Roles of Homologous Recombination in Processing DNA Lesions

Cover picture; Tangled DNA fibres with immunolabelled sites of replication.

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Roles of Homologous Recombination in Processing DNA Lesions

De role van homologe recombinatie in het verwerken van DNA lesies

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In Memory of Maria

Contents

Scope of the Thesis

The abundance of DNA damaging agents poses a constant threat for genome stability. Therefore, cells have evolved multiple mechanisms to repair their DNA. The variety in possible DNA lesions that can occur require specified repair mechanism with the ability to remove particular types of lesions. For example, nucleotide excision repair removes lesions generated by UV-light whereas homologous recombination repairs DNA double-stranded breaks induced by ionising radiation. However, the same type of DNA lesion can be present in the various DNA contexts, resulting in difficulties to detect the damage by proteins of a specified repair system. For example, the UV-light-induced lesion generated at the site of replication fork might not be recognised by nucleotide excision repair proteins because of the DNA structure in which the damage is present, i.e. in double-stranded versus single-stranded DNA. Thus in this case, modification of DNA organisation around the lesion, required for damage recognition, can be performed by proteins with the ability to change DNA conformation. Some of the proteins of homologous recombination, such as Rad54, have the ability to alter DNA topology, therefore they could act upon DNA lesions induced by UV-light at sites of replication forks. Resulting modification of DNA arrangement neighbouring the lesion allows damage detection and subsequent removal by nucleotide excision repair proteins. This thesis describes the cellular behaviour of the mammalian DNA double-stranded break repair protein, Rad54 upon induction of DNA lesions by UV-light. Additionally, the effect on cellular metabolism of inactivation of the Rad54 protein in nucleotide excision repair-deficient cells is discussed. In addition to this study, biochemical and genetic analysis of recently identified *Rad54* paralogue, *Rad54B*, is included in the thesis. Finally, the consequences of incorrect DNA damage repair, reflected by severe clinical cases, are discussed. **Chapter** 1 provides an overview of mechanisms of replication and homologous recombination as well as presents the established evidence for the possible roles of homologous recombination in supporting replication in both, prokaryotic and eukaryotic cells. **Chapter 2** describes the analysis of the functionality of a *Rad54* knockin system generated in both nucleotide excision repair -proficient and -deficient mouse embryonic stem cells. **Chapter 3** examines the difference in nuclear distribution of the Rad54 protein induced by either ionising radiation or UV-light. Furthermore, the discrepancy in Rad54 foci formation between nucleotide excision repair-proficient and -deficient effect cells in response to UV-light is presented. Finally, UV-light-induced alterations of cell cycle progression of both cell lines are discussed. **Chapter 4** demonstrates the effect of inactivation of the Rad54 protein in nucleotide excision repair-deficient cells on the survival, accumulation of structural chromosomal aberrations and the replication restart upon UV-light treatment of these cells. **Chapter 5** presents an analysis of the biochemical properties of the mammalian *Rad54* paralogue, *Rad54B*. Additionally, the examination of the sensitivity to DNA-damaging agents of cells and mice deficient in these genes is included. **Chapter 6** provides an overview of the possible consequences of malfunction of DNA repair pathways, which may result in chromosomal rearrangements including translocations. Moreover, diagnostic and prognostic applications of specific translocations are considered.

CHAPTER I

Role of Homologous Recombination in Restart of Replication Examples from Bacteria and Eukaryotes

Manuscript in preparation

ABSTRACT

The abundance of factors with the potential to induce DNA damage poses cells with the risk of losing their genomic integrity. Additionally, the duplication of genomic DNA, crucial for division of most types of cells, provides a further challenge to the integrity of the genome. Specific repair systems that guard genomic integrity have been revealed in both prokaryotic and eukaryotic cells. Recently, the importance of the interplay between some of these DNA repair systems and the DNA replication for cell survival has been implicated in all domains of life.

Replication in bacteria

In bacteria, DNA replication of the single chromosomal copy is initiated from a unique DNA replication origin, *oriC* [1, 2]. Bacterial origins of replication possess at least two conserved DNA elements: the repeats of nine base pair consensus sequences termed the DnaA boxes and the regions that are highly AT rich [3-5]. The DnaA boxes are target sequences for DnaA protein (Table 1) that can bend DNA in an ATP-dependent manner [6, 7]. A key step in replication initiation at origins is the binding of DnaA protein to DnaA boxes and subsequent unwinding of the DNA duplex. The single-stranded (ss) DNA formed in this way is stabilized by a ssDNA binding protein (SSB) [8]. Once the 'melted' origin–DnaA complex has formed, the replicative helicase DnaB can be loaded. This reaction requires another protein, DnaC, which interacts with and facilitates loading of the helicase DnaB onto the ssDNA region of the melted origin [9, 10]. Subsequently, the primase DnaG synthesises a RNA primer [11-13]. Next, a subunit of DNA polymerase III (Pol III) holoenzyme, the β clamp replication processivity factor, encircles the RNA-DNA heteroduplex and recruits the replicative Pol III [11-16]. Once initiated, replication of the bacterial chromosome proceeds very fast, up to 1000 nucleotides/sec, which is approximately 20 times faster than the speed of replication in eukaryotes [17, 18].

Homologous recombination in bacteria

Homologous recombination (HR) is a process involved in ssDNA gap and DNA double-stranded breaks (DSBs) repair (Figure 1). SsDNA gap repair begins when the RecQ helicase unwinds DNA at the gap and the RecJ 5' to 3' nuclease processes the unwound DNA (Figure 1A) [19, 20]. Subsequently, the gaped DNA is recognised by the RecFOR proteins, which bind to the 5' dsDNA-ssDNA edge and facilitate nucleation of RecA on the ssDNA [21]. Additionally, the RecFOR proteins, which primary function is ssDNA gap repair, provide an alternative way to repair DSBs in case of inactivation of RecBCD (discussed below). Next, the RecA filament searches for a homologous sequence on a dsDNA template and invades it, forming a D-loop. Then, the DNA helicase RuvAB complex or the RecG helicase extends the region of DNA heteroduplex by branch-migrating the crossover point. Finally, RuvC protein, which is a part of a complex with the RuvAB proteins recognises and cleaves the formed Holliday junctions (HJs) resulting in resolution of the joint molecule [22].

Alternatively, when a DSB has occurred, linear duplex DNA is processed at the DSB to produce the 3' ssDNA overhangs, which are subsequently coated by RecA protein (Figure 1B). Resection of the ends involves both helicase and endonuclease activity of the RecBCD complex [22]. The nuclease activity is regulated by the *cis*-acting element Chi (χ)-sequence [23, 24]. In order to protect the integrity of its genome each bacterium has its own χ-sequence and RecBCD system. This provides a mechanism to recognise its own DNA and to prevent recombination of foreign DNA into the genome. Upon recognition of the χ-sequence, the 3' to 5' nuclease activity of the RecBCD complex is attenuated, whereas the 5' to 3' nuclease activity is activated resulting in

generation of ssDNA 3'-overhangs, which are required for the process of HR. Interestingly, RecBCD also directs the loading of RecA onto χ-containing ssDNA [23-27]. The RecA protein assembled on the ssDNA end catalyses the strand exchange reaction leading to a D-loop formation, which can then be further processed as in case of ssDNA gap repair [28, 29].

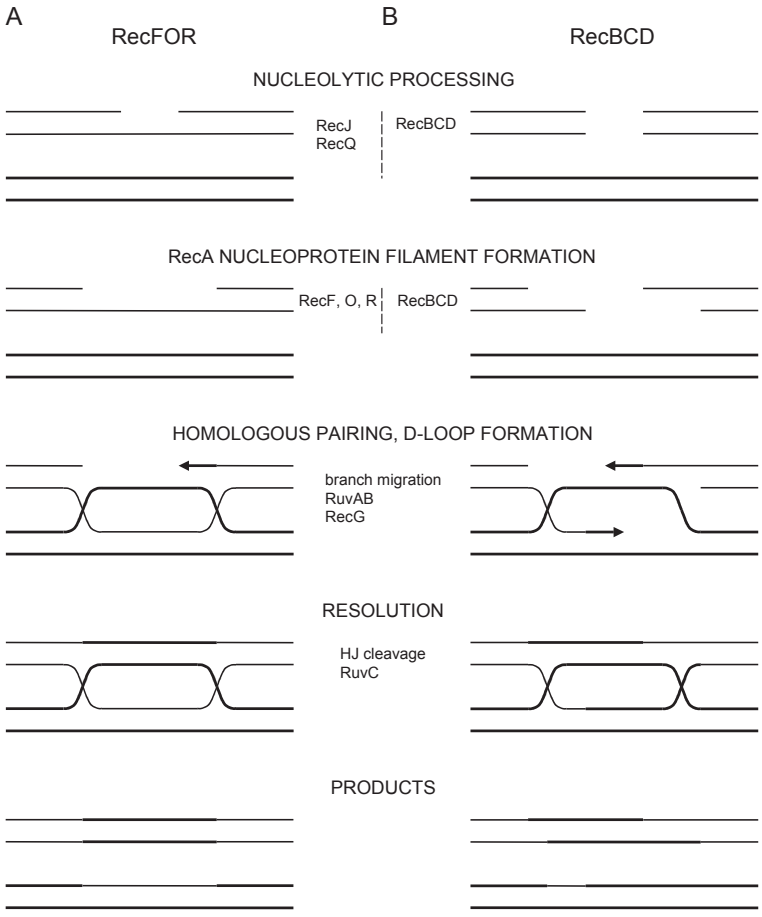

Figure 1. **HR pathways in bacteria**.

(**A**) Model for RecFOR-mediated gap repair. The process begins when the RecQ helicase and RecJ nuclease proteins extent the gap. Next, the RecFOR complex recognises and binds to the 5' end of the ds-ssDNA junction and subsequently recruits RecA protein that nucleates on the ssDNA. The resulting RecA filament searches for a homologous sequence in intact duplex DNA and invades it creating a D-loop. Simultaneously with DNA synthesis this structure is extended by branch migration activities of RecG helicase and RuvAB complex. Finally, the structure-specific endonulease, RuvC resolves the HJs. (**B**) Model of RecBCD-mediated DSB repair. First, ssDNA overhangs are created by the combination of the RecBCD helicase and endonuclease activities. Additionally, RecBCD recruits RecA onto ssDNA overhangs containing 3' tails. The subsequent steps of repair are similar to the RecFOR mediated ssDNA gap repair.

Source and the type of damage with a potential to block DNA replication

The presence of only one origin of replication in the bacterial genome poses a great problem for the accuracy of genome duplication. In order to avoid any loss of genetic material during replication, in principle, once started, replication has to continue until the terminating sequences (*ter*) are encountered [30]. However, even under normal growth conditions, it is very unlikely that replication can proceed undisturbed until its natural termination site. The presence of various DNA damages in the chromosome, such as template base modification or breaks in the phosphodiester backbone of the DNA can frequently stall a replication fork even under normal growth conditions [31]. Obviously, the risk of encountering DNA damage by the replication fork is remarkably increased in cells exposed to DNA-damaging agents such as UV-light or ionising radiation. Both, helix-distorting lesions; cyclobutane pyrimidine dimers (CPDs) or pyrimidine-(6,4)-pyrimidone adducts (6-4PPs) induced by UV-light or DSBs caused by ionising radiation are potentially lethal for the cell [32, 33]. In order to overcome these problems, bacteria evolved various repair mechanisms for stalled/collapsed DNA replication forks [31, 34]. Even though the exact molecular mechanism of repair remains yet to be elucidated, there is existing evidence that some proteins of HR are recruited to the sites of such collapsed replication forks [35, 36].

The above-mentioned UV-light-induced DNA lesions are normally removed by nucleotide excision repair (NER), however 6-4PPs more efficiently than CPDs [32]. On the other hand, when the lesion occurs at a site of replication fork the activation of HR both preceding and following damage removal via the NER pathway might be essential. Additionally, in the absence of functional NER the activity of HR might facilitate the bypass of the damage in order to continue the replication. The roles of HR in damage bypass and in supporting NER in damage removal will be discussed in the following sections.

Differential effects on replication fork progression of lesions on the leading versus lagging strand template

Lesions altering only a single DNA strand, caused by either endogenous DNA-damaging agents or UV-light, can inhibit DNA synthesis by blocking the replicative DNA polymerase (Figure 2). This can result in stalling and collapsing of DNA replication forks. Depending on whether the lesion occurs on the template of the leading or the lagging strand, the resulting DNA structure of the altered replication fork is different. When the lesion is located in the template of the leading strand the progression of the DNA Pol III holoenzyme is blocked leading to the replication fork arrest (Figure 2A) [37]. The DNA lesion does not always block the DNA helicase-primase complex, which can still progress independently of the DNA polymerase, which will result in complete destruction of the replisome structure [38]. Annealing of the nascent ssDNA strands together with unwinding of the original template can result in a fork regression and formation of a HJ-like structure referred to as a chicken foot [39]. Putatively, enzymes involved in HR, specifically RecG, can catalyse the

required remodelling of the DNA structure [35, 40-42]. The fate of the generated recombinational intermediate will be discussed in the following paragraphs. Alternatively, the lesion can be located on the lagging-strand template (Figure 2B), which blocks DNA synthesis by DNA Pol III at the lesion. Nevertheless, the β clamp together with the clamp loader subunit γ of Pol III, can translocate the halted polymerase onto the next RNA primer [37, 43]. Therefore, the progression of the DNA replication fork can continue leaving a ssDNA gap behind [43]. In most cases these ssDNA gaps are repaired by RecFOR-mediated HR [22].

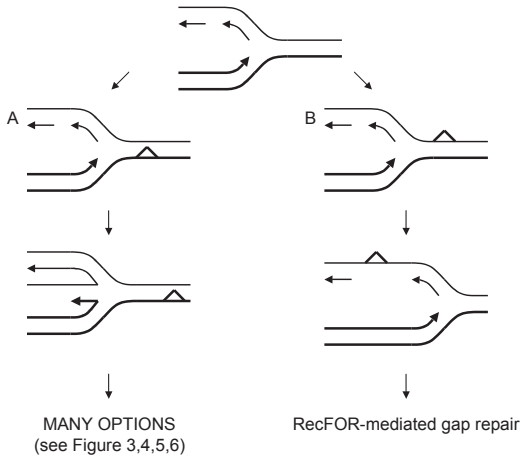

Figure 2. **Different effect on fork progression of DNA damage present in the template of the leading or the lagging strand template.** (**A**) DNA damage blocking progression of a DNA polymerase on the leading strand template (thick lines) results in fork regression and pairing of nascent DNA strands yielding a chicken foot-like or HJ-like DNA structure. (**B**) When the lesion is present on the template of the lagging strand (thin lines) a ssDNA gap will arise, which can be subsequently repaired by Rec-FOR-mediated HR .

NER and HR

One of the proposed mechanisms of stalled replication fork restart depends on the cooperation between the processes of HR and NER [21, 44, 45]. In this case, the lesion is removed from the DNA by the proteins of the NER pathway [45]. However, the proteins of NER, the UvrAB complex in *E. coli*, can recognise the lesion only in the context of dsDNA [46, 47]. Therefore, it has been proposed that the stalled DNA replication fork has to regress in order to provide dsDNA around the DNA lesion site [48]. This can be achieved when two nascent ssDNA strands anneal together and parental strands reanneal until the DNA lesion is placed back in the context of dsDNA (Figure 3A). Only then can the UvrABC complex recognise and subsequently remove the lesion from the DNA. Since DNA synthesis on the leading strand is disrupted by the lesion earlier than the synthesis on the lagging strand an imbalance between the lengths of both nascent ssDNA strands can arise. The extension of ssDNA of the nascent lagging strand can be removed by the exonucleolitic activities of the RecQ and RecJ proteins, because both *recQ* and *recJ* null mutants, although not UV-light sensitive, show a decreased rate of replication recovery upon UV-light treatment [21, 49].

The possibility of remodelling the DNA structure around the lesion in a mechanistically different manner is underscored by the phenytopes of strains mutated in members of RecF-mediated HR

pathway. Genetic and biochemical approaches showed that *recA*, *recF*, *recO* and *recR E. coli* mutants are sensitive to UV-light and show a decreased efficiency of recovery of DNA replication after the treatment [44, 50]. In these mutants a high level of the degradation of nascent ssDNA occurs upon UV-light. Additionally, *recO* cells fail to maintain the branched structures of collapsed replication forks [51]. All of these data suggest that the RecFOR proteins together with RecA play an important role in the maintenance of the branched structure of the collapsed replication fork, which might result not only from fork regression but also during RecF-mediated gap repair (Figure 3B) [45].

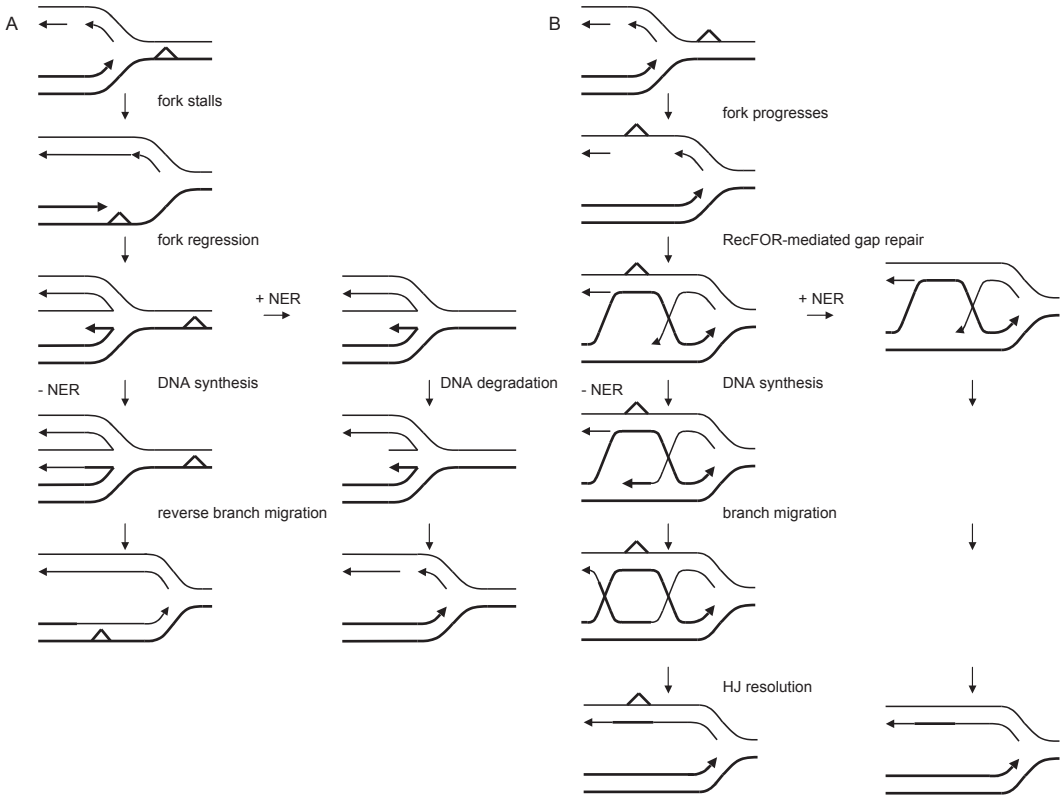

Figure 3. **The potential roles of HR in processing UV-light-induced DNA damage in the presence or absence of NER**.

