as methyl methane sulfonate) and agents that cause interstrand DNA cross-links (e.g. cisplatin, mitomycin C). We wondered what the Rad54 response would be if instead of applying DNA damaging agents, the cells were exposed to HU, a replication inhibitor, which can indirectly cause DSBs. To visualize Rad54, we used cell line #27, which expresses a Rad54HA fusion protein under the endogenous promotor (see chapter three). The Rad54HA fusion protein is fully functional in correcting the DNA repair defect of *Rad54KO* cells and can easily be detected by immunofluorescence using an  $\alpha$ HA antibody (18). Cells treated for 24 hours with 1 mM HU displayed Rad54 foci, similar to cells fixed two hours after receiving a dose of 12 Gy of IR (Figure 1A). Mock treated cells also showed Rad54 foci (~25% of the population), but treatment with HU or IR significantly increased the number of Rad54 foci positive cells (>70%) as shown in Figure 1B. Concentrations lower than 1 mM HU also induced Rad54 foci (data not shown). To test the specificity of the  $\alpha$ HA antibody, we used both Rad54 wild type and KO cell lines (both lacking for the Rad54HA protein). This did not reveal any staining (data not shown).

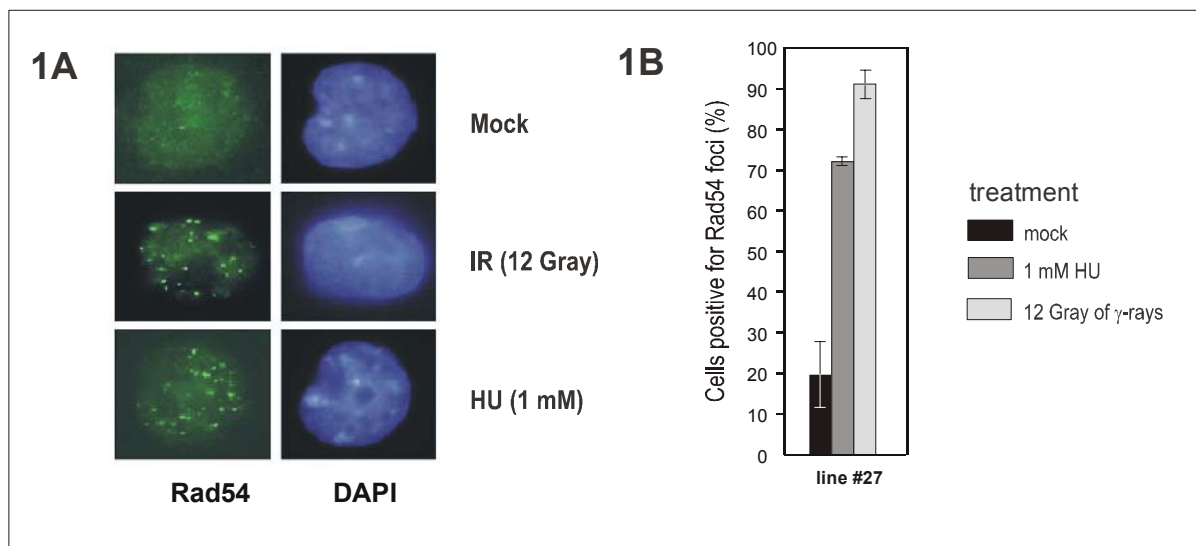


Figure 1. Treatment with HU induces Rad54 foci similar to treatment with IR. A, pictures of cells mock treated, irradiated (12 Gray) or treated with 1 mM HU and stained to detect Rad54. B, Bar graph showing the percentage of Rad54 foci positive cells in cell populations directly after treatment with HU, two hours after treatment with IR, or mock treated.

### Rad54 is required for cell survival following HU treatment

To test whether the HU-induced Rad54 foci are biologically relevant, we investigated whether the lack of Rad54 correlated with HU sensitivity in *Rad54KO* ES cells. Cells were treated with increasing doses of HU for 24 hours, after which the cells were allowed to form colonies in HU free media for five to seven days. *Rad54KO* cells were slightly more sensitive (2-3 fold) to HU, compared to wild type and Rad54 heterozygous cells (Figure 2). As treatment with HU can lead to DSBs close to the stalled replication fork (10) this observation suggested that the sensitivity was possibly caused by inefficient DSB repair due to the absence of the Rad54 protein.

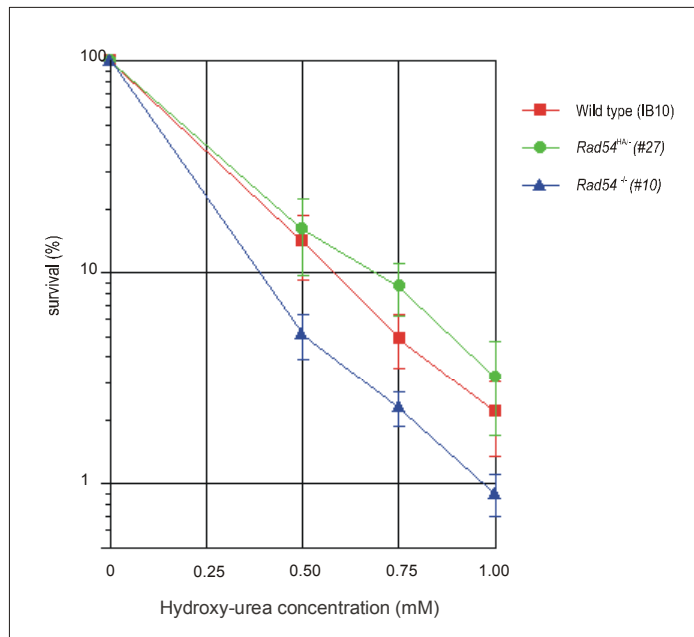


Figure 2. Rad54KO cells are sensitive to HU. The cell lines used are Rad54KO cell line, #10 (triangles), the heterozygote cell line #27 (circles) and the wild type cell line IB10 (squares). The cells were exposed to the indicated concentration of HU for 24 hours and grown for 5-7 days, the surviving colonies were fixed and counted. The Rad54KO line is 2-3 fold more sensitive compared to the wild type and heterozygous line.

### Different kinetics for HU-induced Rad54 foci compared to IR-induced Rad54 foci

As the DNA damaging agent IR directly induces DSBs in DNA, while HU indirectly induces DSBs due to processing of stalled replication forks, we investigated whether this difference in DSB induction was reflected by different foci behavior. We fixed the IR treated cells two hours after irradiation, as our previous studies (chapter three, Figure 3) showed that the maximum level of Rad54 foci positive cells is reached after two hours, and is independent of the given dose (up to 12 Gy). In addition, cells were fixed and stained for Rad54 at 6 and 24 hours after irradiation. For comparison, cells were treated with 1 mM HU for 24 hours and samples were taken at different time points, ranging from 0 to 2 hours after the HU was washed away. Upon the IR treatment Rad54 foci persisted in cells for more than 24 hours (Figure 3A). In contrast, the HU-induced Rad54 foci showed a different kinetics of disappearance. They disappeared at a much faster rate than IR-induced Rad54 foci (Figure 3A). Cells treated with 1 mM HU showed a decrease of foci positive cells from >70% down to nearly background levels within two hours. Our previous studies (chapter three; (18)) and the data shown in Figure 3B applying a range of different doses of IR (2-12 Gy) show that it takes cells much more time to return Rad54 foci levels to background, even when treated with lower IR doses. An IR dose as low as 2 Gy, still required four hours for half the cell population to lose its Rad54 foci. Thus, the majority of HU-induced Rad54 foci may be involved in a (slightly) different process from IR-induced Rad54 foci, as they disappear much quicker.

The observation that two hours after release from HU, there are still more Rad54 positive cells (33.0%) than in the mock treated population (25.3%) (Figure 3A), could be explained if some of the stalled replication forks are converted into DSBs needing HR (and thus Rad54) for repair. Consequently, these foci remain much longer.

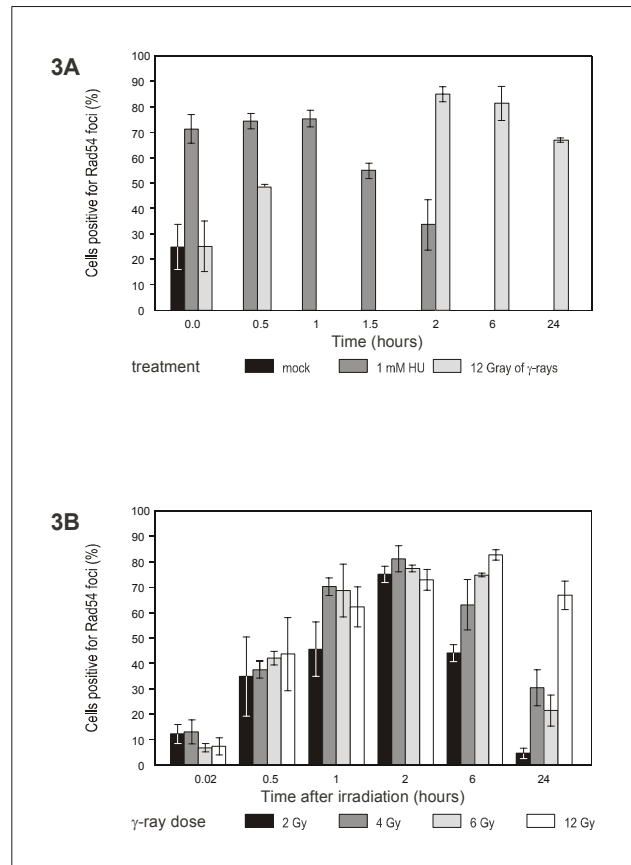


Figure 3. Different kinetics of HU and IR-induced Rad54 foci disappearance. A, At doses of 1 mM HU and 12 Gray of IR, HU-induced Rad54 foci disappear within 2 hours, while those induced by IR persist for >24 hours as reflected by the percentage of Rad54 foci positive cells within the treated populations. Cells were exposed for 24 hours to HU, then washed and then given fresh medium without HU. Samples were taken within 2 hours after refreshing the medium. Cells to be treated with IR were grown for 24 hours, then irradiated, then washed and then fresh medium was given. Samples were taken within 24 hours after irradiation. B, at lower doses of IR, IR-induced foci can disappear, but that this process takes significantly more time than the disappearance of HU-induced foci.