(**A**) When the lesion occurs in the template of the leading strand regression of the replication fork moves the damage back into dsDNA. After lesion removal by NER, RecQ and RecJ degrade the 5' extension of the nascent lagging strand. Replication can restart after moving the junction back to a fork, putatively carried out by the RecG helicase. However, in the absence of NER the nascent leading strand can be elongated using the nascent lagging strand as a template to the extend allowing lesion bypass. Reverse branch migration results in fork reconstitution. (**B**) When the lesion is present in the lagging strand template the replication fork need not to stop, since new Okazaki fragments can be initiated downstream of the

lesion, resulting in ssDNA gap at the lesion. RecFOR-mediated strand exchange places the lesion in dsDNA, which can be now removed by NER. Subsequently, the ssDNA gap can be repaired by RecFOR-mediated HR. In the absence of damage repair, RecFOR can mediate the lesion bypass by a similar strand exchange reaction.

Bypass to leave lesions unrepaired

Simple bypass of the DNA damage appears to be the easiest way to continue replication in cases when the DNA damage repair mechanisms are neither available nor capable of removing the damage in the context of a stalled replication fork. Lesions in the DNA can be bypassed in three mechanistically different manners (Figure 4). The first possibility is the use of intact, nascent complementary ssDNA as a template, ignoring the original, damaged one. When a stalled replication fork is transformed into a chicken foot the nascent lagging strand can be used as a template for DNA synthesis (Figure 3A and 4A) [35, 52-54]. Alternatively, the ssDNA gap caused by a replication block can be filled by transient displacement of a nascent ssDNA from a sister chromatid duplex, which is subsequently used as a template for DNA synthesis (Figure 3B) [55, 56]. Both DNA intermediates formed either during fork regression or gap repair have the potential to serve as substrates for reconstitution of the replication (see below).

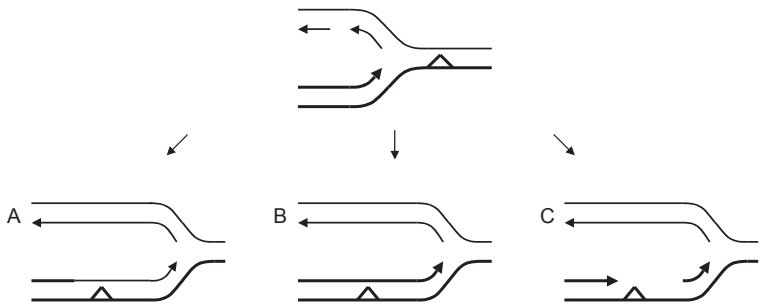

Figure 4. **Possible mechanism of bypassing UV-light-induced DNA damage.**
The different final DNA products are shown in **A**, **B** and **C**. For simplicity of the Figure, DNA damage present in the leading strand template only is considered. (**A**) Product of bypass as detailed in Figure 3A. (**B**) Product of translesion DNA. (**C**) Lesion bypass mediated by DnaB repriming results in the presence of ssDNA gaps at the site of the lesion. Further details are provided in the text.

Another way to bypass the lesion is to replace DNA Pol III that is blocked at the lesion by one of the specialised translesion polymerases, Pol IV or Pol V [57, 58]. These polymerases can incorporate nucleotides opposite the damage, however their inaccuracy increases the risk of point mutations. It has been shown *in vitro* that efficient Pol V-mediated bypass requires both RecA and the β clamp replication processivity factor stably loaded on the primer template [59-62]. RecA forms

a nucleoprotein filament on the ssDNA region generated by transient progression of the replicative helicase downstream from the blocked Pol III [63]. When dissociated from the arrested replication machinery the β clamp becomes available for interaction with Pol V and tethers it to the site of damage. After PolV catalysed DNA synthesis bypassing the lesion, the regular replicative polymerase replaces Pol V and continues replication in an error-free manner (Figure 4B) [64]. However, even in *E. coli*, models of translesion DNA synthesis (TLS) are still actively developing because a recent *in vitro* study demonstrated that the most efficient translesion synthesis occurs when RecA forms a filament on ssDNA oriented *in trans* to the DNA lesion [65]. Thirdly, a new potential way of restarting replication downstream from the damage has been proposed recently. In this case replication is restarted on both the leading and the lagging strand by priming them by DnaG primase and subsequent DNA synthesis (Figure 4C and see below) [66].

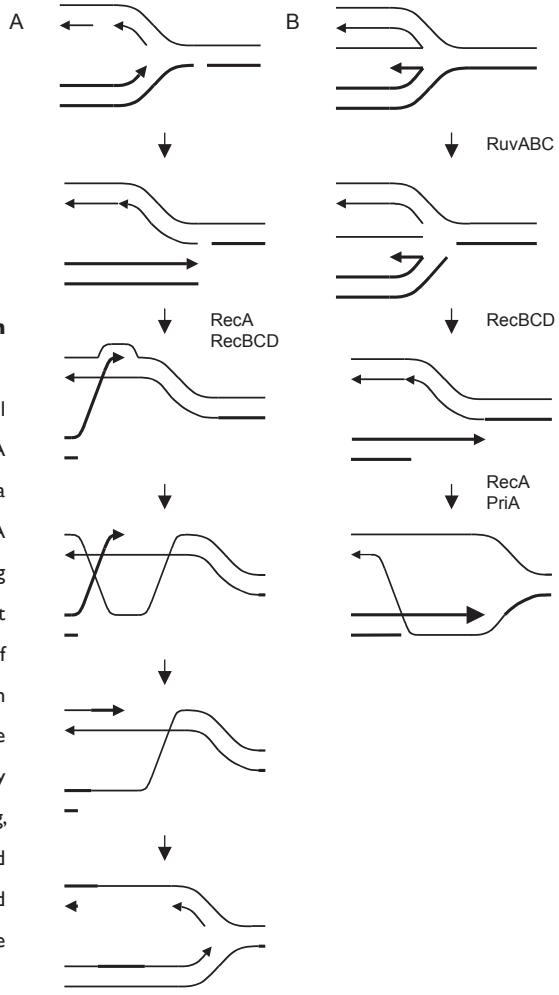

Figure 5. **Generation of a DSB at a replication fork.**

(**A**) Replication through a nicked or gaped template will result in the generation of a free dsDNA end. The dsDNA end is recessed by the RecBCD endonuclease resulting in a ssDNA overhang, which is coated by RecA protein. The RecA nucleoprotein filament invades the sister chromatid forming a D-loop. This DNA intermediate has a potential to restart replication using PriA. After HJ resolution the structure of a replication fork is re-established. (**B**) Alternatively, when a fork has regressed (due to a DNA damage) its HJ-like structure can be converted into a one-ended DSB by specified structure-specific endocluease. After processing, the 3'-tailed end can be coated by the RecA protein and the nucleoprotein filament can invade the sister chromatid resulting in a D-loop. From this structure PriA can promote replication restart (see Figure 7)

Generation of a DSB

Alternatively to UV-light-induced lesions, a DSB can occur at the site of the replication fork (Figure 5). At least two major models of the mechanism of DSB formation have been proposed. In the first model the DSB is induced by the progression of DNA replication fork through the DNA template containing a ssDNA break or a ssDNA gap (Figure 5A) [67, 68]. In the second model, a DSB is deliberately generated at a site of a stalled replication fork by cellular, structure-specific, endonucleases (Figure 5B). Screening of various mutants revealed that the stalled DNA replication fork is regressed, leading to the formation of a HJ-like structure [35, 52, 54]. This structure is subsequently cleaved by the HJ resolvase, RuvABC, resulting in transformation of a regressed DNA replication fork into a DSB, which is an intermediate for the repair process [54].

Processing of a chicken foot structure

The chicken foot DNA structure, which is formed during replication fork regression due to replication block on the template of the leading strand, is a very important intermediate for the replication recovery. Recently, apart from RecG, NER helicase, UvrD, has been also implied in mediating this modification [69, 70]. The potential carried by this DNA arrangement is reflected in the variety of possible modifications of this structure (Figure 6). It is commonly acknowledged that fork regression results in replisome disassembly. The only so far known origin-independent recombination restart is possible from D-loop or replication fork DNA structures [40, 71]. Therefore, in order to resume replication, the chicken foot has to be further rearranged. This four-way branched DNA junction is a possible target for nucleases and proteins with a potential to alter the topology of DNA.

Regression of stalled forks resulting in a chicken foot provides also a model for cooperation between HR and NER. In this case, after the DNA lesion has been removed by NER proteins, HR is involved in reconstitution of the replication fork. In the model of direct rescue, RecG helicase reverses the HJ-like structure restoring the replication fork (Figure 6A). In another case, a forked DNA structure is acquired by endonucleolitic cleavage of the dsDNA end of a chicken foot by RecBCD proteins (Figure 6B). Alternatively, direct cleavage of the HJ-like structure by the RuvABC endonuclease results in formation of a free dsDNA end that can subsequently be processed by RecBCD into a ssDNA overhang (Figure 6C). Once coated with RecA, the nucleoprotein filament invades homologous duplex DNA and replication can be reconstituted from the D-loop intermediate. A further model assumes that the excess of nascent lagging strand is removed by a combination of the exonucleolitic and helicase activities of RecQ and RecJ (Figure 6D).

There are two proposed mechanisms of lesion bypass via the chicken foot. In the first scenario after the nascent leading strand is elongated to the extent allowing to bypass the lesion, proteins with branch-migrating activities such as RecG can move the HJ in the direction of replication re-creating in this way the replication fork, which in turn serves as a target for PriA (Figure 6E). In

the second scenario, the free dsDNA end is recombined back into parental DNA duplex, resulting in formation of a double HJ (Figure 6F). This modification protects the structure from direct cleavage by RuvABC, which would result in release of a free dsDNA end. In this manner the risk of an inappropriate recombination between the dsDNA end formed at the fork and homologous sequences elsewhere in the genome is reduced. Subsequently, the cleavage of HJs by RuvABC generates the forked structure.

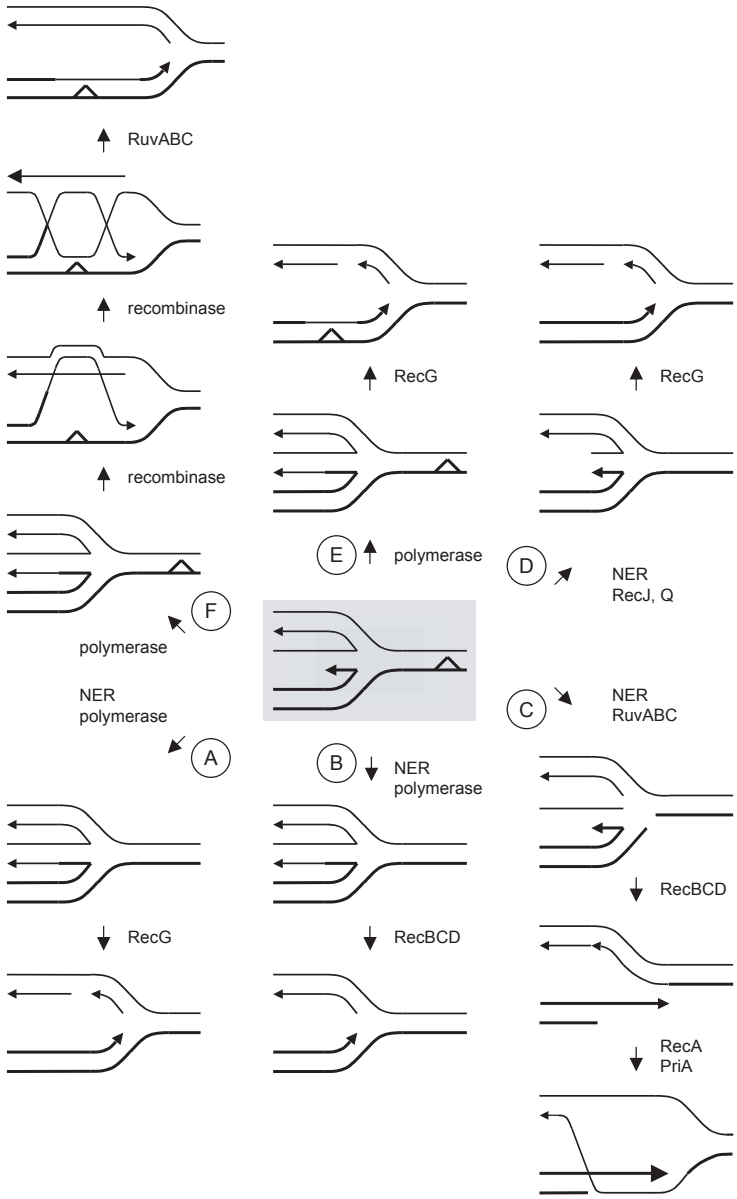

Figure 6. **Potential fates of a chicken foot-like DNA structure**.

The UV-light-induced lesion present in the template of the leading strand causes the replication fork to regress. In bacteria this process is mediated by the RecG helicase and the resulting chicken foot-like structure has the potential for further modifications. (**A**) Direct rescue of replication requires activity of the RecG helicase, which can reverse the regressed fork after damage removal by NER. (**B**) Following damage removal by NER, RecBCD can degrade the free dsDNA end leading to a structure from which a fork can be reconstituted. (**C**) Cleavage of the HJ-like structure after damage removal will result in fork collapse. RecBCD mediated HR results in restoration of a structure with the potential to reinitiate DNA replication. (**D**) Alternatively, following damage removal, the protruding nascent lagging strand can be removed by the joint activities of the RecQ and RecJ proteins. Reversion of the resulting branched structure can result in reconstitution of a replication fork. (**E**) In the absence of damage repair, the nascent lagging strand can be used as a template to elongate the leading strand. When the leading strand is long enough to bypass the lesion, RecG can restore the structure of replication fork. (**F**) In the last scenario of the lesion bypass, the dsDNA end is recombined back into the parental duplex and a double HJ is formed. This protects the chicken foot-like structure from direct cleavage, which would endanger illegitimate recombination. Resolution of the double HJ restores the replication fork.

Reconstruction of the replication fork

As described above, involvement of the proteins of HR in restart of a stalled/collapsed replication fork leads to formation of a D-loop, which is an intermediate in the process of HR, or to formation of forked DNA structures. The requirements of a D-loop to restore disrupted replication can be explained by the properties of the PriA protein, which is an example of a direct link between recombination and replication. Under normal growth condition 50% of *recA* and 90% of *priA* null mutants are not viable [72]. These phenotypes are probably due to a defect in the recovery of damaged replication forks. Biochemical analysis showed that PriA binds to DNA in a structure-dependent manner. D-loops, R-loops and open origins of replication of bacteriophage ϕX174 are the known targets for the protein [44, 72-74]. The interaction of PriA protein with the replication proteins DnaB, DnaC, DnaG, PriB, PriC, and DnaT has also been shown for bacteriophage ϕX174 replication [75-78]. In case of a stalled/collapsed replication fork PriA can bind to RecA and RecFOR generated D-loop DNA structures. Once bound to a D-loop it recruits, with help of PriB and DnaT, the DnaB-DnaG replisome complex. Therefore, PriA recruits DNA replication factors that are required for the reconstruction of the replisome (Figure 7). However, recently an alternative possibility of replication reconstitution has been proposed based on *in vitro* experiments. According to the suggested model, loading by a replication restart system of a single hexamer of the DnaB helicase on the lagging-strand template is sufficient for coordinated priming by the DnaG primase of both the leading and lagging strand [66]. This model suggests the existence of a less complex shortcut to restart replication explaining the high speed of replication recovery in UV-light irradiated cells. Nevertheless, the high recovery speed is gained at the cost of generating ssDNA gaps in the lagging-strand as well as persistence of UV-light-induced DNA damage (Figure 4C).

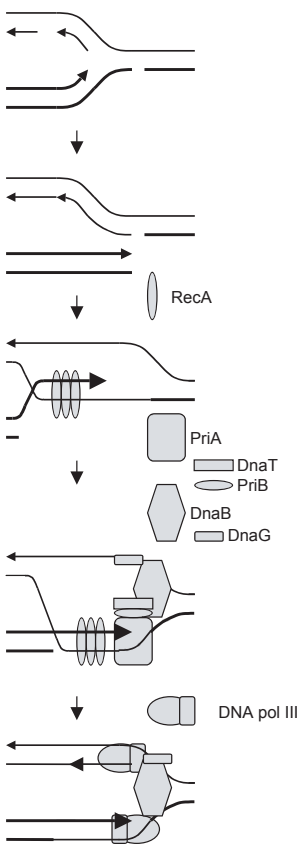

Figure 7. **PriA mediated replication restart from a D-loop intermediate.**

When a collapsed replication fork arises due to a nick in a template RecBCD-mediated HR can transform it into a D-loop. PriA recognises and binds to this structure and subsequently loads the replicative helicase DnaB, which is followed by binding of DnaG primase. DNA synthesis by Pol III can then continue. DnaB loading onto the lagging strand template requires the presence of PriB, PriC and DnaT accessory factors. Inappropriate loading of DnaB onto ssDNA is prevented by its interaction with DnaC.

process	protein		function
	E. coli	Eukaryotes	
DNA replication	SSB	RPA	ssDNA binding protein
	DnaA	ORC-Cdc6-Cdt1	initiator of DNA replication
	DnaB	Mcm2-7, Cdc45	replicative helicase
	DnaC	ORC-Cdc6-Cdt1	helicase auxiliary protein
	DnaG	?	primase
	?	pol α	polymerase, extends RNA primers
	Pol III (DnaE)	pol δ, pol ε	replicative polymerase
	β (DnaN)	PCNA, GINS	replication processivity factor
	γ	RFC	clamp loader
	Pol IV, Pol V	pol η	translesion polymerase
	PriA, PriC	?	helicase, origin independent replication initiatior
	PriB, DnaT	?	PriA auxiliary protein
	?	Mcm10	component of replication fork possibly stabilising pol α
	?	geminin	inhibitor of Cdt1 in metazoans
	?	Cdk1	kinase, in yeasts inhibits Cdt1, Cdc6, ORC, Mcm(2-7)
	?	DDK	Cdc7-Dbf4 kinase, regulates assembly of GINS onto origin of replication
NER	UvrAB	Xpa, Xpc, Xpe,	damage recognition and verification
	UvrC	Ercc1-Xpf, Xpg	endonuclease
	UvrD	TFIIH (Xpb, Xpd)	helicase
HR	RecA	Rad51	recombinase
	RecBCD	?	DNA helicase with an associated ssDNA endonuclease activity
	?	Mre11 complex	
	RecFOR	Rad54 (?)	stabilisation of recombinase on ssDNA
	RecQ	BLM, WRN	3'-5' recombination-specific helicase
	RecJ	?	5'-3' exonuclease
	RuvAB	BLM, WRN, Rad54	branch-migration
	RuvC	Mus81/Eme1 (?), Rad51C/Xrcc3 (?)	HJ nuclease

Table I

Replication in eukaryotes

Duplication of the genome in eukaryotic cells requires more complicated mechanisms than in bacteria. During S phase, the period of the cell cycle when the genome is replicated, DNA is more prone to damage than during the other cell cycle phases [79]. Thus, it is important to keep it as short as possible. To achieve this goal, eukaryotic cells have evolved DNA replication machinery that allows fast, yet precise duplication of their chromosomal DNA. In contrast to bacteria, eukaryotic replication is initiated from multiple origins of replication, typically 10^3-10^5 per genome [80, 81]. To ensure replication accuracy, processivity of eukaryotic polymerases is around 20 times less than bacterial polymerases [82]. Additionally, complex control mechanisms prevent re-replication of the genomic DNA from already fired origins [83].