### Rad54 is necessary for the prevention of HU-induced DSBs

Since HU-induced Rad54 foci behave different from DSB repair-associated IR-induced Rad54 foci we hypothesized that one of Rad54's many functions (chapter four; (19)) could be to prevent the conversion of stalled replication forks into DSBs. Otherwise the HU-induced foci should persist as long as IR-induced foci. To address this issue, we performed pulsed field gel electrophoresis (PFGE) to measure the amount of DSBs, in the form of broken chromosomal DNA, induced by HU in Rad54-proficient and Rad54-deficient cell lines after exposure to different concentrations of HU for 24 hours. The amount of DSBs is much higher in the *Rad54KO* cells compared to wild type cells. Thus, absence of Rad54 in HU-treated cells leads to a higher amount of DSBs, suggesting that Rad54 is necessary for the repair and/or prevention of HU-induced DSBs (Figure 4).



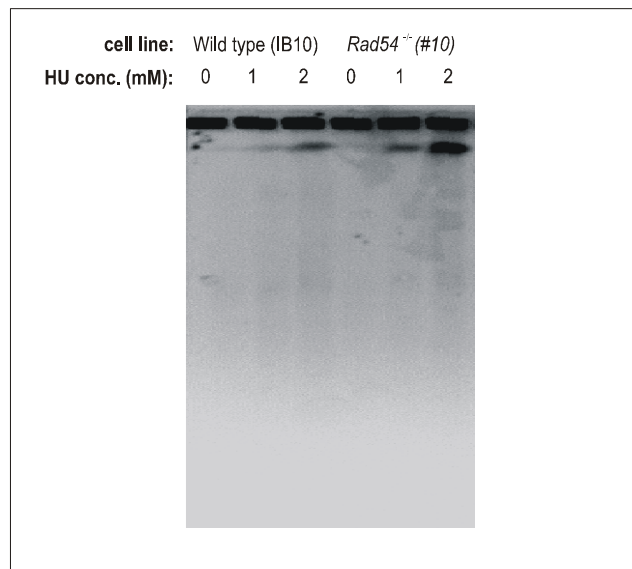


Figure 4. Absence of Rad54 increases the amount of HU-induced DSBs. Pulsed field gel electrophoretic analysis of chromosomal DNA from HU-treated cells. Treatment with HU causes DSBs in both Rad54 proficient (IB10) and *Rad54KO* (#10) cells in a concentration dependent manner. However, the amount of DSBs is significantly higher in the *Rad54KO* cell line.

## DISCUSSION

Replication is one of the important processes in a cell's life and though it is known that prokaryotic recombination proteins have a share in this (9, 11, 12) most research on eukaryotic recombination proteins is focused on their role in meiotic recombination and in DSB repair (for a review see Symington, (16)). Recent reports however, indicate the importance of these proteins in eukaryotic DNA replication. Segurado and co-workers found that *Schizosaccaromyces pombe* yeast strains deficient for the Rad51 and Rad54 homologues rhp51 and rhp54, respectively, activate their replication origins very inefficiently and accumulate abnormal replication intermediates (15). The latter suggests that rhp54 has a direct role in replication, by promoting replication initiation and prevention and/or processing of abnormal replication intermediates. None of this type of data has been found in mammalian cells, but Lundin and colleagues found that the HR-deficient *Xrcc3* mutant hamster cells (irs-1SF) are sensitive to HU, which stalls replication forks due to dNTP depletion and are sensitive to thymidine, which slows fork progression (10).

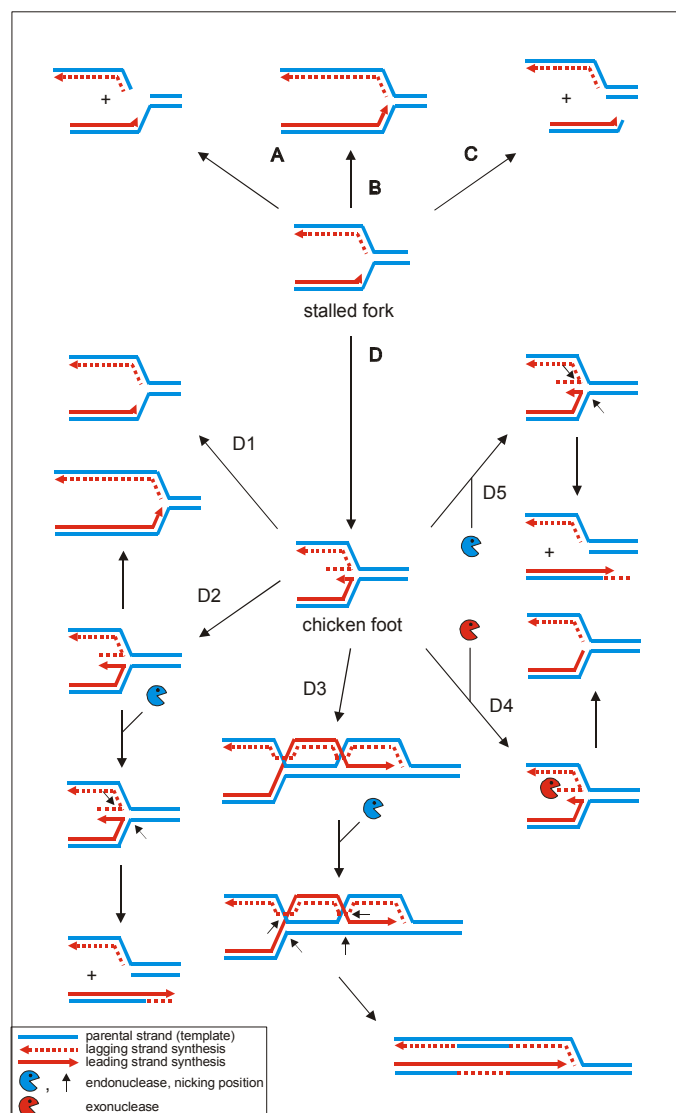


Figure 5. Possibilities to resolve stalled replication forks. An overview of the cell's possibilities to deal with a stalled replication fork. The stalled fork could break (A, C), resume replication (B) or be converted into a chicken foot (D), which also offers multiple options to solve the structure (D1-D5). For an extensive explanation see text (Discussion).

For our studies of the response of HR proteins to stalled replication forks we analyzed the effect of HU on the Rad54 protein and in Rad54-defective cells. Cells have different options to deal with a stalled replication fork as shown in Figure 5. Stalled replication forks can break either at the lagging or leading strand templates (Figure 5A, 5C, respectively) resulting in a DSB. DSB formation likely involves the activity of structure-specific endonuclease, such as Mus81/Eme1 (6, 7). These DSBs could be repaired via HR, a process in which Rad54 is involved (chapter four, (19)). Instead of breaking, the replication fork can also be reinitiated and resume as shown in Figure 5B in case normal conditions are restored. Studies in prokaryotes revealed that stalled forks can also be converted into so-called chicken foot structures (Figure 5D, see chapter one, section five and references therein for a more extensive explanation). These structures arise by regression of the replication fork during which the newly synthesized daughter strands anneal to each other. Replication forks in HU-treated cells undergoing this option have to re-regress the chicken foot into a normal fork structure prior to resumption of DNA synthesis (Figure 5D1). Equally (Figure 5D2), a cell could choose to first replicate on the annealed daughter strands until they are of approximately similar length. Thereafter, the chicken foot can be re-regressed into a replication fork (Figure 5D2-upper). However, it is also possible that the structure is recognized as a recombination intermediate and therefore will be processed by Holliday junction specific nucleases (Figure 5D2-lower). The cleaved molecule will then be repaired via HR. The chicken foot itself can also be recognized by the nucleases (Figure 5D5), cleaved and processed by HR. Furthermore, this intermediate could be acted upon by HR proteins directly (Figure 5D3). This can lead to strand invasion and exchange of different parts of the parental and daughter strands. Recently, evidence has been found for the existence of DNA replication-dependent X-shaped DNA molecules between sister chromatids (1). The recombination processes will end with cleavage of the different junctions and after sealing of the nicks replication can continue. This intra-molecular recombination can also start from the initial intermediate shown in Figure 5D2. Another possibility is that exonucleases degrade the daughter strands rendering a normal fork structure from where replication can be re-initiated (Figure 5D4).

Our data show that absence of HR protein Rad54 leads to increased sensitivity to HU. HU indirectly causes cytotoxic DSBs (Figure 4) due to processing of stalled replication forks. This could explain both the induction of Rad54 foci in wild type cells (Figure 1) and the sensitivity of *Rad54KO* cells to HU (Figure 2). The increased amount of DSBs in the *Rad54KO* cells (Figure 4) could reflect the inability of the mutant cells to repair DSBs. However, our data also show that HU-induced Rad54 foci are shorter lived than IR-induced Rad54 foci, while both treatments cause DSBs (Figure 3). The difference in kinetics could (at least partially) be explained by the different nature of the induced DSBs. IR-induced DSBs break a DNA molecule in two parts with two double-stranded ends around the break (see Figure 6, left panel). Both ends need to engage a template molecule in order to be repaired by HR. HU-induced DSBs originating from stalled forks or chicken feet only have one part with a double-stranded end, the other broken part does not contain a double-stranded end but might contain a single-stranded gap (see Figure 6, right panel). These one-ended breaks are much more simple to repair by HR than the two-ended breaks. Thus, HU-induced DSBs might be less complex and possibly easier and faster to repair than the IR-induced DSBs. This could explain the observation that HU-induced Rad54 foci disappear faster than IR-induced foci (Figure 3).

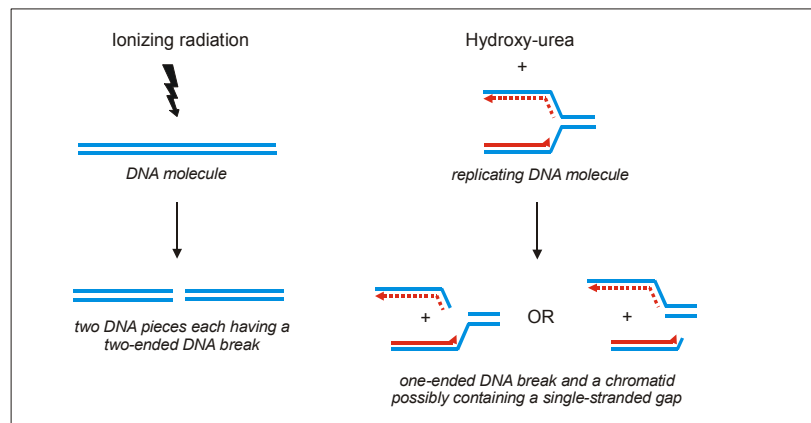


Figure 6. Differences in the nature of DSBs induced by IR and HU. IR (left panel) induces 'complex' breaks: a DNA molecule hit by IR will break in two parts, each of which will have a double-stranded end (two-ended DNA break). For repair via HR, both pieces need to find and engage a template molecule. Alternatively, NHEJ can simply ligate the ends together after appropriate processing. HU-induced (right panel) DSBs are close to the (stalled) replication fork and originate from a break in the leading or lagging strand template. The result in a one-ended DNA break and a chromatid possibly containing a single-stranded gap (3).