Once in a cell cycle, licensing the origins of replication

In bacterial cells, control of replication initiation is relatively easy when compared to eukaryotes, due to the presence of only a single origin. The presence of multiple replication initiation sites and the vast size of the eukaryotic genome require a more efficient control mechanism. Therefore, in eukaryotic cells the control of accuracy of replication is a multilevel process that happens during the entire cell cycle. The earliest event involved in initiation of replication is origin licensing, a process that allows an origin of replication to begin DNA replication at that particular site. Licensing of origins of replication begins already in G1 phase by assembling a pre-replicative complex (pre-RC) (Figure 8). First, the origin recognition complex (ORC) binds to replication origins at the expense of ATP hydrolysis [80]. In *S. cerevisiae* ORC recognises a highly conserved 11 base pair sequences known as the autonomously replicating sequence (ARS) consensus sequences (ACSs or A elements) [84]. The ARSs are non-coding DNA regions of approximately 100-200 bp in length containing, additionally to ACS, less conserved sequence motifs [85, 86]. However, finding a correlation between a DNA sequence and an origin of replication in eukaryotes other than *S. cerevisiae* remains difficult [87]. Subsequently, ORC recruits the initiation factors Cdc6 and Cdt1. Only then can the heterohexameric minichromosome maintenance (Mcm2-7) complex, containing the presumed replicative helicase activity, be loaded [88-92]. In the absence of Mcm2-7 replication cannot proceed [93-95]. To maintain genome stability it is pivotal that loading of Mcm2-7 is both stable and regulated. Regulation of assembly of Mcm2-7 complex onto DNA is performed by Cdc6 and Cdt1. Cdc6 and Cdt1, together with ORC, act as a clamp loader [96]. Cdc6 hydrolyses ATP, which is crucial for the opening and re-assembly of the Mcm2-7 ring around DNA. This can only occur when bound to ORC and in the presence of an origin, ensuring in this way that Mcm2-7 is not loaded on a DNA distinct from the origin [97, 98]. Cdt1 can directly bind the Mcm2-7 heterohexamer, possibly recruiting it to the DNA [98-100]. Control of Mcm2-7 activation only once at the origin per cell cycle is obtained by downregulation of the activity of its auxiliary proteins. Once the Mcm2-7 complex is bound to the origin ORC, Cdc6 and Cdt1 become redundant [101-104]. Therefore, inactivation of

ORC, Cdc6 and Cdt1 at the end of G1 prevents illegitimate association of Mcm2-7. In yeast cells cyclin-dependent kinase (CDK) Cdk1 phosphorylates Cdc6, which in turn triggers its degradation at the G1-S-phase transition [105-108]. Phosphorylation of ORC bound to DNA maintains its inactive state through S and G2 phase [109]. Promoted by CDK in S, G2 phase and early mitosis nuclear export of Cdt1 and Mcm2-7 prevents their access to chromosomal DNA [110, 111]. In contrast, the role of CDK in downregulating origin licensing is marginal in mammalian cells. Major control comes from downregulation of Cdt1 activity in S and G2 phase, which is obtained by binding of its inhibitory protein, geminin (Figure 8) [99, 100, 104, 105, 112]. The transition from G1 to S phase involves the conversion of pre-RCs into replication forks. Initiation requires origin unwinding, stabilisation of ssDNA and loading of DNA replicative polymerases. Though pre-RCs are loaded onto all origins, not all of them fire at the same time. Distinguishing between origins that fire early in S phase from those fired in late S phase is ascertained by activity of CDKs and Dbf-dependent kinase (DDK), which are only coupled to origins that are activated.

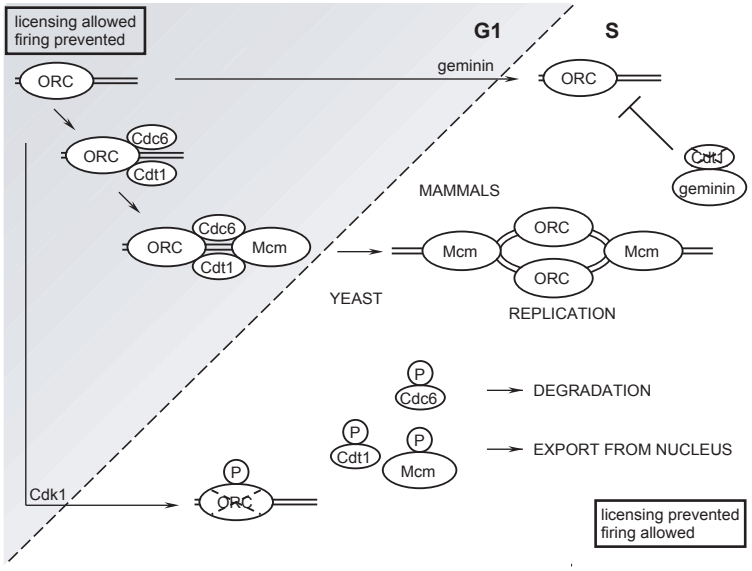

Figure 8. **Mechanism of regulation of origin licensing in eukaryotic cells.**

G1 phase is indicated by the grey background, S phase by the white background and the transition from G1 to S phase is marked by the dashed line. The sequential assembly of replication factors in G1 phase, common for both yeast and mammals, is schematically depicted. First, ORC binds to origins of replication. Next, it recruits Cdc6 and Cdt1, which then in turn load the putative replicative helicase complex Mcm2-7. Replication begins in S phase by origins opening by Mcm2-7 helicase (middle right hand section). Depending on the organism, mechanisms preventing re-replication differ. In mammalian cells (upper right hand section) geminin binds to Cdt1 preventing its binding to ORC. In yeast (lower right hand section) Cdk1 phosphorylates ORC inactivating it. Additionally, phosphorylation of Cdt1 and Mcm2-7 by Cdk1 triggers their export from the nucleus, whilst phosphorylation of Cdc6 induces its degradation

The earliest event of replication initiation is recruitment of Mcm10 to the pre-RC. Next, Mcm10 employs Cdc45, which is indispensable for DNA unwinding by Mcm2-7 and subsequent loading of replicative polymerases [113-118]. Phosphorylation of Mcm2-7 by DDK is facilitated by physical interaction of Mcm10 with both proteins [119]. An additional role of Mcm10 in replication elongation is probably to retain Cdc45 in elongating forks [120-122]. The Cdc45 that interacts with components of pre-RC (Mcm2-7) and polymerases physically links initiation and elongation factors [123-126]. Further origin unwinding is stimulated by stabilisation of ssDNA by replication protein A (RPA) [117, 127]. Subsequently, RNA primase and polymerase (pol) α are recruited and synthesise short RNA primers for both leading and lagging strands synthesis [128, 129]. Finally, pol α is replaced by the replicative polymerases; pol δ and pol ε, which have higher processivity and possess proofreading exonuclease activity [14, 130]. In metazoans pol δ is topologically linked with DNA by proliferating cell nuclear antigen (PCNA), whilst pol ε interacts with GINS (go ichi ni san) that both encircle DNA [131-136]. Recently, it has been shown that the interaction between the Mcm(2-7) complex and Cdc45 as well as GINS is essential for DNA unwinding activity [94, 137, 138]. Additionally, in yeast, loading of a polymerase processivity factor onto DNA requires the activity of replication factor C (RFC) [134, 139, 140]. Although pol δ and pol ε are both essential and play non-redundant roles, pol δ is probably the main polymerase for both leading- and lagging strand synthesis because pol ε fails to stably replicate DNA in the absence of pol δ [141]. Recently, temporal separation of activities suggest a dominant role of pol α and pol ε in early S phase and pol δ in late S phase of the cell cycle [142].

Homologous recombination in eukaryotes

Compared to bacteria the process of HR in eukaryotes is both more complicated and less explored. Already the mechanism of sensing the damage and recruitment of the repair machinery to the site of the lesion are revealed only to limited extends. According to current model, initially in response to a DSB, the ATM protein kinase is activated by autophosporylation [143]. Once activated, ATM phosphorylates multiple substrates including BRCA1, p53, p53 binding protein 53BP1 and histone H2AX [144]. However, the ability of ATM to phosphorylate those substrates is enhanced in the presence of the Mre11–Rad50–Nbs1 (MRN) complex [145-147]. Therefore it is likely that ATM activation involves recruitment of MRN to sites of DSBs and subsequent recruitment and activation of ATM by MRN. Another set of proteins present at a DSB early after damage induction and with a potential to activate ATM include 53BP1, and MDC1 [148, 149]. The 53BP1 checkpoint protein senses changes in chromatin structures, occurring at sites of DSB and subsequently activates ATM [143]. Thus, it is possible that ATM senses the damage in an indirect way. Subsequently, ATM phosphorylates H2AX, generating γ-H2AX, which in turn recruits chromatin remodeling complexes to sites of DSBs where they are able to change the chromatin arrangement to allow repair factors to gain access to, and repair, the lesion [150]. MDC1 may play a role in controlling the rate of H2AX

phosphorylation thus putatively limiting changes in chromatin conformation to the proximity of a site of a DSB [151-153]. Additionally, interaction of BRCA1 with a SWI/SNF complex suggests its role in the chromatin-remodeling step [154]. However, the precise interplay of the proteins involved in DSB sensing remains to be elucidated.

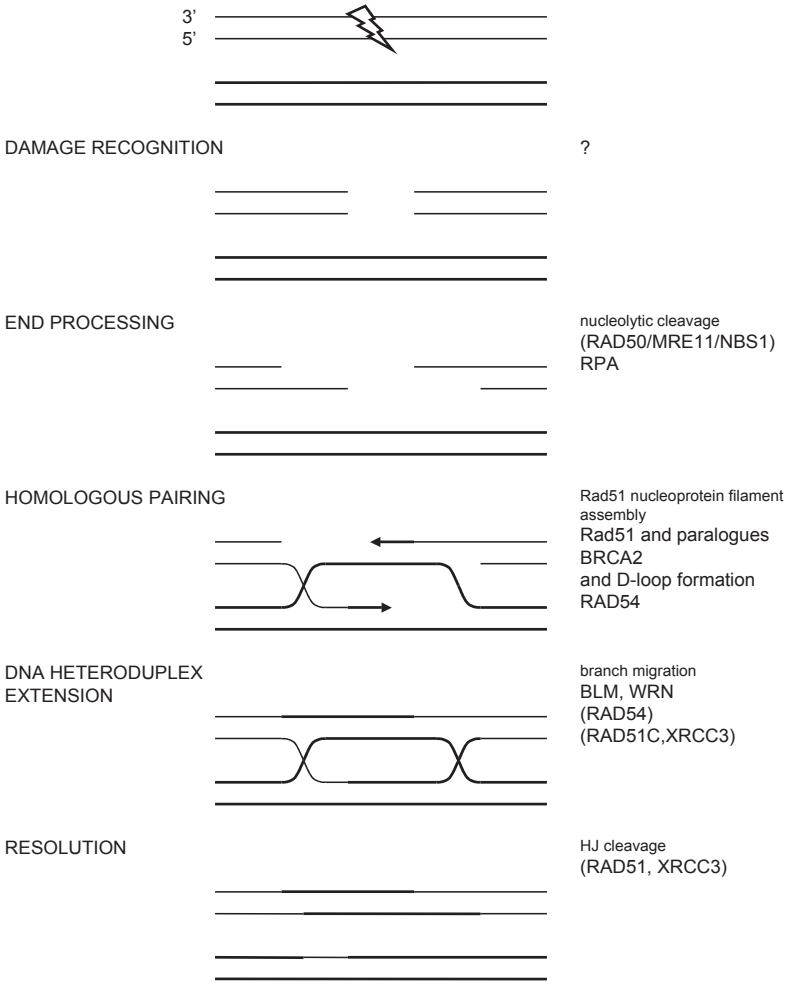

Figure 9. **HR in eukaryotic cells.**

Stages of the process of HR are shown on the left. In the middle section thin lines represent the broken DNA, thick lines symbolise the intact homologous template. Arrows indicate DNA synthesis. The proteins of HR involved in each stage of the process are listed on the right. When a DSB is induced in one of the sister chromatids nucleolytic processing results in formation of 3' DNA overhangs which are subsequently coated by the Rad51 recombinase. The search for homology and invasion of homologous DNA by the DNA tail(s) results in formation of a D-loop. Homologous pairing of the displaced DNA strand with the complementary sequence of the damaged chromatid and continued DNA synthesis results in creation of a double HJ. Resolution of the HJs and ligation completes the repair process. Proteins whose activity at respective stages of the HR are predicted only are listed in parenthesis.

After damage recognition, ends of a DSB are nucleolytically processed resulting in 3'-ssDNA overhangs (Figure 9). The role of the Rad50/Mre11/Nbs1 complex in this step is unclear, however there are indications that the complex tethers two ends of a DSB together as well as having the potential to assist in the resection [155, 156]. Subsequently, 3'-ssDNA overhangs are coated by the RPA protein, which is then replaced by the Rad51 protein resulting in formation of a nucleoprotein filament. In yeast, the Rad52 protein promotes the nucleoprotein filament formation by mediating between the RPA protein on the ssDNA and Rad51 [157]. In mammalian cells BRCA2 could facilitate loading of Rad51 onto RPA-coated ssDNA [158, 159]. Next, the Rad51 filament searches for and invades the homologous DNA duplex forming a D-loop DNA intermediate. The Rad54 protein has the ability to stimulate this step [160, 161]. The formed four-way DNA HJs can move along DNA extending the DNA heteroduplex. In human cells this process is mediated by specialised helicases BLM and/or WRN that can unwind the four-way DNA junctions such as HJs [162]. However, recent biochemical experiments show that Rad54 can also branch migrate the HJs [163]. Subsequently, the missing DNA context is restored using the intact DNA template for DNA synthesis. Finally, HJ resolution terminates the process of repair. Identification of proteins involved in this step in eukaryotic cells has remained unsuccessful. However, recently, the Rad51 paralogues RAD51C and XRCC3 have been associated with a HJ resolution activity [164].

HR-dependent restart of replication

In contrast to two-ended DSB repair by HR little is known about its role in restart of replication in eukaryotes. Nevertheless, recently emerging evidence suggests that similarly to bacteria HR is activated upon replication arrest also in eukaryotic cells [165, 166]. Additionally, based on the resemblance of some of the DNA structures generated at stalled replication forks and during HR, the potential role of HR in supporting replication can be predicted. Below, the known activities of HR proteins in restart of replication in eukaryotic cells will be discussed.

UV-light and HR

The role of HR in dealing with UV-light-induced DNA damage in eukaryotic cells is not yet very clear. This type of DNA lesion is either repaired by NER or bypassed by TLS [32]. NER is a multistep process consisting of damage recognition and verification, incision of the damaged DNA strand and subsequent filling of the missing DNA content. This process involves activity of numerous proteins including Xpa, which acts early in the NER process and is thought to be involved in damage verification [167-169]. Consequently, Xpa-deficient cells are totally NER-deficient and hypersensitive to UV-light [170]. Interestingly, in *Xpa*$^{-/-}$ cells levels of sister chromatid exchange (SCE), an event correlated with recombination, are increased upon DNA damage induction, when compared to wild type cells [171]. This observation suggests that in NER-deficient cells UV-light activates HR. Alternatively, the DNA damage can be bypassed by TLS, which involves activities of specific

translesion DNA polymerases that can synthesise short stretches of DNA through a template tract containing a lesion [172]. TLS is an important process even in cells with functional NER because xeroderma pigmentosium variant (XP-V) cells, in which the translesion polymerase (pol) η gene is mutated are sensitive to UV-light [173, 174]. Remarkably, UV-light-induced replication arrest leads to an accumulation of the Mre11/Rad50/Nbs1 complex and phosphorylated histone H2AX, γ-H2AX in nuclear foci at sites of stalled replication forks in these cells [175, 176]. Additionally, in human cells the RAD51 protein colocalises with the translesion pol η upon UV-light treatment. This suggests that unrepaired UV-light-induced damage requires, apart from pol η, also RAD51. Alternatively, RAD51 protein might be recruited to arrested replication forks that are recombination substrates, while pol η is recruited to the same sites for its bypass activity [177]. However, biochemical data showed that human pol η binds with higher efficiency to D-loops than to replication fork structures. Moreover, the interaction of pol η with RAD51 recombinase stimulates pol η-mediated D-loop extension. These results indicate that at stalled replication forks pol η can promote translesion synthesis as well as reinitiate DNA synthesis by HR repair [178]. This dual role of pol η has been supported by analysis of HR-mediated DSB repair in pol η-deficient chicken cells. The level of HR-mediated repair was reduced 10-fold when compared to wild type cells [179]. Therefore, similarly to bacteria, HR may play a role in processing UV-light-induced DNA damage at the site of replication forks.

Break-induced replication

The substrate specificity of Mus81-Eme1 and XPF-ERCC1 endonuclease, which cleave 3'-flap DNA structures and three-way branched junctions containing two ssDNA arms, respectively, indicate their capability to process stalled replication forks before they have regressed to form a HJ [180-183]. The resulting one-ended DSBs at a stalled replication forks can become a substrate for HR-mediated break-induced replication (BIR), which represents one of the possible mechanisms of repair of a collapsed replication fork.

In yeast BIR is a nonreciprocal recombination-dependent replication process, which begins when one end of a DSB invades a template and sets up a replication fork (Figure 10) [184]. Created in this way the D-loop migrates down the template with the assistance of branch-migrating enzymes [184, 185]. Additionally, this process requires at least some enzymes that are involved in normal DNA replication, such as the pol α/primase complex [186]. Since the invading 3' DNA end can easily be extended, the requirement for the pol α/primase complex suggests the presence of lagging strand synthesis in the D-loop created replication fork. Therefore, this mechanism can be employed by a cell to recover collapsed replication forks, which create only one DSB end and in this way become perfect substrates for BIR. Genetic analysis of various yeast mutants revealed that BIR requires the recombination protein Rad52 [187]. Additionally, the efficiency of the process greatly depends on Rad51 protein, because in the absence of Rad51 the level of BIR events drop significantly [188, 189]. The analysis of Rad51-deficient strains revealed that Rad51-independent BIR

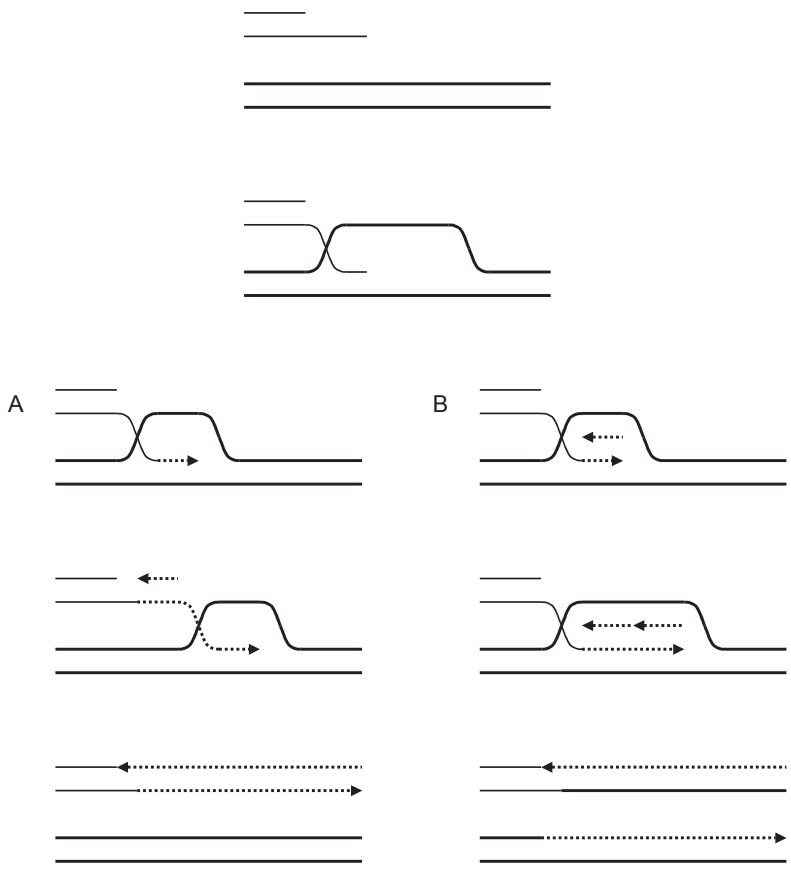

Figure 10. **Mechanisms for BIR.**

A broken chromosome end can be transformed into a 3' overhang by 5' to 3' exonuclease activity. This allows strand invasion carried out by various recombination proteins. (**A**) The 3' end of the invading strand initiates DNA replication leading to a migrating D-loop. The newly synthesised DNA strand can be used as a template for DNA synthesis. (**B**) alternatively, following the 3' end invasion semiconservative DNA replication can occur. The created HJ will be resolved as replication continues. A combination of both variants of the models depicted in **A** and **B** is also possible.

is not initiated randomly in the genome. A small *cis*-acting DNA sequence facilitates the majority of BIR events. It has been proposed that it acts as a suppressor of cellular nucleolitic activities, analogously to the bacterial sequence χ. However, in Rad51-mediated BIR this sequence is unnecessary [190].

Fork regression

Fork reversal has been proposed as serving a number of useful purposes, including providing room for the repair of DNA lesions, a mechanism for template switching and a substrate for enabling fork collapse and subsequent recombination-dependent restart of replication (Figure 6). Using electron microscopy and 2D gels the -like structure that would result from fork regression has been detected in wild type yeast cells in which replication was prematurely disrupted [191, 192]. This supports the notion that a stalled replication fork can indeed be transformed into a HJ-like structure. In bacteria transformation of a stalled replication fork into a chicken foot probably requires the activity of the RecG helicase [193]. Similarly, human cells mutated in the BLM gene, which encodes a homologue of the bacterial RecQ helicase, show genomic instability due to their inability to properly remodel stalled replication forks [194, 195]. The DNA unwinding activity of the BLM protein might be necessary to reverse a stalled fork into a chicken foot-like structure. However, recently the potential role of BLM also in reversal of the regressed fork has been proposed. In this case reconstitution of a replication fork includes the generation of double HJs, which are then dissolved by the BLM protein in combination with topoisomerase. This mechanism of lesion bypass is favourable, especially in mammalian cells in which repeated sequences are abundant. This is because the alternative, direct cleavage of the chicken foot-like structure by specialised endonucleases, can result in release of a free dsDNA end that can illegitimately recombine somewhere else in the genome, thereby posing the cell with the risk of losing genomic stability. Activity of another member of the RecQ family, the WRN protein, in maintenance of stalled replication forks has been anticipated in human cells. Following DNA replication arrest, WRN associates and colocalises in an MRE11-dependent manner, with the MRE11 complex at sites also including the DNA polymerase processivity factor PCNA [196]. Consequently, Werner Syndrome cells carrying a mutated WRN gene have a slower rate of repair associated with DNA damage-induced in S phase and reduced induction of RAD51 foci [197]. These findings support the hypothesis that WRN as well as BLM could be involved in correct resolution of recombinational intermediates that arise from replication arrest due to either DNA damage or replication fork collapse [162, 198, 199].

In yeast stabilisation of replicative polymerases at the site of a stalled replication fork requires the presence of the yeast homologue of RecQ, Sgs1 [200-202]. In its absence, RPA dissociates from ssDNA near the stalled fork [202]. Moreover, an accumulation of aberrant DNA replication intermediates is observed in yeast *sgs1* mutant cells [203]. Therefore, the recombinational helicases can play an important role not only during fork regression and reversal of created intermediate but might also be necessary for the maintenance of the structure of the regressed fork.

D-loop formation

The accumulation of unrepaired chromosomal breaks during S phase together with the increase of replication blockage in cells depleted of Rad51 support the notion that blocked replication forks can subsequently be transformed into D-loops [188, 204]. Similarly as in bacteria, this can be achieved in a DSB-dependent and –independent manner. The resolution of HJs by a specified endonuclease can result in release of a one-ended DSB, which after processing can be a substrate for the Rad51 recombinase (Figure 6E) [180, 205, 206]. This possibility is in agreement with findings that CHO cells overexpressing Rad51 are resistant to treatment with agents inducing DSBs at replication sites [207]. Additionally, Rad51 can catalyse D-loop formation when a DSB is generated by fork processing through a ssDNA break or gap (as discussed in the BIR section). Moreover, CHO cells overexpressing Rad51 are more resistant than wild type cells to treatment with thymidine, which slows replication but does not induce DSBs [207]. This suggests that invasion of a homologous DNA duplex by a free ssDNA stretch can depend on the activity of the Rad51 protein. Reconstitution of a replication fork performed in this manner avoids generation of a potentially deleterious DSB (Figure 6B).

Resolution

In order to continue DNA metabolism HJs formed upon replication arrest require resolution. The human RAD51 paralogues, RAD51C and XRCC3, were shown to be associated with HJ resolvase activity and branch migration activities [164]. Additionally, BLM and RAD51 interact with each other, thus it is very likely that Rad51 activity is not only limited to D-loop formation but that it is also involved in resolution of HJs [208]. Therefore it is possible that, similarly to bacteria, also in eukaryotic cells stalled replication forks can reverse resulting in a HJ containing structure, which in turn can serve as an intermediate for further repair.

Concluding remarks

The data summarised here clearly shows the existing gap in understanding of crucial cellular processes between bacteria and eukaryotes. Division of HR into two sub-pathways, RecFOR- and RecBCD-mediated, was demonstrated only in the bacteria. Depending on whether a DSB or a ssDNA gap occurs, RecA is loaded onto the ssDNA by RecBCD or RecFOR proteins, respectively. Elaborate and mechanistically distinct controls of loading Rad51 onto different substrates for HR are likely to exist in eukaryotes as well. Yet research in this area is still trying to come to grips with the many proteins and their enzymatic activities that are likely to be involved, before a clear division into pathways can be made. Similarly, the elegant mechanisms of origin-independent PriA-mediated restart of replication revealed in bacteria await eukaryotic counter parts.

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CHAPTER 2

Functional analysis of a Rad54-GFP knockin construct in mouse embryonic stem cells

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ABSTRACT

Gene targeting is a widely used technique that allows the definition of *in vivo* functions of various genes by inactivating them. Since its creation the procedure has undergone multiple modifications. Besides deleting genes, this method can also be used to modify endogenous loci through the knockin approach. Using this variant of gene targeting a modified version of a gene of interest is expressed under the control of its endogenous promoter. Rad54 is an important protein for the repair of DNA double-stranded breaks by homologous recombination (HR) and can be used as a cellular marker for HR. Here we report the establishment of a *Rad54-GFP* knockin system. In addition to creating *Rad54-GFP* knockin cells in an otherwise wild type background, we also generated the knockin in the context of a nucleotide excision repair-deficient background. This gives us an opportunity to investigate the role of Rad54 protein in cellular processes other than HR, such as, for example, the restart of stalled replication forks due to the presence of UV-light-induced lesions (described in Chapter 3). In this chapter we present results of multiple control experiments that verified the reliability of the *Rad54-GFP* knockin system created.

INTRODUCTION

The advent of reverse genetics has given great insights into the function of genes involved in mammalian DNA repair. Deletion or (over)expression of numerous genes has provided (often crucial) evidence for their function [1]. For example, inactivation of Rad54 in mouse embryonic stem (ES) cells revealed its importance in the process of homologous recombination (HR), which is one of the processes involved in the repair of ionising radiation-induced DNA double-stranded breaks (DSBs) [2]. Importantly, the ionising radiation-hypersensitive phenotype of *Rad54*$^{-/-}$ ES cells exposed the significant role of HR-mediated DSB repair in mammalian cells. This was contrary to the existing dogma that in *S. cerevisiae* and mammals two different repair pathways contribute to ionising radiation resistance. The role of HR in guarding of *S. cerevisiae* cells against ionising radiation was underscored by the sensitivity of the HR mutants to the treatment. However, in mammalian cells the only mutants sensitive to ionising radiation known at the time were those in which another DSBs repair pathway, non-homologous end-joining was impaired whilst the HR mutants were lethal. A number of further aspects of DNA repair were revealed by the *in vivo* analysis of proteins implicated in the process by fusing them to tags that can be observed in living cells. For instance, fusion of a green fluorescent protein (GFP) tag to the members of the Rad52 family exposed the dynamic behaviour of these proteins in living cells, both in the absence and presence of DNA damage [3, 4]. However, in these studies the cDNA constructs containing an ectopic promoter were transfected into cells resulting in higher than endogenous levels of expression of the tagged proteins. To address this problem we employed a modification of gene targeting using knockin constructs. The knockin method relies on proper integration of the tagged gene in the endogenous locus. Consequently, the modified gene is controlled by its natural regulatory elements and the possibility of proper regulation of the expression of the tagged gene is enhanced.

In order to be able to follow HR events in cells we used Rad54 protein as a cellular marker of recombination processes. In contrast to other recombination knockouts such as Rad51 or its paralogues, which are lethal, the Rad54 knockout is viable yet sensitive to ionising radiation [2, 5-10]. Additionally, it is easy to follow the cellular behaviour of Rad54 protein since it forms readily observable foci upon ionising radiation treatment [11].

In this study we were able to demonstrate that the modified *Rad54* allele encodes a protein that protects cells from ionising radiation. Additionally, Rad54-GFP does not interfere with cellular sensitivity to UV-light. Finally, Rad54 foci detected directly by GFP fluorescence colocalise with the foci detected by anti-Rad54 antibodies. Therefore, we conclude that *Rad54-GFP* encodes a functional variant of *Rad54*.

EXPERIMENTAL PROCEDURES

DNA constructs

A *RAD54–GFP* targeting construct was made to obtain expression of tagged *Rad54* from the endogenous promoter upon targeted integration. To obtain expression of Rad54 with a carboxy-terminal GFP tag a *GFP* gene was cloned behind the *hRAD54* cDNA. This *GFP*-tagged cDNA was subcloned into pPGK–p(A) to add a polyadenylation signal. The *Rad54–GFP* cDNA was cloned in front of a puromycin resistance gene under the control of a phosphoglycerate kinase (PGK) promoter. Using linkers, a downstream *Sfu* I site was introduced. Digestion of this construct with *Sfu* I yielded a fragment containing the 3'-terminal part of the *Rad54–GFP* cDNA spanning exons 4 to 18 and the puromycin resistance cassette. This fragment was subcloned into the unique *Sfu* I site in exon 4 of a 9 kb *Eco* RI fragment of the mouse *Rad54* genomic sequence containing exons 4–7 in pBluescript II KS. The construct is schematically depicted in Figure 1A.

ES cells culture and gene targeting

Wild type (C57Bl/6) and *Xpa*$^{-/-}$ ES cells were cultured in BRL-conditioned medium supplemented with 1000 U/ml leukaemia inhibitory factor. To generate ES cells expressing Rad54-GFP, 20 μg of *Pvu* I-linearised *Rad54-GFP* knockin construct was electroporated into approximately 10^7 ES cells in 500 μl ES cell medium. Selection with 1 μg/ml of puromycin was started 24 h after electroporation. After 8–10 days, puromycin-resistant clones were isolated. Screening for homologous recombinants was performed using DNA blot analyses of *Stu* I-digested DNA with a probe containing exons 7 and 8. In case of homologous targeting apart from an endogenous band of 9.0 kb a 6.5 kb doublet band appears. Protein extracts from the targeted clones with the expected hybridisation pattern were subsequently analysed for the expression of the *GFP*-tagged protein. To generate *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells, the *Rad54-GFP* knockin construct was also electroporated in *Xpa*$^{-/-}$ cells.

Immunoblots

For whole cell protein extracts, cells were trypsinised, pelleted, resuspended in electrophoresis sample buffer, and sonicated. Whole cell extracts were electrophoresed through SDS-PAGE gels and blotted to nitrocellulose. The blots were hybridised with anti-Rad54 [2] or anti-GFP antibodies (Roche).

Fluorescence-activated cell sorting (FACS) analysis

Wild type (C57Bl/6) ES cells, *Rad54*$^{+/GFP}$ ES cells and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells were trypsinised and resuspended to single cell suspension in phosphate buffered saline (PBS). They were fixed for 15 min with 1% *para*-formaldehyde at room temperature. After permeabilisation with 0.1% Triton X-100 and RNase treatment, cells were analysed in a Becton Dickinson FacsCalibur on

a green fluorescence (FL1) versus forward scatter (FSC-H) plot. In a fluorescence (GFP) histogram, non-GFP- and GFP-expressing cells appear as separate peaks.

Immunofluorescence and confocal microscopy imagining

Cells were trypsinised and seeded at approximately 25% confluency on glass coverslips coated with 0.1% gelatin. Twenty-four hours later, cells were irradiated with 12 Gy of ionising radiation. Afterwards, cells were washed and incubated in fresh medium at 37°C for 2 h and then fixed with 2% *para*-formaldehyde in PBS, pH 7.4,for 15 min. The Rad54 protein was detected by rabbit polyclonal anti-Rad54 immunoglobin (1:100) [2]. For the purpose of visualisation of the immuno-conjugated protein alexa 594-conjugated goat anti-rabbit immunoglobin (1:1000; Molecular Probes) was used. Both immunoglobins were diluted in PBS with 0.15% glycine and 0.5% bovine serum albumin. Nuclear staining patterns were visualised with a Zeiss confocal laser scanning microscope LSM 510 META. To detect GFP fluorescence the images were recorded with a 488 nm Ar-laser, immunostained Rad54 was detected by 543 nm HeNe-laser.

Colony survival curves

Sensitivity of ES cells to increasing doses of DNA-damaging agents was determined as described previously [2]. Briefly, cells were plated in 6 cm dishes, at various dilutions. After 12-16 h, cells were irradiated with a single dose in the range of either 0-8 J/m^2 of UV-C (254 nm) or 0-8 Gy of ionising radiation. Subsequently, cells were grown for 7-10 days, fixed, stained and colonies were counted. All experiments were performed in triplicate.

RESULTS

Expression of GFP tagged Rad54 from the endogenous promoter

We designed a knockin construct for *Rad54* such that a GFP tagged Rad54 protein would be expressed from its endogenous locus. This construct was generated by fusing exons 4-18 of the *hRAD54* cDNA to a 3'-terminal *GFP* tag, followed by a poly(A) signal and a puromycin selectable marker gene. This fragment was subcloned into exon 4 of a 9-kb *Eco* RI genomic *Rad54* fragment (Figure 1A). The knockin construct was targeted into wild type and *Xpa*$^{-/-}$ ES cells. Colonies obtained after the selection were screened for the presence of the appropriate integration event using DNA blotting (Figure 1B). Cells were obtained which contained one *Rad54* wild type allele and one *Rad54* knockin allele as evidenced by the appearance of the appropriate bands on DNA blots (see Experimental Procedures). These cells are referred to as *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$. Expression of *Rad54* from the knockin allele resulted in the production of Rad54-GFP protein from the endogenous *Rad54* promoter. This offered two major advantages. The tagged protein should mimic the endogenous expression level and could easily be detected by direct fluorescence.

Therefore, we next examined the expression level of *Rad54-GFP*. The *Rad54-GFP* encodes a protein of 1021 aa with a predicted molecular weight of 112.3 kDa whilst the wild type Rad54 protein contains 747 aa with a predicted molecular weight of 84.6 kDa. Proteins of the expected molecular weight for Rad54 and Rad54-GFP were detected in extracts from *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells by immunoblotting using affinity-purified anti-hRad54 antibodies (Figure 1C). Furthermore, the relative protein levels produced by the *Rad54* wild type allele and the *Rad54* knockin allele are indistinguishable in both cell lines analysed (Figure 1C). The tagged protein was also detected using anti-GFP antibodies (Roche). No degradation or other protein-GFP fusion products could be observed (Figure 1D). Both tagged and untagged Rad54 were absent from *Rad54*$^{-/-}$ ES cells and the tagged protein could not be detected in wild type and *Xpa*$^{-/-}$ ES cells (Figure 1C and D). We conclude that the expression level of tagged protein in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells is similar. Moreover, the GFP fluorescence signal is derived by intact Rad54-GFP fusion protein. Subsequently we used FACS analysis to investigate Rad54-GFP in the cell population. Distribution of fluorescent positive cells within the *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa* $^{-/-}$ cell populations was similar confirming that the Rad54-GFP protein expression levels in both ES cell lines are comparable (Figure 1E). In addition, the presence of a single fluorescence peak for each cell line indicates that the tagged protein is homogenously expressed in the respective cell populations. Additionally, no overt differences in growth rates, morphology of the cells as well as in the differentiation state between generated cell lines were observed.

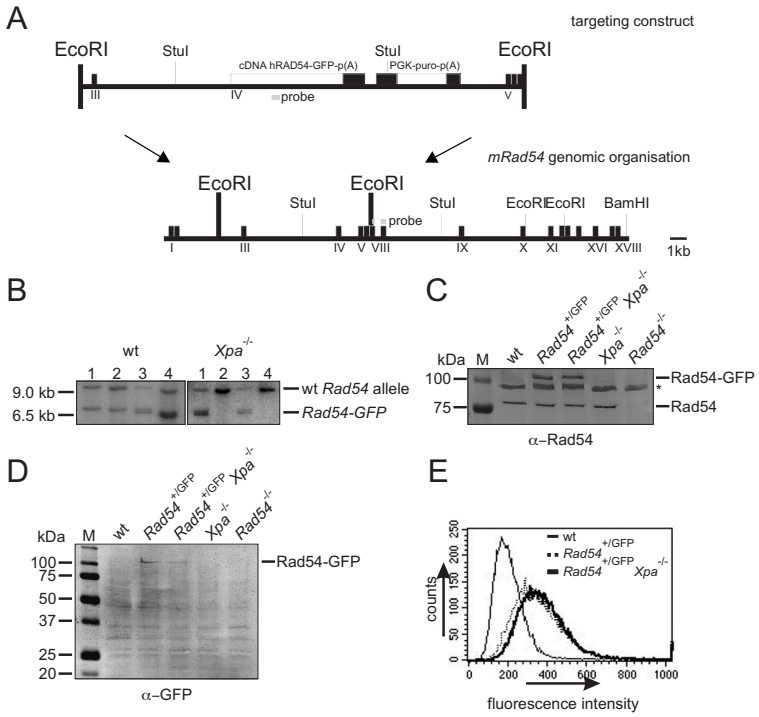


Figure 1. **Generation of ES cells expressing Rad54-GFP from the endogenous *Rad54* locus.**

(**A**) *Rad54-GFP* knockin targeting construct. Structure of the genomic *Rad54* locus and gene targeting construct. The lower line represents the approximately 30 kb mouse *Rad54* genomic locus. All 18 exons of the *Rad54* cDNA are indicated by black boxes. The targeting construct (upper line) was generated by fusing the human *RAD54* cDNA from exon 4 up to, but not including the stop codon, to *GFP* and a downstream p(A) sequence followed by a puromycin selection marker. Absence of a promoter and the first three exons from the targeting construct results in expression from the endogenous *Rad54* promoter only after homologous targeting. The DNA probe used in blotting experiments is indicated by grey bars and detects exon 7 and 8. Positions of diagnostic *Stu* I restriction endonuclease sites are indicated. (**B**) DNA blots to detect the *Rad54-GFP* knockin construct in wild type and *Xpa*$^{-/-}$ ES cells. For each targeted cell line four clones are shown. Genomic DNA isolated from clones transfected with the *Rad54-GFP* knockin construct was digested with *Stu* I restriction endonuclease. DNA blotting using the indicated probe identified homologous integration events. The position of wild type (9.0 kb) and targeted locus (6.5 kb) are indicated. (**C**) Immunoblot of protein extracts from ES cells used in this study. The expression of Rad54-GFP was confirmed by immunoblot analysis of whole cell extract with anti-Rad54 or (**D**) anti-GFP antibodies. Positions of wild type and GFP-tagged Rad54 are indicated. Extracts of wild type, *Rad54*$^{-/-}$ and *Xpa*$^{-/-}$ cells were used as controls. The nature of the protein cross-reacting with the Rad54 antibody, indicated by asterisk is unknown. (**E**) FACS analysis of the level of expression of Rad54-GFP in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells. A wild type none GFP containing cell line was used as a control. Shown is fluorescence intensity versus the number of cells. The expression of the GFP results in the shift of the fluorescence peak to the right relative to the peak of non-fluorescent cells.