A more speculative explanation for the difference between the kinetics of HU and IR-induced Rad54 foci disappearance could be a dual role of Rad54 itself, rather than a difference in the nature of the DSBs. In this case Rad54, besides its involvement in DSB repair, could be a possible involvement in the replication process itself. Absence of Rhp54 in *S. pombe* leads to inefficient activation of replication origins (15) and it could be that Rad54 has a role in re-initiation of the stalled replication fork. Besides inefficient origin activation Segurado and colleagues also observed an accumulation of abnormal replication intermediates in the *rhp54Δ* mutant (15). Thus, it is possible that mammalian Rad54 may be involved in processing or preventing abnormal replication intermediates. The mild phenotype of *Rad54KO* mice suggests that in unchallenged cells this role of Rad54, if any, would be minor (4, 5). However, Rad54's involvement could be revealed in cells in which replication is stressed by HU. The consequence for cells lacking Rad54 and treated with a replication inhibitor such as HU may be much bigger in terms of replication restart and of processing (or prevention) abnormal replication structures than in terms of repairing the DSBs, which are merely a side product of replication inhibition. Data supporting a role for mammalian Rad54 in processing stalled replication forks is provided by the PFGE experiments reported here (Figure 4). Rad54-proficient cells show much less DSBs when treated for 24 hours with 2 mM HU than Rad54-deficient cells. This is most likely not a consequence of the DSB repair defect of *Rad54KO* cells. Cells were harvested immediately after HU exposure, and were not allowed to recuperate in HU-free medium. Thus, even though cells were either Rad54-proficient or Rad54-deficient and therefore HR-proficient or deficient, respectively, they never had the time to repair the DSBs before they were killed and analyzed by PFGE, since it is unlikely that HR can take place when dNTPs, required for HR-mediated DSB repair, are depleted due to the HU exposure. However, even though the repair abilities were not a relevant factor, the Rad54-proficient cells still contained considerably less DSBs than the Rad54-deficient cells. Therefore the most reasonable explanation for this observation would be that the presence of Rad54 under these HU-treated and dNTP-starved conditions would avoid that stalled forks and other abnormal replication structures would be converted into DSBs. Lack of Rad54 would lead to

collapse of the replication fork and subsequent processing of the abnormal replication structure could result in a DSB, which is reflected in the increased amount of DSBs in the *Rad54KO* ES cells. This would also explain why Rad54 foci positive cells disappear much faster in a HU-treated than in an IR-treated cell population (Figure 3); HU-induced foci reflect Rad54 involvement in replication-associated processes rather than in DSB repair processes. A function in processing (abnormal) replication structures for vertebrate Rad54 proteins could also explain why Takata and co-workers found increased levels of spontaneous strand breaks in Rad54 deficient chicken cells (17). The absence of Rad54 not only comprises the inability to repair DSBs arising from stalled forks, but also increase the number of stalled forks and abnormal replication structures ending up in a DSB. As recombination proteins can both prevent DSB formation (during replication processes) and repair DSBs, and as long as it is better to prevent than to cure, it is of interest to determine whether the main function of recombination proteins is to avoid DSB generation by ensuring proper replication, rather than in repairing DSBs via recombination.

## EXPERIMENTAL PROCEDURES

### Cell culture

Mouse ES cells were cultured on a 1:1 mix of DMEM and BRL (Buffalo Rat Liver) cell cultured medium, enriched with 10% fetal calf serum, 0.1 M non-essential amino acids, 50 $\mu$ M  $\beta$ -mercaptoethanol and 500 U/ml leukemia inhibitor factor. Cell cultures were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere humidified to 95-100%. The cell lines tested were wild-type IB10, *Rad54KO* #10, and the *Rad54* heterozygous lines #18 and #27. The latter contains a HA-tagged *Rad54* knock-in construct under the endogenous promoter (see chapter three).

### Cell survival assays

Various dilutions of a cell suspension were seeded onto gelatinized 60 mm dishes, pre-incubated in medium for four hours (at 37°C) after which HU was added to the indicated concentrations (see Figure 2). Cells were exposed to HU for 24 hours, after which the cells were washed in phosphate buffered saline (PBS) and incubated for 5-7 days, fixed in methanol and stained in coomassie brilliant blue. The sensitivity was determined by comparing the colony forming ability of ES cells. Each experiment was performed in triplicate and error bars in Figure 2 are standard errors of the mean based on two independent experiments.

### Immunofluorescence

ES cells were plated onto gelatinized glass slides at sub-confluent densities. Cells were given fresh medium + 1 mM HU (HU treated cells) or medium + PBS (mock treated cells). After 24 hours of incubation (at 37°C) cells were washed twice in PBS (or irradiated using a <sup>137</sup>Cs source, and then washed with PBS), and supplied with fresh medium. Samples were collected at indicated time points, washed twice in PBS and fixed with 2% para-formaldehyde + 0.2% Triton-X100 in PBS for 10 minutes at 20°C. Permeabilization continued after fixation using 0.2% Triton-X100 in PBS for an additional 35 minutes at 20°C. Samples were washed twice in incubation buffer (0.5% BSA + 0.05% tween-20 in PBS at 20°C) and incubated for one hour (20°C) with primary (Rat $\alpha$ HA antibody, Roche Diagnostics GMBH clone 3F10) or secondary antibody (Goat $\alpha$ Rat-Alexa488 conjugated, Molecular Probes) in incubation buffer. Samples were washed five times with incubation buffer in between the incubations and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in Vectashield<sup>R</sup> mounting medium. Cells were considered to be positive for Rad54 foci when containing  $\geq 3$  foci/nucleus,  $\geq 200$  cells/sample were counted. Error bars in Figures 1B and 3A show the standard error of the mean based on three independent experiments. Results shown in Figure 3B are based on two independent experiments, and the conditions for Figure 3B are as described in chapter three.