The *Rad54-GFP* knockin construct encodes a functional variant of Rad54

To test whether *Rad54-GFP* encodes a functional variant of Rad54 we investigated the ability of the fusion protein to protect cells from ionising radiation. We have shown previously that expression of Rad54 from a single allele is sufficient to protect cells from ionising radiation because *Rad54*$^{+/-}$ ES cells are as resistant to the treatment as wild type cells [2]. Therefore, here we analysed the functionality of the Rad54-GFP fusion protein in response to ionising radiation in cells expressing Rad54 from the knockin allele only. For that purpose, we generated a *Rad54*$^{-/GFP}$ ES cell line by targeting the remaining *Rad54* wild type allele with a *Rad54-GFP* knockin construct in the *Rad54*$^{+/-}$ cell line (data not shown). Subsequently, the ionising radiation sensitivity of the *Rad54*$^{-/GFP}$ ES cell line was determined. While the *Rad54*$^{-/-}$ ES cells were hypersensitive to ionising radiation compared to *Rad54*$^{+/-}$ cells, the *Rad54*$^{-/GFP}$ ES cells displayed a similar sensitivity to the treatment as *Rad54*$^{+/-}$ cells (Figure 2A). We conclude that the *Rad54-GFP* knockin allele encodes a Rad54-GFP fusion protein that protects cells against ionising radiation as efficiently as the wild type Rad54 protein.

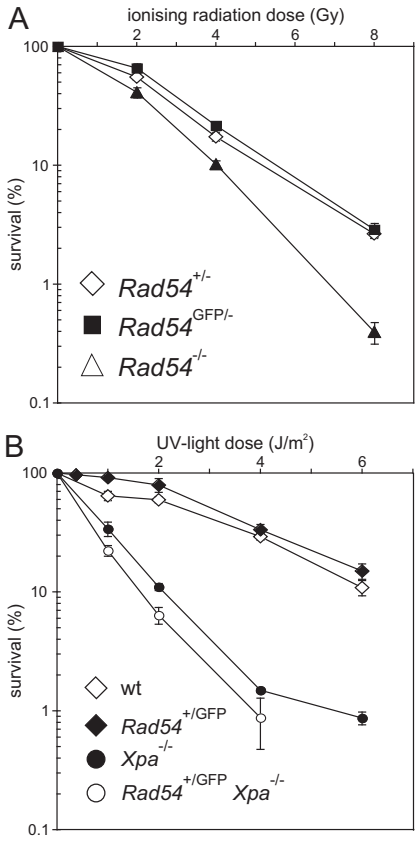


Figure 2. **Effect of ionising radiation and UV-light on survival of wild type, *Xpa*$^{-/-}$, *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells.**

Clonogenic survival of ES cells treated with (**A**) ionising radiation or (**B**) UV-light. After treatment with increasing doses of DNA-damaging agents cells were grown for 7-10 days, fixed, stained and colonies were counted. The ability to form colonies is plotted as function of increasing dose of DNA damage. Error bars represent standard error of the mean (SEM). Genotypes of analysed cell lines are indicated.

In order to exclude the possibility that the presence of GFP tagged Rad54 in *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells could interfere with their sensitivity to UV-light we checked the ability of *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cell lines to form colonies after treatment with increasing doses of UV-light. Both wild type as well as *Rad54*$^{+/GFP}$ cells were only mildly, and equally, sensitive to increasing doses of UV-light. However, colony formation was severely reduced for both *Xpa*$^{-/-}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cell lines (Figure 2B). We conclude that the presence of the *Rad54* knockin allele has no influence on the resistance of the analysed cell lines to UV-light.

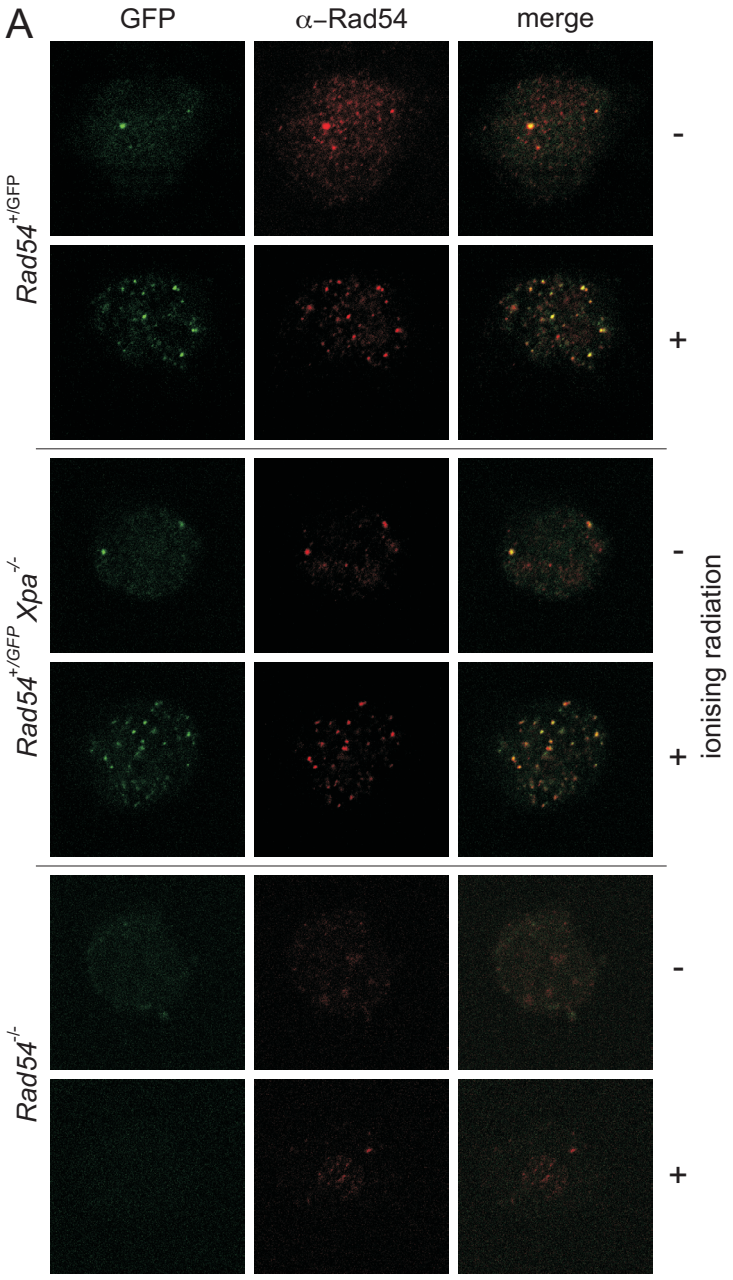
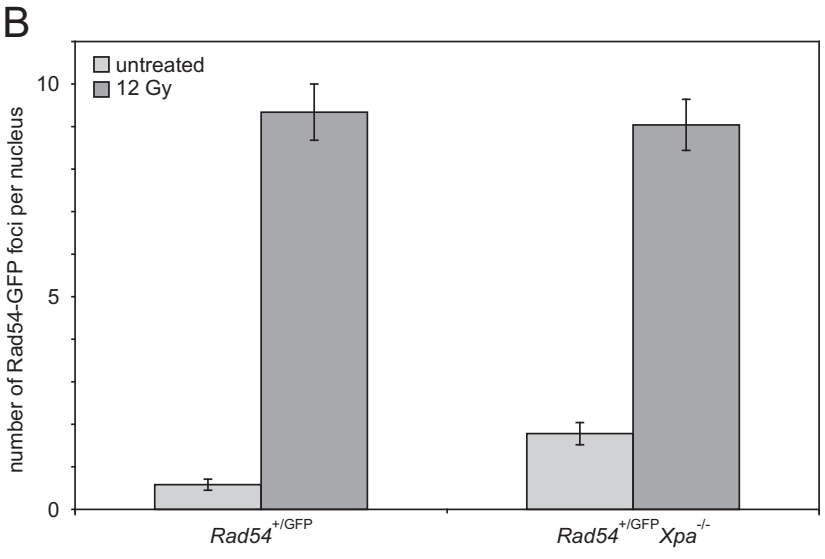


Figure 3. **Ionising radiation induces equal Rad54 foci formation in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells.**

(**A**) Confocal image of ES cells expressing Rad54-GFP. Shown are *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells and *Rad54*$^{-/-}$ cells untreated (-) and 2 h after treatment with 12 Gy of ionising radiation (+). Cells were fixed and stained with anti-Rad54 antibodies under conditions that did not disturb the detection of fluorescence from GFP. The fluorescence signal from Rad54-GFP



is shown in the column on the left. The signal detected by the Rad54 antibody is shown in the centre column. The column on the right displays the merged images, in which yellow demonstrates colocalisiation. (**B**) Quantification of the number of Rad54-GFP foci per nucleus, before (light grey bars) and after irradiation (dark grey bars). At least fifty nuclei were examined per data point. The error bars represent standard error of the mean (SEM)

Rad54 foci formation in *Xpa*$^{-/-}$ ES cells after ionising radiation treatment

We have previously shown by immunofluorescence that after ionising radiation treatment mouse Rad54 redistributes within the nuclear compartment and can be detected as bright foci [11]. To determine whether the Rad54-GFP encoded from the knockin construct retains this ability in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells, we exposed both cell lines to 12 Gy of ionising radiation. Two hours post treatment cells were fixed with 2% *para*-formaldehyde and immunostained with affinity-purified anti-hRad54 antibodies as described [11]. Appearance of Rad54 foci was analysed with a confocal microscope (Figure 3). As previously shown for wild type cells [11], Rad54 foci were clearly induced by ionising radiation in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells. Similar staining patterns were observed when detecting the Rad54 protein through either the GFP signal or anti-Rad54 antibodies. We conclude that Rad54-GFP functions analogous to Rad54 in terms of DNA damage-induced foci formation.

In the same experiment we also analysed the number of Rad54-GFP foci formed in *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells after treatment of the cells with ionising radiation. Interestingly, the number of spontaneously formed foci in untreated cells was higher in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells compared to *Rad54*$^{+/GFP}$ cells; on average 1.8 and 0.6 foci/cell, respectively (Figure 3B). On the other hand, the induction of foci formation upon ionising radiation treatment was similar in both cell lines; on average 9.0 and 9.3 foci/cell, respectively (Figure 3B). From this experiment we conclude that ionising radiation-induced Rad54 foci formation is unaffected by the absence of the Xpa protein from ES cells.

DISCUSSION

The role of Rad54 protein in DSBs repair has been shown in many different ways by many investigators, reviewed in [12-17]. Specifically, Rad54 is involved in protecting cells from ionising radiation, as *Rad54* $^{-/-}$ ES cells are hypersensitive to the treatment [2]. Additionally, upon challenging cells with DNA-damaging agents, Rad54 redistributes within nucleus into distinct foci [11,18]. This provides a well-established control system than can be used to analyse properties of newly generated mutants of Rad54 or to study Rad54 in the context of different genetic backgrounds. We used this knowledge to confirm the functionality of Rad54-GFP protein at the cellular level in DSBs repair.

In this study we used a *Rad54-GFP* knockin construct that contains a 5'-terminal part of *Rad54* fused to GFP. When homologously recombined into its endogenous locus the gene is expressed from its endogenous promoter, which should result in natural level of expression of Rad54-GFP. Targeting of only one allele of Rad54 with Rad54-GFP provided an internal control for the relative amount of tagged versus untagged Rad54 protein in these cells. Therefore, we compared the quantities of proteins encoded by both the endogenous and the knockin allele. As shown on the immunoblot in Figure 1C the intensity of bands corresponding to Rad54 and Rad54-GFP is similar. Furthermore, there is no difference in Rad54-GFP levels between Xpa-proficient and -deficient cell lines. This shows that in both generated cell lines Rad54-GFP is most likely expressed at its endogenous level. Additionally, the expression of Rad54, as well as Rad54-GFP, is unaffected by the absence of the Xpa protein.

In order to assure that within the cell populations Rad54-GFP is homogenously expressed, we checked the fluorescence intensities of GFP by FACS analysis in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells. The presence of one fluorescence peak only for each examined cell population confirms homogenous expression of GFP tagged Rad54. Additionally, the fluorescence peaks of both the Xpa-deficient and -proficient cells overlap, confirming again the same content of Rad54-GFP protein in these cell lines. Using anti-GFP antibodies we were able to detect only a single band whose size corresponds to Rad54-GFP. We conclude that the analysed cells do not contain free GFP or truncated proteins that might arise from proteolytic degradation or abnormal transcription/translation of the Rad54-GFP fusion protein.

To verify that Rad54-GFP encodes a protein that is functional and does not interfere with cellular metabolism, we investigated the resistance of the generated cells to DNA-damaging agents. It has been shown before that *Rad54*$^{-/-}$ ES cells are sensitive to ionising radiation and that this sensitivity can be rescued by expression of cDNA encoded Rad54 [2,3]. In order to analyse whether the Rad54-GFP protein can rescue the phenotype of *Rad54*$^{-/-}$ ES cells, we analysed the sensitivity of *Rad54*$^{-/GFP}$ ES cells to ionising radiation. In contrast to *Rad54*$^{-/-}$ cells, that are hypersensitive to the treatment, *Rad54*$^{-/GFP}$ cells are as sensitive to ionising radiation as wild type cells. This confirms

that the GFP tag does not interfere with the abilities of Rad54 protein to perform repair of ionising radiation-induced DNA damage. Additionally, we showed that Rad54-GFP present in both, wild type and *Xpa*$^{-/-}$ cells does not influence the sensitivity of these cells to UV-light. Thus, we conclude that the *Rad54-GFP* knockin construct encodes a functional protein that does not disturb the cellular processes analysed here.

We generated cells expressing Rad54-GFP protein to perform diverse fluorescent microscopy analyses. These cells, apart from a GFP tagged Rad54 protein also express wild type, unmodified Rad54 protein, that cannot be detected by direct fluorescence. In order to exclude the possibility of a bias between wild type and tagged Rad54 protein in response to DNA damage we visualised Rad54 in cells challenged with ionising radiation using direct detection of GFP fluorescence combined with detection of Rad54 protein by antibodies. Images obtained from confocal microscopy analysis show that Rad54 foci detected by direct GFP signal fully correspond to these visualised by anti-Rad54 antibodies. Additionally, the number of Rad54 foci formed after ionising radiation treatment is the same in wild type and *Xpa*$^{-/-}$ cells with the interesting exception of untreated cells. This implicates that under normal culture conditions, HR appears to be activated in the absence of functioning NER. This phenomenon will be discussed in the Chapter 3.

We conclude that the *Rad54-GFP* knockin system established here is fully representative of the wild type situation and can be used for further investigations. Endogenous expression control of the GFP tagged Rad54 yields physiological levels of Rad54-GFP protein and overcomes the intrinsic limits of analysis of fixed cells. Therefore, it is possible to analyse the dynamics of the Rad54 protein by fluorescence recovery after photobleaching experiments in response to various DNA damage. Additional introduction of a further, differently tagged, protein provides an opportunity to investigate DNA damage-induced subnuclear changes in localisation of the two proteins in the context of living cells. Further expansion of this knockin system, for example by deleting other genes, will make it possible to define more precisely the discrete stages of the process of HR.

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CHAPTER 3

Rad54-mediated response to UV-light treatment of mouse embryonic stem cells

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ABSTRACT

Rad54 is a member of the Rad52 protein family that acts in the process of homologous recombination (HR). HR is a process responsible for repair of a range of DNA lesions including DNA double-stranded breaks (DSBs), which can be generated by ionising radiation. Additionally to DSBs repair, HR plays a role in restarting stalled replication forks, as shown in *E. coli*. Another DNA repair process, nucleotide excision repair (NER), removes UV-light-induced DNA lesions. Here, using Rad54-GFP knockin mouse embryonic stem (ES) cells, we investigated the distribution of the Rad54 protein in both wild type and NER-deficient ES cells in response to UV-light-induced DNA damage. Our data suggest that HR is activated by UV-light, particularly in NER-deficient ES cells, which is reflected by redistribution of Rad54 into foci upon the treatment. Both subnuclear localisation and appearance of UV-light-induced Rad54 foci differs from those induced by ionising radiation. We suggest that activation of HR by UV-light is required to support disrupted replication rather than to repair two-ended DSBs.

INTRODUCTION

The integrity of genomic DNA is constantly being threatened by various endogenous and exogenous damaging agents. Therefore it is important for cells to protect their DNA. For that purpose cells have evolved numerous elaborate pathways responsible for repair of damaged DNA [1]. Failure of any of the crucial components of the repair machinery can leave the cell with a mass of unrepaired DNA lesions. Accumulation of these lesions can hinder vital DNA metabolism such as replication and transcription. Incomplete duplication of chromosomes can result in their unbalanced distribution into daughter cells during cell division, giving rise to translocations, deletions or duplication of chromosomal fragments. The disturbance of the delicate balance in organisation of DNA in a nucleus can have dangerous consequences for the cell and the whole organism. It can lead to disturbed signalling pathways, disabled control of cell cycle checkpoints, effect apoptosis, or cause cell immortalisation and transformation into tumours [2-5].

Different types of damage are removed from DNA by different, specialised repair pathways [1]. For example helix-distorting lesions, generated by UV-light, are predominantly removed by nucleotide excision repair (NER). Another DNA repair pathway, homologous recombination (HR) removes DNA damage that includes affects both strands of the helix, such as ionising radiation induced DNA double-stranded breaks (DSBs). The importance of NER and HR for maintenance of genomic integrity is underscored by a number of rare human hereditary diseases, that are associated with defects in these DNA repair pathways. Severe symptoms, such as malignant skin cancer, mental and physical retardation or premature aging are diagnosed in patients harbouring altered genes of NER components [6-8]. Mutations in one of the HR genes, Brca2 cause hereditary breast and ovarian cancers [9, 10]. In addition, mutations in the Rad50/Mre11/Nbs1 complex, also involved in HR lead to the radiosensitive and cancer prone disorders, ataxia telangectasia-like disorder and Nijmegen breakage syndrome [11-14].

The NER pathway removes lesions from the DNA that distort the regular structure of a DNA molecule, such as UV-light-induced cyclobutane pyrimidine dimers (CPDs) or pyrimidine-(6,4)-pyrimidone adducts (6-4PPs). During removal of the DNA lesion by NER the altered DNA helix is recognised by Xpc and Xpe proteins and subsequently unwound by the TFIIH complex [15, 16]. The resulting single-stranded DNA (ssDNA) is coated by replication protein A (RPA), which results in stabilisation of the open DNA structure and stimulation of binding of Xpa protein to the site of damage [17, 18]. Next, the Xpa protein that freely diffuses in the cell, binds to the marked damage site [7, 19]. In this way, the damage-containing DNA strand can be recognised by the structure-specific nucleases, Xpg and Xpf-Ercc1, resulting in the excision of the damaged nucleotides, after which the generated gap is filled by the DNA replication machinery and the ensuing nick is sealed by DNA ligase. Inactivation of the protein acting early in the process, Xpa, completely abolishes the capacity of the NER pathway to execute the repair process. Consequently,

mice lacking Xpa are totally NER-deficient and develop skin cancer when exposed to UV-light [20]. Additionally, *Xpa* knockout ES cells are UV-light sensitive [21].

HR is one of the major pathways for repair of DSBs and ssDNA gaps. DSBs occur during normal chromosomal metabolism such as DNA replication or crossing over in meiosis. Another, and dangerous for cell functionality, source of DSBs are external damaging factors like ionising radiation and a number of chemical agents [22, 23]. However, under certain circumstances, the recombination activity of HR proteins can be induced without the presence of a DSB. For example, the bacterial RecFOR protein complex of HR is engaged in ssDNA gap repair [24]. During HR several distinct processes can be identified: (1) initiation; DNA ends processing into 3' overhangs and Rad51 nucleoprotein filament formation, (2) search for homologous DNA and DNA strand exchange; invasion by the Rad51 nucleoprotein filament of the homologous dsDNA template and joint molecule formation, (3) DNA heteroduplex extension; branch migration and (4) resolution [24]. The formation of a joint molecule between the damaged DNA and the homologous repair template is a crucial step in HR that is mediated by Rad51 and stimulated by Rad54. Rad54 is thought to transiently unwind a duplex DNA substrate and thereby help in the homology search by Rad51 nucleoprotein filament [25-28]. It has been demonstrated that Rad54 also plays a role at the late steps of HR, after the joint molecule has been formed successfully. The extension of the heteroduplex, branch migration and replication of a missing stretch of DNA all require transient modification of DNA structure. Finally, after the break has been fixed, repair proteins have to be removed from the repaired DNA site. The ability of the Rad54 protein to disassemble Rad51 filament formed on dsDNA suggests that by tracking along the DNA molecule it can also remove other residual proteins [28, 29].

Duplication of genomic DNA inevitably involves replication of a DNA molecule that contains lesions. The DNA lesions left unrepaired before the arrival of replication machinery can lead to a replication block, which, if not eliminated, can subsequently result in disruption of the replication process [30]. Consequently, prematurely blocked replication leaves the cell with genomic DNA that has been only partially duplicated. Distribution of such asymmetrically copied genome during cell division alters the DNA content in at least one of daughter cells. In order to prevent such potentially deleterious events cells have evolved mechanisms protecting the integrity of a genome during the process of replication.

HR is proposed to act upon DNA damage present at the site of a replication fork so as to support the resumption of replication. In bacteria ssDNA gaps are repaired by RecFOR-mediated recombination. These DNA structures are very likely to arise at sites of stalled replication forks [31]. Therefore, HR mediated by the RecFOR proteins could be involved in restart of disturbed replication [24]. Additionally, replication of a DNA template containing a ssDNA nick or gap results in generation of a DSB at the site of a collapsed replication fork. However, this leads to generation of only one end of a dsDNA molecule [32]. This contrasts with a two-ended DSB

generated by ionising radiation, which is therefore also a substrate for an alternative DSB repair pathway; non-homologous end-joining. The mode of action of HR proteins, that can fix a break with one dsDNA end only, implies their involvement in the repair of a collapsed replication fork. The majority of the information about the mechanism of resolution of a blocked replication fork comes from studies of various *E. coli* mutants that are impaired in replication and HR. It has been shown that viability of cells with disturbed replication strongly depends on the HR. Many mutants in which both replication and HR have been impaired are not vital [33]. This indicates the involvement of proteins of HR in the restart of a stalled or collapsed replication fork. In contrast to repair of two-ended DSBs in higher eukaryotes, not much is known about the mechanism of repair of damage with only one dsDNA end. Recently, evidence implicating an important role of the Rad51 protein in one-ended repair events has come from observations in yeast and chicken cells. The level of break-induced replication, which represents one of the possible mechanisms of repair of a collapsed replication fork, is severely diminished in *rad51*Δ yeast cells [34]. Additionally, accumulation of unrepaired chromosomal breaks during S phase was observed in *Rad51*$^{-/-}$ DT40 cells [35]. These observations suggest that Rad51 might be indispensable for restarting replication in eukaryotic cells. A possible role could be in the formation of a D-loop at the site of a stalled replication fork as has been proposed for RecA, the bacterial Rad51 homologue [36]. In the process of HR, Rad51 together with other proteins, like Rad54, mediates this step *in vitro* [26]. It is then possible that a replication-related activity of Rad51 in eukaryotic cells involves formation of an analogous structure.

To elucidate the role of HR in the repair of stalled replication forks we investigated changes in subnuclear localisation of Rad54 protein in cells in which replication has been prematurely blocked by UV-light-induced DNA lesions. These lesions are likely to be removed from the genome by the NER pathway. In order to increase the possibility of replication fork stalling upon UV-light irradiation, we included NER-deficient, *Xpa*$^{-/-}$ ES cells apart from NER-proficient cells. In both cell lines one allele of the genomic Rad54 was replaced with the Rad54-GFP knockin construct (Chapter 2). Here we demonstrate that upon UV-light treatment Rad54 more readily forms foci in NER-deficient cells when compared to NER-proficient cells. Additionally, the subnuclear redistribution of Rad54 caused by UV-light differs from the one induced by ionising radiation and resembles the pattern formed by a protein correlated with replication sites, proliferating cell nuclear antigen (PCNA). Moreover, UV-light-induced Rad54 foci are smaller and of a different shape than those induced by ionising radiation. In spite of these differences we detect another HR protein, Rad51 as well as γ-H2AX in the UV-light-induced Rad54 foci, as it happens for Rad54 foci generated by ionising radiation. Therefore, we conclude that under certain circumstances in ES cells, UV-light-induced DNA damage activates HR.

EXPERIMENTAL PROCEDURES

ES cells culture

Rad54$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells were cultured in BRL-conditioned DMEM medium containing 10% FCS and supplemented with 1000 U/ml leukaemia inhibitory factor.

Immunofluorescence and confocal microscopy imaging

Cells were trypsinised and seeded at approximately 25% confluency on glass coverslips coated with 0.1% w/v gelatin. Thirty-six hours later, the cells were irradiated with γ-rays (8 or 12 Gy) or UV-C (254 nm, 1 J/m^2). Afterwards, the cells were washed with PBS and incubated in fresh medium at 37°C for the indicated time and then fixed with 2% *para*-formaldehyde in PBS, pH 7.4, for 15 min. Cells were permeabilised with 0.1% Triton X-100 in PBS, and the phosphorylated form of H2AX (γ-H2AX) was detected with polyclonal anti-γ-H2AX immunoglobin (1:1000, Upstate Biotechnologies). To detect Rad51 protein rabbit polyclonal anti-Rad51 immunoglobin was used [22, 37]. For visualisation of the immuno-conjugated proteins alexa 594-conjugated goat anti-rabbit immunoglobin (1:1000; Molecular Probes) was used in both cases. All immunoglobins were diluted in PBS with 0.15% glycine and 0.5% bovine serum albumin. Nuclear staining patterns were visualised with a Zeiss confocal laser scanning microscope LSM 510 META. Images were recorded with a 488 nm Ar-laser to detect Rad54-GFP and 543 nm HeNe-laser to detect the immunostained proteins.

Analysis of foci appearance

The area of DNA damage-induced foci was calculated as π x (A x B / 2) where A and B represent two longest perpendicular axis of a focus. The length of the axis of a focus in a randomly selected confocal plane was determined using LSM software (Zeiss). The shape of a focus is represented as a ratio between these two axes. In case of a circle this value becomes 1, whilst values bigger than 1 represent an ellipsoid shape. Both parameters were determined for thirty individual foci.

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

For cell cycle analysis *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells (60–70% confluency) on 6 well culture dishes were treated with UV-C (254 nm, 1 J/m^2). At different time points after treatment, cells were collected and fixed with 70% ethanol. After a minimum of 2 h on ice, cells were washed with PBS and resuspended in 400 μl PBS containing 0.1% Triton X-100, 0.1 mg/ml propidium iodide and 0.1 mg/ml RNase. Cells were incubated overnight and analysed on a Facscan (Becton Dickinson) on a red fluorescence (FL2) versus forward scatter (FSC-H) plot.

RESULTS

Rad54$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells respond differently to UV-light radiation

In order to investigate whether HR is activated upon the induction of UV-light-induced DNA damage when NER is defective, we followed the behaviour of the Rad54 protein in *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells upon UV irradiation. Most untreated *Rad54*$^{+/GFP}$ cells showed a relatively homogenous nuclear localisation of Rad54-GFP with a small portion of the cells displaying a limited number of foci (Figure 1A, panel 1). Interestingly, the untreated *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells exhibited more foci compared to the *Rad54*$^{+/GFP}$ ES cells (Figure 1A, panel 5). As described in Chapter 2, ionising radiation induces equally bright foci in *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells. In contrast, exposure of cells to 1 J/m^2 of UV-light significantly elevated Rad54 foci formation in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells (Figure 1A, panels 6-8), while *Rad54*$^{+/GFP}$ ES cells showed an attenuated response in foci formation (Figure 1A, panels 2-4). To exclude the possibility that *Rad54*$^{+/GFP}$ cells were slower in foci formation, we followed the cells over a 6 h time course. Numerous foci were observed in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ 2 h following treatment, whilst *Rad54*$^{+/GFP}$ cells showed a moderate increase in the number of foci over the time span analysed.

In order to specify differences in Rad54-mediated response to UV-light between *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cell lines we determined for each cell line the percentage of cells containing more than 5 Rad54 foci. The NER-deficient cells already showed an increased number of Rad54 foci in the absence of UV irradiation when compared to NER-proficient cells (Figure 1B, see also Chapter 2). In both cell lines percentages of cells positive for Rad54 foci increased after exposing cells to 1 J/m^2 of UV-light. However, induction of Rad54 foci by UV-light was on average 2-fold lower in NER-proficient (30-40 %) cells than in NER-deficient cells (70 %) (Figure 1B). This tendency was sustained between 2 and 8 hours after treatment. Twelve hours after treatment, the number of Rad54 foci-positive *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells decreased to 45 % while in *Rad54*$^{+/GFP}$ ES cells the value remained around 40 %. A further decrease in the number of Rad54 foci-positive cells in both cell lines was observed 24 h after treatment. Remarkably, in both cell lines analysed, the initially low percentages of cells with Rad54 foci was not reached even at the latest timepoint.

Not only did Rad54 foci form preferentially in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells compared to Xpa-proficient cells, but also their size and distribution pattern differed from those induced by ionising radiation. Ionising radiation induces foci that are relatively evenly distributed in the nucleus, whereas in a number of cells foci formed after UV-light treatment were localised close to the periphery (see arrows in Figure 1A). This particular foci pattern is reminiscent to that of PCNA foci, observed during DNA replication. PCNA is a sliding clamp that confers processivity to replicative DNA polymerase. PCNA displays a homogenous nuclear staining in cells in the G1 and G2 stages of the cell cycle and forms small foci throughout the nucleus in early S phase. Foci are

closer towards the nuclear periphery in mid-S phase and form larger accumulations late in S phase, often near the nucleolus [38-41]. Since PCNA foci can be used as a marker for replication events, the UV-light-induced Rad54 foci pattern in NER-deficient cells, resembling subnuclear distribution of PCNA, suggests localisation of Rad54 to sites of replication.

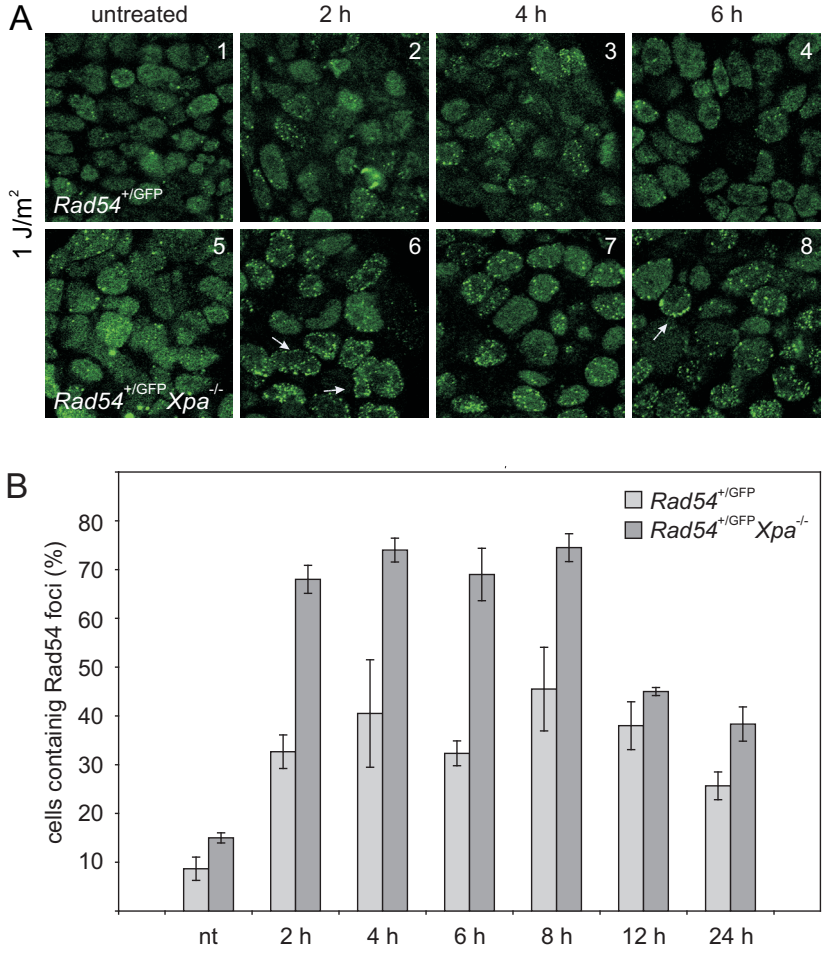

Figure 1. **Number of Rad54-GFP foci is increased in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells compared to *Rad54*$^{+/GFP}$ cells after UV-light treatment.**

(**A**) Confocal images of *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells after treatment with 1 J/m^2 of UV-light. Cells were fixed at the indicated times. (**B**) Quantification of detected foci. The bars represent percentage of cells containing more than 5 Rad54 foci. In light grey shown are *Rad54*$^{+/GFP}$ ES cells, in dark grey *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells. Error bars represent standard error of the mean (SEM). The time of analysis after treatment is indicated below the graph. At least 50 cells were analysed for each cell line.

UV-light induces smaller Rad54 foci compared to ionising radiation

Next we investigated in detail the difference in foci size induced by ionising radiation and UV-light. Using exactly the same confocal microscope settings we collected images of a single confocal plane of cells exposed to either ionising radiation or UV-light. Since foci formation after UV-light treatment was observed mostly in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ and not in *Rad54*$^{+/GFP}$ ES cells we analysed *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells. The average size of ionising radiation-induced foci was 3-fold larger (total area: 0.23 ± 0.1 μm^2) than that of the foci induced by UV-light (0.084 ± 0.034 μm^2). In addition, their shapes also differed. As assessed by axis ratio (see Experimental Procedures) ionising radiation-induced foci had a ratio close to 1, whilst the axis ratio of foci formed after UV-light was closer to 2 (Figure 2B). This indicates that ionising radiation induces nearly round foci, while UV-light-induced foci are more of an ellipsoid shape.

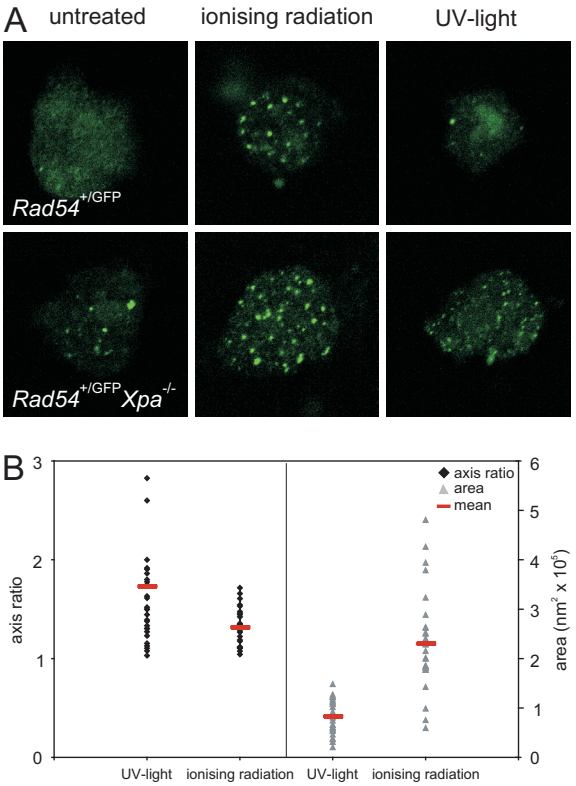

Figure 2. **Different effect of UV-light and ionising radiation on Rad54 foci appearance in ES cells.**

(**A**) Confocal images of *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells fixed untreated and 2 h after treatment with either UV-light or ionising radiation. The images were taken using exactly the same confocal settings to depict the difference in size and shape of induced foci. (**B**) The differences in both size (represented by area) and shape (represented as 'axis ratio') of Rad54 foci formed after different treatment are illustrated. The treatment is indicated below each data series. Only Rad54 foci formed in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells were analysed. Each data series contains measurements from 30 foci.

Colocalisation of HR proteins Rad54 and Rad51 with UV-light-induced DNA damage in *Xpa*$^{-/-}$ ES cells

We have shown that the Rad54 protein is involved in DSB repair induced by ionising radiation [37,42-44]. However, predominantly in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells, we were also able to detect Rad54 foci following UV-light treatment which is known to induce DNA damage distinct from DSBs. In order to determine whether DSBs are present at the site of UV-light-induced Rad54 foci we investigated whether Rad54 colocalises with phosphorylated histone H2AX (γ-H2AX), which can be used as a

marker of DNA damage, including DSBs [45]. As shown in Figure 3, extensive γ-H2AX accumulation occurs in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells (Figure 3, panel 15) compared to *Rad54*$^{+/GFP}$ ES cells (Figure 3, panel 6) following UV-light treatment. Importantly, we also find that Rad54 colocalises with γ-H2AX (Figure 3, panel 18). Additionally, UV-light-induced γ-H2AX distribution resembles that of the PCNA subnuclear pattern (Figure 3, panel 15).

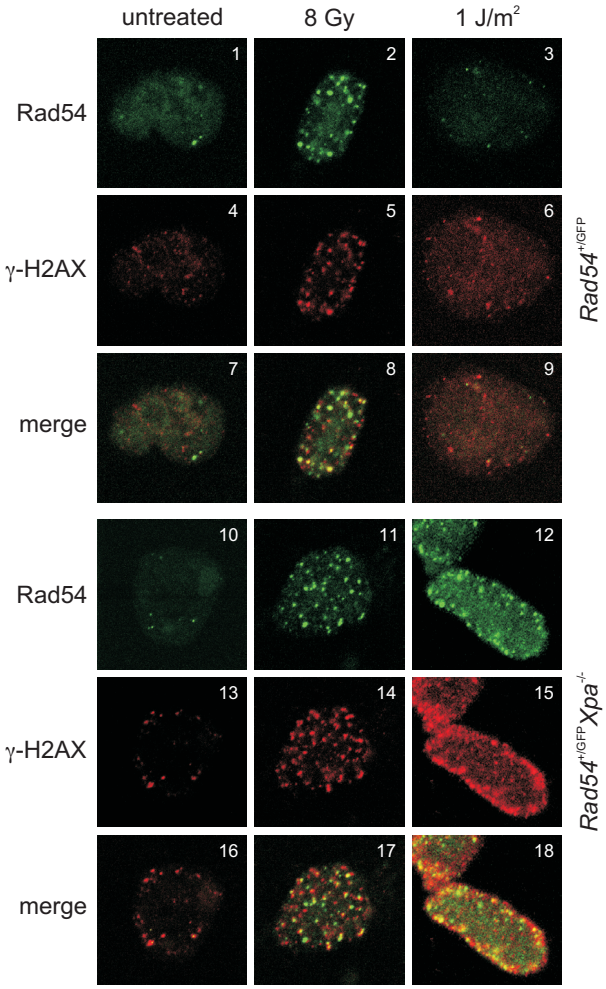

Figure 3. **Induction of γ-H2AX foci formation in *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells after UV-light treatment.**

Confocal images of fixed *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells untreated and 2 h after treatment with either 1 J/m^2 of UV-light or with 8 Gy of ionising radiation. γ-H2AX foci were detected using rabbit polyclonal antibodies (Upstate). The 'merge' pictures visualize colocalisation of Rad54 and γ-H2AX.