### Pulsed Field Gel Electrophoresis

Sub-confluent cultures of ES cells were treated with HU for 24 hours. Cells in the plates were harvested by trypsinization, counted and 10<sup>6</sup> cells were incorporated into each agarose insert. The agarose inserts were incubated in lysis buffer (100 mM EDTA, 1% sodium lauryl sarcosyne, 0.2% sodium deoxycholate, 1 mg/ml proteinase K) at 50°C for 48 hours and thereafter washed four times in TE buffer (10 mM Tris-HCl-pH 8.0, 1 mM EDTA-pH8.0). Pulsed field gel electrophoresis occurred for 23 hours at 13°C in 250 mM TBE using the Biometra Rotaphor apparatus, essentially as described (14), with the following parameters: Voltage-180V to 120V log; angle from

120° to 110° linear; interval 30s to 5s log. The gel was subsequently stained with ethidium bromide and analyzed.

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## SUMMARY

Chromosomes are the carriers of our genome. All the information for a cell's survival and propagation is stored there in the base sequence of the DNA. Unfortunately, our DNA is under continuous attack from DNA damaging agents, of which some are produced during a cell's own metabolic processes, while others may be of exogenous origin. DNA damage leads to mutations if not (or incorrectly) repaired and, depending on the nature of the mutation, can lead to cancer or other diseases. Our cells suffers 10,000-1,000,000 DNA lesions a day, but still many of us do not develop cancer or, if so, at a relatively late age. This illustrates the importance of the various DNA repair mechanisms in our body: without it we would not be able to survive for long.

The research described in this thesis is about the response of proteins involved in homologous recombination, one of the DNA repair pathways, to DNA damage in mammalian cells. Recombinational repair is involved in the repair of a special class of DNA lesions: double strand breaks (DSBs) and interstrand cross-links (ICLs). These are a very toxic class of lesions, i.e. even one DSB could be lethal to a cell (if left unrepaired). DSBs can also be repaired by another pathway, called non-homologous end-joining. The critical differences between end-joining and recombination is that during end-joining the broken ends of a DNA molecule are stuck together, which is not necessarily error-free. Homologous recombination on the other hand, uses an undamaged, identical piece of DNA as a template, makes a copy of it in order to repair the damaged molecule and is error-free. However, this is not an easy task, as the template, usually the sister chromatid or homologous chromosome, must be found. Then the broken molecule must pair and one (or both) of the recessed ends must invade the template molecule. These steps, named homologous pairing and strand exchange/invasion are considered to be the critical steps of homologous recombination. Key proteins involved in these processes are the in **chapter one** described Rad52 group of proteins, which includes Rad51 and Rad54, the central proteins of this thesis. Equally, the nature and importance of DNA lesions, the various DNA repair pathways and the two other functions of recombination are described in this introductory chapter.

To study the proper functioning of Rad51 after DNA damage, we made use of one particular feature of the protein which is described in **chapter two**: it redistributes into nuclear foci as a response to treatment with DNA damaging agents that cause DSBs and ICLs. Since Rad52 and the Rad51 paralogues XRCC2 and XRCC3 are also involved in recombinational repair we screened the respective mutant cell lines to assess the hierarchy and order of these proteins relative to Rad51. As the XRCC2 and XRCC3 mutants were unable to form Rad51 foci in response to induced DNA damage, we concluded that the XRCC2 and XRCC3 proteins are essential for proper Rad51 functioning after DSB and ICL induction. A Rad52KO mutation did not lead to ablation of Rad51 foci, but using a cell line expressing a Rad52GFP construct we could demonstrate that both Rad51 and Rad52 form damage inducible foci and partially co-localize. Thus, though Rad52 is involved, it is not essential for Rad51-associated DSB repair in mammalian cells. We also screened other mutant cell lines, known for their sensitivity for DSB or ICL inducing agents to detect whether the gene products they were mutated in were essential for Rad51-associated repair. The Snm1 mutant and V-H4 cell line were able to form Rad51 foci, but the VC-8 cell line

was unable to form foci. Complementation studies revealed that the mutation involved the breast cancer associated gene Brca2.

We also looked for interactions between Rad51 and Rad54, as described in **chapter three**. We found that Rad54 also forms foci as a response to DSB inducing ionizing radiation (IR). In addition, we tested different types of DNA damaging agents to see which ones could induce Rad51 and Rad54 foci. Not only do these damage-inducible foci co-localize, but the proteins do physically interact, which we could only detect after inducing DSBs. We provided evidence that DNA damage-induced Rad51 foci are less stable in the absence of Rad54. From results of a topological assay we conclude that Rad54 is capable of introducing supercoiling in dsDNA by translocating along it, which could be important for the formation or stabilization of Rad51-mediated joint molecule formation.

The mechanistic and biochemical functions of Rad54 are further discussed in **chapter four**. In this chapter, our own results and recent biochemical data of others are put in perspective and argue that Rad54 is a much more versatile protein than previously thought. Originally considered as an accessory protein whose mechanistic functions had not been properly clarified we discuss that it could have important functions throughout the three main stages of recombination; during pre-synapsis, when it stabilizes Rad51 nucleoprotein filaments, during joint formation or synapsis, and post-synapsis when it could remove the recombination proteins after the reaction.

DSBs can be formed by treating cells with DNA damaging agents. However, DSBs also occur during DNA replication, another process in which homologous recombination is involved (see chapter one, section 1.5). The DSBs could occur in the vicinity of stalled replication forks and we investigated this in closer detail in **chapter five**. Using hydroxy-urea (HU), a replication inhibitor, and looking for Rad54 foci formation as an indicator for DNA damage, we found that Rad54 foci are formed indeed when replication is stalled. Formation of these foci was biologically relevant, as we observed that Rad54KO cells are sensitive to HU. However, the number of foci positive cells disappears much faster in HU-treated cell populations than in IR-treated populations, indicating that the HU-induced foci probably reflect a process different from the usual DSB repair. Although we found accumulation of HU-induced DSBs in the absence of Rad54, we think that it is not due to a defect in HR-mediated DSB repair because this process is likely to be very inefficient in the presence of HU, even in DSB-proficient cells. Possibly, a function of Rad54 is to regress stalled replication forks, thereby preventing collapse, such that they can be processed and restarted without the need for double-strand breakage.

## SAMENVATTING

Chromosomen zijn de dragers van ons erfelijk materiaal. Alle informatie die onze lichaamscellen nodig hebben om te leven, te functioneren als cel en als onderdeel in een bepaald lichaamsdeel en over te dragen op een volgende generatie is hierin opgeslagen. Het is zo belangrijk, dat de cel van elk chromosoom twee kopieën heeft. Chromosomen zijn, onder andere opgebouwd uit DNA, waarin de erfelijke eigenschappen zijn opgeslagen in een specifieke volgorde van bouwstenen. Het DNA staat constant bloot aan beschadiging. Zo vinden er elke dag tussen de 10.000 en 1.000.000 DNA beschadigingen in onze cellen plaats als gevolg van blootstelling aan schadelijke stoffen, maar ook als gevolg van biologische processen in ons lichaam waarbij eveneens schadelijke stoffen ontstaan. DNA schade moet dus elke dag op grote schaal gerepareerd worden en ook nog nauwkeurig: ongerepareerde of onnauwkeurig gerepareerde schade leidt tot veranderingen in ons DNA die we mutaties noemen. Mutaties staan aan de basis van kanker, een van de meest verspreide ziektes in de moderne wereld. Hoe belangrijk DNA herstel is voor ons lichaam blijkt wel uit het onderzoek naar DNA herstel: vele mutaties die DNA herstel mechanismen aantasten zorgen al voor dood in het embryonale stadium en andere, minder ernstige mutaties hebben kanker of andere symptomen tot gevolg. Zonder DNA herstel mechanismen zouden wij waarschijnlijk een minder lang en minder prettig leven hebben.

Dit proefschrift beschrijft mijn studie over de reactie van de recombinatie eiwitten, Rad51 en Rad54, op DNA schade in zoogdiercellen. Homologe recombinatie is een DNA herstelmechanisme dat dubbelstrengsbreuken (DSB) en kruisverbindingen in het DNA herstelt. Het zijn zeer schadelijke types DNA schade, waarbij een laesie in een cel al de dood tot gevolg kan hebben voor deze cel. Deze types DNA schade worden onder meer veroorzaakt in de kliniek tijdens röntgen en chemotherapie, want de bedoeling van deze therapieën is om zoveel schade aan het DNA van tumorcellen toe te dienen dat ze sterven, maar de naburige gezonde cellen niet. Zo blijkt dus dat het onderzoek naar DNA herstel niet alleen belangrijk is voor het begrijpen van het ontstaan van kanker, maar ook voor het genezen ervan.

**Hoofdstuk één** geeft een overzicht weer van het belang van DNA herstel, de niveaus van onderzoek, de types DNA schade, de bijbehorende herstelmechanismen en andere functies van homologe recombinatie, een bepaald soort DNA schade herstelmechanisme dat centraal staat in dit proefschrift. Ook wordt daar dieper ingegaan op het herstel van DSBs, waar behalve homologe recombinatie ook een ander, minder precies herstelmechanisme beschikbaar voor is. Homologe recombinatie, een nauwkeurig herstelmechanisme, maakt gebruik van een onbeschadigd, identiek stuk DNA (de homoloog) om het beschadigde stuk te repareren. Hierbij moet, na vaststellen van de schade en het voorbereiden van de beschadigde stukken, eerst het identieke stuk DNA worden opgespoord. Daarna wordt het ontbrekende of beschadigde stuk gekopieerd van de homoloog, waarna beide DNA moleculen weer uit elkaar gaan. De recombinatieherstel eiwitten Rad51 en Rad54 spelen een rol bij het opsporen, kopiëren en uit elkaar gaan van het gebroken DNA molecuul en zijn homoloog.