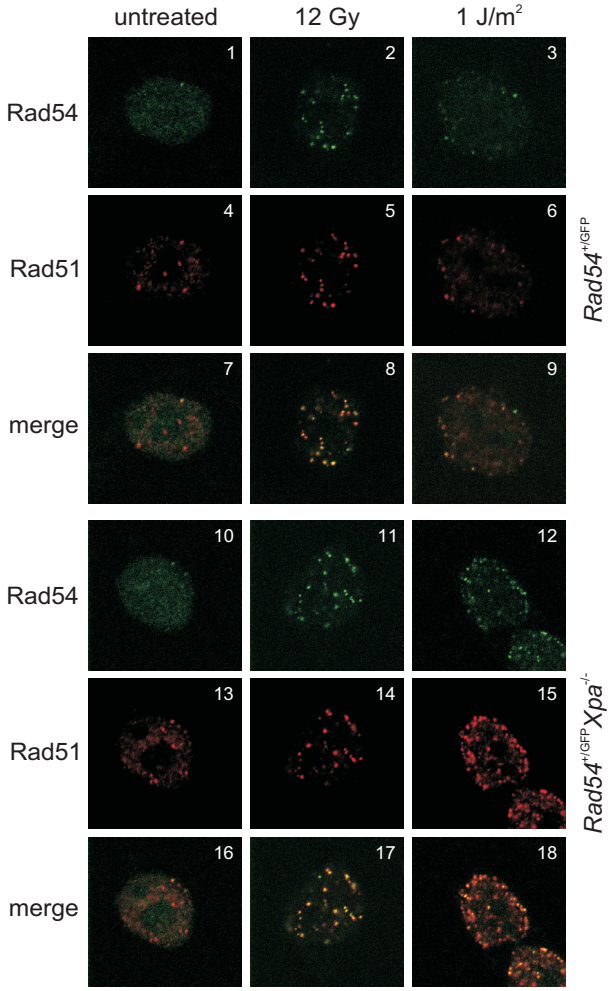

Figure 4 **Response of Rad51 protein to UV-light treatment in *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells.**

Confocal images of fixed *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells untreated and 3.5 h after treatment with 1 J/m^2 of UV-light or with 12 Gy of ionising radiation. Rad51 foci were detected using rabbit polyclonal antibodies. The 'merge' pictures visualize colocalisation of Rad54.

To further investigate the hypothesis that HR plays a role in repair of UV-light-induced lesions, we examined Rad54 colocalisation with Rad51, another protein involved in the process of HR. In cells exposed to ionising radiation, which induces DSBs Rad54 colocalises with Rad51 [37] (Figure 4, panels 8 and 17). As in the case of γ-H2AX, Rad51 forms a PCNA-like distribution pattern after UV-light treatment in *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells (Figure 4, panel 15). We find that Rad51 colocalises with Rad54 in UV-light-induced foci (Figure 4, panel 18).

Rad54$^{+/GFP}$*Xpa*$^{-/-}$ cells are delayed in exit from S-phase after treatment with a low dose of UV-light

In order to provide the time for repair, cells challenged with DNA-damaging agents are often delayed in cell cycle progression. Consequently, the arrest is usually more severe in cell lines in which DNA repair pathways have been disturbed. For example, after ionising radiation treatment *Rad54*$^{-/-}$ ES cells remain arrested in G2 phase longer than wild type cells (data not shown) and exposing *Xpa*$^{-/-}$ ES cells to UV-light causes longer delays in cell cycle progression than it does in wild type cells [46]. Exposure of *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells to 1 J/m^2 of UV-light-induced strong Rad54 foci formation in a pattern that closely resembles that of PCNA in the mid-S phase of the cell cycle. We were unable to observe such an intense staining in *Rad54*$^{+/GFP}$ control cells. Therefore, we analysed whether the difference in the ability of Rad54 foci formation is reflected by cell cycle progression of *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cell lines after treatment with 1 J/m^2 of UV-light. Both cell lines responded initially (at 8 h) by depletion of cells from G1 and G2 phase, however at the 12 h time point *Rad54*$^{+/GFP}$ ES cells already started to recover, visible as the reappearance of G1 and G2 peaks on the DNA profile whilst the majority of *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells remained accumulated in mid-S phase (Figure 5). In contrast to *Rad54*$^{+/GFP}$, *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells failed to fully restore the G1 pool even at 24 h after treatment.

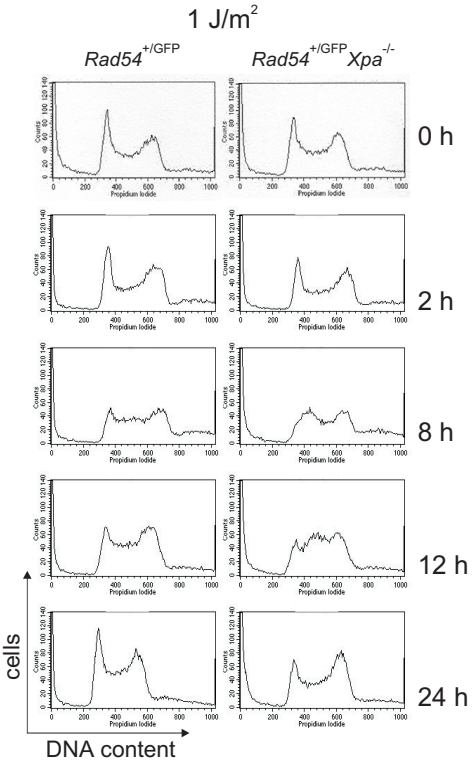

Figure 5 **FACS analysis of the effect of UV-light on cell cycle progression of ES cells.**

Cells of the indicated genotype (either *Rad54*$^{+/GFP}$ or *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$) were irradiated with 1 J/m^2 of UV-light and fixed at indicated time points. Shown on the *x*-axis is the DNA content of the cells (stained with propidium iodide) and on the *y*-axis the number of cells. Genotypes of ES cells are shown on the top. Time (h) is shown next to the panels.

DISCUSSION

The mechanisms by which HR can support stalled replication have been addressed in some detail in *E. coli* (for details see Chapter 1). Analysis of various bacterial HR mutants revealed that in order to restart prematurely blocked replication caused by UV-light, bacteria activate RecF-mediated recombination. One important aspect of the recovery of stalled replication is the protection of the structure of a stalled replication fork from degradation and collapse. Extensive degradation of nascent DNA and failure to recover replication after UV-light treatment has been observed in *RecF*$^{-}$, *recR*$^{-}$ and *recO*$^{-}$ cells thus these proteins play important roles in maintaining the structure of stalled replication forks [47, 48]. On the other hand, selective degradation of the nascent DNA is required for fork recovery after UV-light treatment. It is very likely that at the site of a replication fork the UV-light-induced lesion is present in ssDNA and cannot be recognised by the NER proteins. Therefore, the conversion of a lesion-containing site to a form that is accessible to NER enzymes can be accomplished by the cooperative action of RecJ (a 5'-3' single-strand exonuclease) and RecQ (a 3'-5' helicase) because cells impaired for RecJ or RecQ, although not hypersensitive to UV-light, show decreased DNA synthesis at early time points after UV-light treatment [48, 49].

In our study we established a murine system in which replication is prematurely blocked by UV-light-induced DNA damage. Our data suggest that unlike ionising radiation induced Rad54 foci, Rad54 foci formed after UV-light treatment resemble the sub-nuclear pattern of PCNA foci. This implies that Rad54 localises to stalled replication forks, which are abundant in NER-deficient cells after UV-light treatment. Additionally we observe that not only the subnuclear distribution of Rad54 foci induced by UV-light is different to that induced by ionising radiation but also the appearance of the foci is different. UV-light-induced foci are smaller and of irregular shape when compared to the ones induced by ionising radiation. This suggests that less Rad54 molecules are required for restarting a stalled replication fork than to repair a DSB. The difference in number can result from the difference in a DNA structure between a stalled replication fork and a random position of a two-ended not replication-associated DSB. Additionally, at the site of a stalled replication fork Rad54, either directly or indirectly, possibly interacts with some proteins of the replisome, which are absent from a two-ended DSB site. Therefore, quantitative requirements for Rad54 in these two aspects of its action may differ. Additionally, it has been shown that S phase replication-associated Rad51 foci have different genetic requirements than Rad51 foci formed at a site of a DSB [50]. In *BRCA2*-defective cells Rad51 still forms foci, presumably at replication sites, but these foci no longer form in response to ionising radiation-induced DSBs. However, the appearance of Rad51 foci was not addressed in this study. These observations suggest that targeting of Rad51 and Rad54 to replication forks may involve a different set of factors than those required for targeting them to DSBs formed at non-replicating DNA. This could possibly be achieved by interactions with components of the replication apparatus.

Furthermore, we were able to show colocalisation of Rad54 with γ-H2AX and another HR protein Rad51. Recently, numerous controversies about the kind of DNA damage highlighted by anti-γ-H2AX antibodies arose. It is very likely that phosphorylation of histone H2AX occurs not only at sites of DSBs but also at ssDNA breaks and gaps [51, 52]. The latter type of DNA structure has been shown to occur at sites of stalled replication forks [31]. Therefore, removal of these DNA structures can require activation of a pathway acting analogously to the bacterial HR RecFOR system, which has a secondary role in repair of ionising radiation-induced DSBs [24]. Finally, we showed that cells impaired for NER are seriously delayed in exit from S phase when treated with UV-light. This is consistent with a greater amount of UV-light-induced replication problems experienced by NER-deficient cells compared to NER-proficient cells.

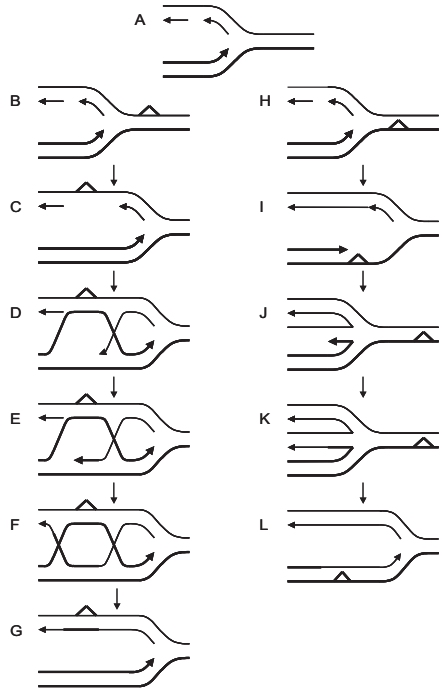

Figure 6 **Possible mechanisms for re-establishing DNA replication forks.**

(**A**) Shown is a replication fork with the leading strand represented by thick lines and the lagging strand depicted by thin lines. Arrow heads indicate 3' ends. (**B**) In the first indicated scenario (left hand panels) UV-light-induced DNA damage (indicated by a triangle) is present on the template of the lagging strand. (**C**) Continued DNA synthesis on the leading strand template and a block in the lagging strand synthesis at the site of the lesion causes formation of a stretch of ssDNA of the lagging strand template because the discontinuous nature of lagging strand synthesis allow a new Okazaki fragment to be initiated downstream of the lesion. (**D**) The downstream nascent lagging strand can invade the homologous duplex DNA consisting of the lagging strand and its template to form a D-loop. The resulting DNA structure in which DNA damage is present in dsDNA provides another possibility for NER proteins to remove the lesion. (**E**) Otherwise, the DNA intermediate can be used to initiate DNA synthesis of the lagging strand using the leading strand template. (**F**) Subsequently, a double Holliday junction (HJ) intermediate can be formed. (**G**) Resolution of the HJs finalises reconstitution of replication fork. (**H**) in the second situation (right hand panels) UV-light-induced DNA damage is present in the template of the leading strand. (**I**) Continued synthesis of the lagging strand and blocked elongation of the leading strand results in unbalanced length of nascent stands. (**J**) Annealing of nascent strands leads to formation of HJ-like four-armed intermediate, which can be cleaved directly by HJ resolvases (not shown). Additionally, fork regression locates UV-light-induced DNA damage into dsDNA affording in this way another chance for the repair machinery to remove the damage. (**K**) Alternatively, in the absence of repair the nascent leading strand can be elongated using the lagging strand as a template in template switch replication. (**L**) Subsequent regression of this structure leads to both bypass of the lesion and the intermediate to re-establish a replication fork.

Induction of Rad54 foci in Xpa-proficient ES cells, however to a lesser extent than in *Xpa*$^{-/-}$ ES cells, suggests that HR is activated even in NER-proficient cells. In this case HR might be required to protect and/or modify the structure of a stalled replication fork so that the DNA damage can be removed by NER proteins [48, 49, 53]. Nevertheless, the importance of HR in underpinning replication increases in cells in which NER has been inactivated. Additionally, the observed effect is probably due to UV-light induced lesions other than CPDs, most likely 6-4PPs, because in murine cells CPDs are not detected as a problem for cellular metabolism and survival and are not removed even in wild type cells.

The ability of Rad54 to assist Rad51 in D-loop formation suggests a model in which Rad54 can stimulate the restart of a stalled or/and collapsed replication fork mediated by Rad51. In one scenario for fork activation, the lesion on the lagging strand template will result in generation of long ssDNA patches (Figure 6C). SsDNA gap recombination, analogous to the RecF pathway in *E. coli* places the lesion back into a dsDNA context (Figure 6D). This reaction could result in displacement of the 3' end of the newly synthesised Okazaki fragment, which can subsequently pair in a Rad51/Rad54 promoted fashion with the leading strand template (Figure 6D). Replication of this structure can result in a double Holliday junction (HJ) intermediate (Figure 6E and F). Resolution and/or branch migration of this structure results in a DNA conformation from which a replication fork can be reconstituted (Figure 6G). In this scenario Rad54 can play multiple roles. For example, it can stabilise Rad51 nucleoprotein filaments [54]. Its ability to translocate on DNA can open up dsDNA making it more accessible for the invading Rad51-filament [25, 26, 55, 56]. The ability of Rad54 to translocate along DNA could play an important role also in later steps of reconstitution of stalled replication forks. HJs formed in the joined molecule have to migrate along DNA in order to fill the gap of missing DNA (Figure 6E and F). This process could be achieved by translocation of Rad54 along dsDNA [25, 29, 57]. Finally, after completing the above-mentioned steps of repair the DNA content will have been restored and the joint molecule can be resolved (Figure 6G). In the process of resolution of a HJ the RecQ family of helicases also plays an important role. Members of this family include the BLM and WRN proteins [58]. Since genomic instability of *BLM*$^{-/-}$ cells results at least in part from the inability to restart collapsed replication forks, the BLM protein was proposed to act at HJs formed after replication blockage [59]. Recently, it has also been shown that the Rad51 homologue Rad51C plays a role in the postsynaptic step of HR. Cell extracts from which Rad51C is lacking does not support branch migration or HJ cleavage of exogenously added DNA substrates [60]. Additionally, the physical interaction between Rad51 and its paralogues and the BLM helicase has been demonstrated [61-63]. Therefore, resolution of HJs formed at sites of collapsed replication can be mediated by RecQ helicase homologues together with Rad51.

In an alternative scenario the damage is located on the leading strand template (Figure 6H). Uncoupling of leading and lagging strand DNA polymerases can result in continued lagging strand DNA synthesis past the lesion (Figure 6I). Subsequent regression results in formation of a HJ

and the lesion would once again be in dsDNA (Figure 6J). Formation of this transient DNA intermediate would also require activities of proteins with the ability to branch migrate DNA. From this intermediate the leading strand can be extended past the position that contains the lesion in the original template by template switch replication of the nascent leading strand (Figure 6K). Branch migration, potentially mediated by Rad54, will result in bypass of the lesion and in a DNA structure from which a replication fork can be reconstituted (Figure 6L) [57].

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CHAPTER 4

Analysis of inactivation of Rad54 in nucleotide excision repair-deficient mouse embryonic stem cells

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ABSTRACT

In nucleotide excision repair (NER)-deficient cells, UV-light-induced DNA damage is left unrepaired and can pose a problem for the cell during DNA replication. The role of homologous recombination (HR), which is involved in DNA double-stranded breaks repair, in rescuing stalled replication fork has been demonstrated in bacteria. Previously, we have shown that, upon UV-light treatment, the HR protein, Rad54, forms foci in *Xpa*$^{-/-}$ mouse embryonic stem cells, which are totally NER-deficient. This suggests that HR is activated by UV-light treatment of mammalian cells. Here, we demonstrate that cells in which both the HR and NER pathways are inactivated, were more hypersensitive to UV-light treatment than cells deficient for NER only. Additionally, these cells showed increased disturbance of progression through S phase after UV-light treatment as well as elevated levels of chromosomal aberrations compared to control cells. Finally, the efficiency of replication recovery after UV-light treatment was reduced in these cells.

INTRODUCTION

Exposure of cells to UV-light causes DNA damage that is distributed randomly throughout the genome [1]. If not repaired these lesions present problems for cells in S phase, because the damage would inevitably block multiple replication forks [2]. Disturbed replication results in genetic rearrangements, mutations or cellular lethality, which can threaten the whole organism [3-7]. In order to counteract these dangers, cells have evolved complicated DNA repair processes [1].

Nucleotide excision repair (NER) is a process that removes UV-light-induced helix-distorting lesions from DNA [8]. This is a multistep process including damage recognition and verification, incision of the damaged DNA strand and subsequent filling of the missing DNA content. Xpa is a protein acting early in the NER process and is thought to play a role in damage verification [9-11]. Xpa-deficient cells are totally NER-deficient and hypersensitive to UV-light [12, 13]. Additionally *Xpa*$^{-/-}$ cells, and specifically *Xpa*$^{-/-}$ mouse embryonic stem (ES) cells, are delayed in exit from S phase after UV-light treatment (Chapter 3) [14]. This suggests that in these cells a major problem caused by UV-lesions is blocked replication. Interestingly, we were able to detect numerous foci of the HR protein, Rad54, in Xpa-deficient ES cells after UV-light treatment (Chapter 3), compared to Xpa-proficient cells.

Homologous recombination (HR) is a process that repairs large single-stranded (ss) DNA gaps and DNA double-stranded breaks (DSBs) [15]. These potentially lethal lesions can arise as a result of cellular metabolism or after exposing cells to external sources of DNA damage like ionising radiation or various chemicals [1]. Proteins of the HR pathway restore DNA integrity in an error-free manner [16]. The repair of a DSB by HR requires the presence of a DNA molecule homologous to the broken one, which serves as a template to restore missing DNA content. Depending on cell cycle phase, different DNA templates are available for the repair machinery. In the S and G2 phases newly replicated sister chromatids are still tethered together by the proteins of the cohesion family and are, therefore, in close proximity to each other [17]. When a DSB occurs in one of the chromatids, the other intact chromatid can be used as a repair template. In this case, the genetic information in a cell would not be altered. However, in the G1 phase, prior to DNA replication, only the homologous chromosome is available. Thus, if in this phase of the cell cycle a DSB generated in chromosomal DNA is fixed by HR, the homologous chromosome will have to be employed as a repair template. However, this can result in loss of heterozygosity, which in turn can result in inactivation of recessive tumour suppressor genes [18, 19].