Als men met behulp van een microscoop het gedrag van deze eiwitten volgt, dan kan men zien dat, na het toedienen van DNA schade (DSBs, kruisverbindingen) deze eiwitten in de celkern samenklonteren tot foci. Van deze eigenschap van Rad51 heb

ik gebruik gemaakt door DNA schade gevoelige mutanten te testen op hun mogelijkheid om toegediende schade te herstellen door middel van homologe recombinatie. Het voordeel is dat men niet alleen het effect van bekende mutaties op recombinatieherstel kan bekijken, maar ook dat van onbekende. Op die manier is ook gebleken dat de mutatie in de stralingsgevoelige hamstermutant VC-8 het borstkankergen2 (BRCA2) aantast. Dit staat allemaal beschreven in **hoofdstuk twee**.

Ook in **hoofdstuk drie** is gebruik gemaakt van de focusformatie van Rad51, in dit geval om het functioneren van het andere recombinatie-eiwit, Rad54, onder de loep te nemen. Het laatste vormt ook foci in antwoord op DNA schade en de focusformatie van beide eiwitten blijkt inderdaad specifiek te zijn voor de typen DNA schade waarvoor recombinatieherstel nodig is. Ook is Rad54 vrij belangrijk voor Rad51 focusformatie: de Rad54KO mutant cellen hebben duidelijk moeite met het vormen van stabiele Rad51 foci in reactie op ioniserende straling. Het Rad54 eiwit blijkt het makelijker te maken voor dubbelstrengs DNA, waarbij beide strengen om elkaar heen zijn gewonden, om lokaal te ontwinden, waardoor er een opening in het dubbelstrengs DNA ontstaat. Dit zou een stabiliserende ofwel stimulerende rol kunnen hebben bij het vormen van de zogenaamde joint molecule (JM), een structuur waarbij het gebroken DNA molecuul het intacte, homologe DNA binnenkomt om een kopie te maken voor het herstel.

In **hoofdstuk vier** worden deze bevindingen, samen met recente resultaten over Rad54 van andere onderzoeksgroepen besproken en in perspectief geplaatst.

In **hoofdstuk vijf** wordt weer gebruik gemaakt van de focusformatie van recombinatie-eiwitten als gevolg van DNA schade. In dit geval is gebruik gemaakt van Rad54 als indicatoreiwit. Echter, in tegenstelling tot de vorige hoofdstukken wordt er geen directe DNA schade in de cellen geïnduceerd. Dit keer wordt een DNA replicatierepeller, genaamd hydroxy-ureum (HU), gebruikt. Zoals uitgelegd in hoofdstuk een (sectie 1.5) spelen recombinatie-eiwitten namelijk ook een rol in de bacteriële DNA replicatie. In zoogdiercellen wordt een soortgelijke rol vermoed, maar harde bewijzen zijn er vooralsnog niet. HU stopt de DNA replicatieprocessen en toediening van dit stofje zet cellen aan tot Rad54 focusformatie. Focusformatie kan geïnduceerd zijn omdat het ontstaan van DSBs een neveneffect is van HU behandeling. DSBs ontstaan vlakbij de plaats waar de DNA replicatie gestopt is. De Rad54KO mutant cellen bevatten inderdaad significant meer DSBs na HU behandeling dan normale cellen. Echter, onder de experimentele condities is DSB herstel moeilijk uit te voeren en dan is recombinatieherstel capabele cel niet in het voordeel op een hersteldeficiënte cel. Dus de opeenhoping van DSBs in de mutant hoeft geen gevolg te zijn van het defect in DSB herstel. Bovendien blijken in HU behandelde cellen de geïnduceerde Rad54 foci veel sneller te verdwijnen dan in bestraalde cellen, zodra de cellen de kans krijgen om de schade te herstellen. Waarschijnlijk zijn de HU geïnduceerde foci gerelateerd aan de stopgezette replicatie, terwijl de stralingsgeïnduceerde foci gerelateerd zijn aan het herstel van de ontstane DSBs. Een replicatiegerelateerde functie van Rad54 zou kunnen liggen in het beschermen of verwerken van de stopgezette replicatie waardoor er minder DSBs ontstaan.

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## CURRICULUM VITAE

T. L. Raoul Tan was born in Amsterdam, the Netherlands. In 1992, he graduated from secondary school (Gymnasium) "Hermann Wesselink College", in Amstelveen, the Netherlands. Thereafter, he started his undergraduate studies (Biology) at Leiden University and spent his third and fourth year in France (Institut J. Monod and Université Paris Diderot) and United Kingdom (Cancer Research UK-Clare Hall Laboratories and Oxford University) as part of his undergraduate training. He obtained his masters degree at Leiden University in 1997 and started his PhD studies on DNA repair at the Erasmus University Rotterdam under supervision of Professors J. Hoeijmakers and R. Kanaar. In 2003 he was employed for a short period as European Program Manager at the Science Next Wave department of Science International-American Association for the Advancement of Science (AAAS) and returned to finish his PhD thesis in Rotterdam.

During his PhD studies he co-founded the now annually returning PhD student conference for the PhD students from the British Cancer Research UK and his Dutch graduate school (Medical Genetics Centre). He was also international correspondent and treasurer of the national Dutch PhD student council (Promovendi Netwerk Nederland) and the first president of the European council for postgraduate students and junior researchers (Eurodoc). As such, he had an important role in the foundation of Eurodoc.

Currently he is employed by the Japanese Siebold Foundation for a joint project with the Erasmus Medical Centre concerning the integration of new scientific developments in information and communication technology.

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Raoul