Rad54 is an important member of a family of proteins engaged in HR [20-23]. Rad54 is a dsDNA-dependent ATPase and this activity is required for its DNA translocation, which results in a change in DNA conformation [24]. This change helps in promoting strand separation of a dsDNA template, which facilitates strand invasion, a key step of HR [25-30]. At a cellular level, after treating cells with ionising radiation Rad54 redistributes into nuclear foci [31-33]. The importance of the Rad54

protein in HR is underscored by the hypersensitivity of *Rad54*$^{-/-}$ ES cells to ionising radiation [34]. Additionally, gene targeting, a measure of HR efficiency, and DNA damage-induced sister chromatid exchange (SCE) are reduced in these cells, when compared to wild type cells [34, 35].

Interestingly, in *Xpa*$^{-/-}$ cells SCE levels are increased after treatment with DNA damaging agents, when compared to wild type cells [36]. This observation, taken together with our demonstration that Rad54 foci form upon UV-light treatment prominently in Xpa-deficient cells (Chapter 3), suggests that, in NER-deficient cells, UV-light activates HR. In order to verify that HR plays a role in processing of UV-light-induced DNA lesions, we additionally deleted *Rad54* from *Xpa*$^{-/-}$ ES cells. Thus, the putative rescue pathway for *Xpa*$^{-/-}$ ES cells is attenuated. Our data show that the *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells are more sensitive to UV-light than are *Xpa*$^{-/-}$ cells. Furthermore, these cells are incapable of replenishing the G2 phase cell population after UV-light treatment. Additionally, the number of structural chromosomal aberrations accumulated in these cells after UV-light treatment is higher than in either *Rad54*$^{-/-}$ or *Xpa*$^{-/-}$ cells. Finally, the efficiency of replication fork restart after UV-light-induced DNA damage is drastically reduced in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells. Therefore, we conclude that HR is involved in dealing with UV-light-induced DNA damage, particularly in NER-deficient cells.

EXPERIMENTAL PROCEDURES

DNA constructs

Construction of m*Rad54* targeting vectors was described elsewhere [34]. Briefly, m*Rad54* was disrupted by inserting a cassette containing either a hygromycin or a puromycin resistance gene driven by PGK or TK promoter, respectively, in the unique *Sfu* I site of exon 4. The resulting *mRad54* constructs are referred to as *Rad54*^{hygro} and *Rad54*^{puro}.

ES cells culture and gene targeting

Wild type (C57Bl/6), *Rad54*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells were cultured in BRL-conditioned medium supplemented with 1000 U/ml leukaemia inhibitory factor. To generate the *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cell line, the *Rad54*^{hygro} targeting construct was electroporated into *Xpa*$^{-/-}$ ES cells. Screening for homologous recombinants was performed using DNA blot analyses of *Stu* I-digested genomic DNA with a probe containing exons 7 and 8. In case of targeted integration of both Rad54 targeting constructs, a 9.0 kb band corresponding to the wild type *Rad54* allele is replaced by two bands; 7.5 kb of *Rad54*^{hygro} and 6.5 kb of *Rad54*^{puro} constructs. After standard selection procedures with 150 μg/ml of hygromicin, a clone containing properly targeted *Rad54* allele was isolated. Second targeting with the *Rad54*^{puro} construct resulted in total disruption of *Rad54* in *Xpa*$^{-/-}$ ES cells, which was subsequently confirmed by analysis of protein extracts from targeted clones.

Immunoblots

To make whole cell protein extracts, cells were trypsinised, pelleted, resuspended in electrophoresis sample buffer, and sonicated. Whole cell extracts were electrophoresed through SDS-PAGE gels and blotted to nitrocellulose. The blots were hybridised with anti-Rad54 antibodies [34].

Colony survival curves

Sensitivity of ES cells to increasing doses of DNA-damaging agents was determined as described previously [34]. Briefly, cells were plated in 6 cm dishes, at various dilutions. After 12-16 h, cells were irradiated with a single dose of UV-C (254 nm) in the range of 0-8 J/m^2. Subsequently, cells were grown for 7-10 days, fixed, stained and colonies were counted. All experiments were performed in triplicate.

Cell cycle analysis by fluorescence-activated cell sorting (FACS)

Wild type (C57Bl/6), *Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells (60% - 70% confluency) were treated with a single dose of UV-C (254 nm, 1 or 8 J/m^2) on 6 well culture dishes. Cells were grown in fresh medium for the indicated times. Subsequently cells were trypsinised and fixed with 70% ethanol. After a minimum of 2 h on ice, cells were washed with PBS and resuspended in

400 µl PBS containing 0.1% Triton X-100, 0.1 mg/ml propidium iodide and 0.1 mg/ml RNase. Cells were incubated overnight and analysed on a FACScan (Becton Dickinson) on a red fluorescence (FL2) versus forward scatter (FSC-H) plot.

Chromosome analysis

Wild type (C57Bl/6) and mutant (*Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ clone G2) murine ES cells were exposed to increasing doses of UV-light. Twenty-four hours after the treatment cells divisions at metaphase were stopped by adding prewarmed (37°C) Colcemid (10 µg/ml) for 15 min. Then cells were washed with PBS, trypsinised and centrifuged (5 min, 1000 rpm). Next, cells were resuspended in prewarmed hypotonic solution (0.075 M KCl), immediately centrifuged, resuspended once more in hypotonic solution (room temperature) and fixed 3 times in a fresh 3:1 acetic acid/methanol (room temperature). For chromosome slides preparation, cells were placed on a clean glass slide, air-dried and stained with Atrebrine. Images were collected using Axioplan fluorescence microscope and structural chromosomal aberrations were analysed by eye. The number of aberrations per metaphase was counted, and the aberrations were classified as chromatid or chromosome type aberrations. Ten metaphases per cell line were analysed for the number of chromosomes, and fifty for the presence of structural aberrations.

Replication Labelling and DNA Fibre Spreads

To investigate the efficiency of replication fork restart after UV-light-induced damage the cells were pulsed with 50 µM IdU for 15 min. Immediately before UV-light treatment, cells were chased with 50 µM thymidine for 15 min and subsequently kept in fresh medium for 2 hours before labelling with CldU for 20 min.

For DNA spreads, the cells were trypsinized and resuspended in ice-cold PBS at 2.5 x 10^5 cells/ml. The labelled cells were diluted 1:8 with unlabelled cells, and 2.5 µl of cells were mixed with 7.5 µl of spreading buffer (0.5% SDS in 200 mM Tris-HCl, pH 7.4, 50 mM EDTA) on a glass slide. After 8 min the slides were tilted at a 15° angle, and the resulting DNA spreads were air-dried, fixed in 3:1 methanol/acetic acid, and refrigerated overnight.

Immunolabelling

The slides were treated with 2.5 M HCl for 1 h, neutralized with 0.1 M $Na_2B_4O_7$, pH 8.5 for 7 min, washed in PBS, and blocked in 1% bovine serum albumin, 0.1% Tween 20 for 20 min. To detect CldU the slides were incubated for 1 h at 37°C with rat anti-bromodeoxyuridine antibody (1:40) (Abcam). Subsequently, after washing with PBS, 0.1% Tween incorporared CldU was identified by Alexafluor 488-conjugated anti-rat antibody (1:200) (Molecular Probes). Following washing with PBS, 0.1% Tween and blocking for 20 min; the mouse anti-bromodeoxyuridine antibody (detects IdU) (Becton Dickinson) was applied for 1 h at 37°C. In order to increase antibody

discrimination between two nucleotide analogues slides were washed with high salt buffer (0.5 M NaCl, 0.3 M Tris-HCl, 0.5 % Tween 20) for 7 min at room temperature. After that slides were rinsed in PBS, 0.1% Tween 20, and then incubated with Cy3-conjugated anti-mouse antibody (1:800) (Sigma) for 45 min at 37°C. Finally, slides were washed in PBS, 0.1% Tween 20 and mounted in PBS/glycerol. Images were collected using a Zeiss LSM Meta 510 confocal microscope. Where indicated cells were exposed to a single dose of 80 J/m^2 of UV-C. Replication events were counted on a DNA fibre of 50 μm. For each experiment 50 fibres were scored for every cell line. Recovery of replication was calculated for each fibre by dividing the number of active replication forks by the sum of both active and stalled replication forks. Experiments were performed in triplicate.

RESULTS

Generation of *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells

The increased level of Rad54 foci in NER-deficient cells compared to wild type cells prompted us to analyse the UV-light sensitivity of cells impaired in both HR and NER. For this purpose, we generated *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells. The Rad54 locus was inactivated in *Xpa*$^{-/-}$ ES cells by targeting the locus first with a *Rad54*^{hygro} knockout construct and subsequently with a *Rad54*^{puro} targeting construct [34]. After each targeting event, positive clones were identified by DNA blot analysis using a probe containing exon 7 and 8 of Rad54 (Figure 1A). Targeting efficiency was 14% for the *Rad54*^{hygo} and 7% for *Rad54*^{puro} targeting constructs. We continued the experiment with two independent clones; C1 and G2. Immunoblots of whole cell extracts of the clones stained with anti-Rad54 antibodies confirmed the abolition of expression of Rad54 protein in these cell lines (Figure 1B).

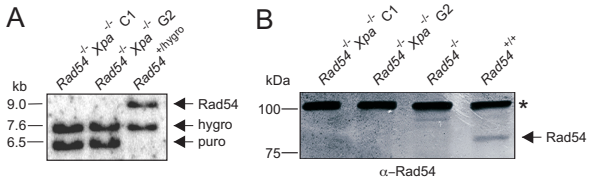

Figure 1. **Disruption of *Rad54* in *Xpa*$^{-/-}$ ES cells.**

(**A**) The *Rad54* locus was deleted from *Xpa*$^{-/-}$ ES cells by subsequent targeting of the *Rad54* alleles by *Rad54*^{hygro} and *Rad54*^{puro} disruption constructs. DNA blot analysis of *Stu* I digested genomic DNA of *Rad54*$^{+/hygro}$ ES cells and two independently obtained *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells (labelled C1 and G2). The position of endogenous *Rad54* and both targeted alleles are indicated on the right. (**B**) Immunoblot confirming abolition of Rad54 protein expression in *Rad54*$^{-/-}$ cells. Whole cell extracts were separated on 6% SDS-PAGE gel and subsequently blotted to nitrocellulose. The blots were hybridised with anti-Rad54 antibodies [34]. Wild type cells and previously obtained *Rad54*$^{-/-}$ cells were used as controls. The position of wild type Rad54 protein is indicated. The nature of the non-specific band of 100 kDa (indicated by asterisk) cross-reacting with anti-Rad54 antibodies is unknown.

Rad54$^{-/-}$*Xpa*$^{-/-}$ cells are more sensitive to UV-light than *Xpa*$^{-/-}$ cells

Xpa$^{-/-}$ ES cells are very sensitive to UV-light treatment [12]. This reflects the difficulties caused by unrepaired DNA damage in these cells. Disruption of yet another DNA repair pathway might pose a greater problem for already challenged cells. Therefore, we investigated the sensitivity of *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells to UV-light. In our experiment wild type cells, as well as *Rad54*$^{-/-}$ cells were equally and only mildly sensitive to UV-light. By contrast, *Xpa*$^{-/-}$ cells were extremely UV-light sensitive. However, *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells were significantly more sensitive to UV-light than were *Xpa*$^{-/-}$ cells.

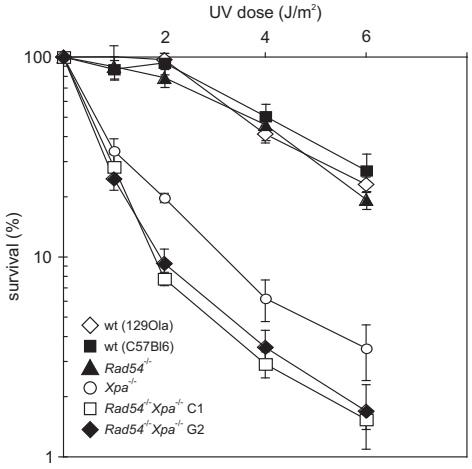

Figure 2. **Effect of UV-light on the wild type, *Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells.**

Clonogenic survival of wild type, *Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and two independent clones of *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cell lines after treatment with increasing doses of UV-light. Following treatment, cells were incubated for 7 - 10 days, and were subsequently fixed, stained and colonies were counted. Percentage of surviving cells after exposure to UV-light is plotted as a function of increased UV-light dose. Error bars represent standard error of mean (SEM).

UV-light dose dependent impairment of S-phase progression

Our data suggest that, upon UV-light treatment, *Xpa*$^{-/-}$ ES cells encounter replication problems that require proteins of HR for their resolution. Since *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells are more sensitive to UV-light than *Xpa*$^{-/-}$ cells, we investigated the cell cycle progression of these cells after treatment with low (1 J/m^2) and high (8 J/m^2) doses of UV-light (Figure 3A and B, respectively). After exposing cells to 1 J/m^2 of UV-light wild type cells responded initially (4 h after treatment) by accumulation of cells in early S phase. Eight hours after treatment cells accumulated in mid-S phase and after a subsequent 2 h (i.e. 10 h after treatment) in late S phase. Restoration of a G1 peak was observed 24 h after treatment (Figure 3A). *Rad54*$^{-/-}$ cells responded initially in the same manner as wild type cells. However, depletion of G1 peak was more distinct 8 and 10 h after treatment. Additionally, *Rad54*$^{-/-}$ cells did not fully restore the G1 peak at the 24 h time point. Surprisingly, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells responded to the DNA-damaging treatment in the same way. The *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells started accumulating in early S phase 4 h after treatment. Further accumulation of cells in S phase, concomitant with the depletion of cells from G1 and G2 phases, continued up to 10 h after treatment. However, none of the cell lines entirely restored the population of G1 phase cells. Most cells remained in the G2 phase even 24 h after treatment.

The number of stalled replication forks is proportional to the dose of DNA-damaging agent applied [37]. Therefore, to analyse cell cycle progression after causing numerous stalled replication forks, we exposed cells to a high dose of UV-light (Figure 3B). Interestingly, in contrast to a low UV-light dose, after treatment with 8 J/m^2 of UV-light all analysed cells were arrested/delayed in different stages of S phase. While challenging with a low dose resulted in accumulation of cells in mid-S phase, the higher dose led to accumulation of cells in early S phase. This difference in cell cycle response to low versus high doses of UV-light was consistent for all analysed cell lines, independently of the mutations that they harboured. However, in contrast to the previous

experiment, treating cells with 8 J/m^2 of UV-light revealed an alteration in cell cycle response between *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells. In the case of *Xpa*$^{-/-}$ cells, depletion of the population of cells in either G1 or G2 phase of the cell cycle was scarcely observable. In contrast, already 8 h after treatment, *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells failed to proceed to the G2 phase, possibly indicating replication-associated problems.

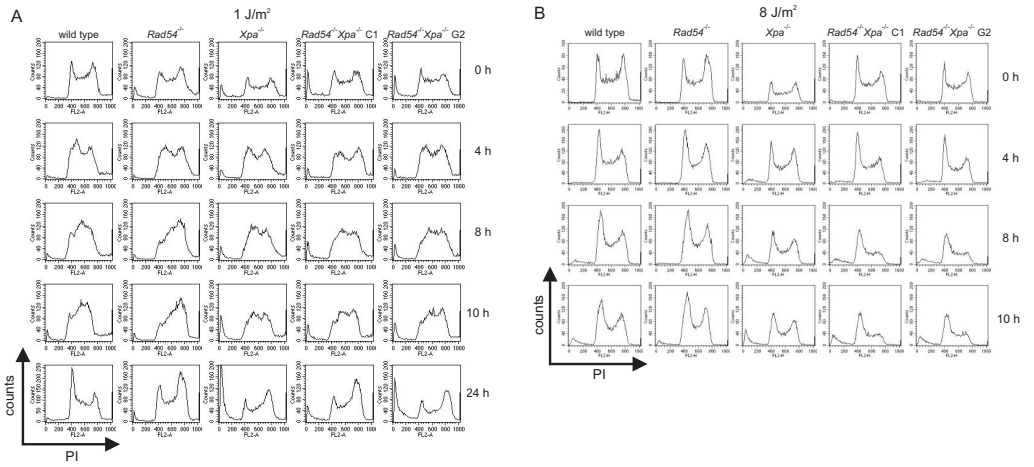

Figure 3. **Effect of UV-light on cell cycle progression of wild type, *Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells.**

FACS analysis of the effect of different doses of UV-light on cell cycle progression of ES cells. Shown on the *x*-axis is the DNA content of the cells (stained with propidium iodide) and on the *y*-axis the number of cells. Genotypes and time of harvest after exposure to UV-light are indicated

Rad54$^{-/-}$*Xpa*$^{-/-}$ ES cells accumulate more chromosomal aberrations than *Xpa*$^{-/-}$ cells after UV-light treatment

One of the hallmarks of an inability of a cell to restart blocked replication is the accumulation of chromosomal aberrations after challenging DNA replication [38]. In order to investigate whether chromosomal aberrations are indeed increased in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells, compared to the control cells, we analysed the accumulation of replication-associated chromosomal aberrations. For this, chromatid gaps and chromosomal breaks were quantified in the genomic DNA after challenging the cells with UV-light. Wild type, *Rad54*$^{-/-}$ and *Xpa*$^{-/-}$ cells showed a similar number of gaps after treatment with increasing doses of UV-light (Table 1). However, treatment of *Rad54*$^{-/-}$ cells with 4 J/m^2 resulted in increased number of chromosomal breaks, compared to the wild type and *Xpa*$^{-/-}$ cells, which is consistent with the inability of these cells to execute DNA damage-induced HR properly [34]. An augmented number of chromatid gaps after treating cells with 2 J/m^2 of UV-light was observed in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells when compared to the other cell lines. Additionally, this treatment gave rise to an elevated amount of chromosomal breaks in these cells, compared to wild type and *Xpa*$^{-/-}$ cells.

genotype	treatment (J/m^2)	average no of gaps in one metaphase	average no of breaks in one metaphase
wild type	0	0.02	0
	1	0.04	0
	2	0	0
	4	0.16	0.04
Rad54$^{-/-}$	0	0	0
	1	0	0
	2	0.04	0.04
	4	0.18	0.14
Xpa$^{-/-}$	0	0.02	0
	1	0.04	0.08
	2	0	0
	4	0.10	0.02
Rad54$^{-/-}$*Xpa*$^{-/-}$	0	0	0
	1	0.02	0.06
	2	0.52	0.18
	4[a]	0.28	0.16

[a]25 metaphases analysed

Table 1. Structural chromosomal aberrations induced by UV-light in wild type, *Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells. Shown are an average number of either chromosomal breaks or chromatid gaps per metaphase. Unless indicated otherwise, the number of each type of aberration was scored in 50 metaphases. Genotypes of cell lines are indicated in the column on the left. The treatment is depicted in the next column. The type of chromosomal aberration scored is shown in a top row.

The stability of replication forks is reduced in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells after UV-light treatment

In order to demonstrate involvement of HR, through Rad54 activity, in removal or bypass of DNA damage at sites of stalled replication forks, we employed a technique to analyse individual chromosomal fibres. Using this strategy, it is possible to investigate a single replication fork in a mammalian cell [39-42]. It has been shown that a dose of 50 J/m^2 of UV-light induces one cyclobutane pyrimidine dimer (CPD) in a 4.4 kb plasmid DNA [37]. In murine cells, CPDs are poorly repaired without having a significant effect on cellular survival. Hence, the main DNA damage posing a problem for replication appear to be pyrimidine-(6,4)-pyrimidone adducts (6-4PPs). It has been established

that CPDs induced by UV-C occur in DNA 1.4 – 3 times more frequently than 6-4PPs [43, 44]. In our study, we scored replication events on a DNA fibre of 50 μm, which corresponds to approximately 100 kb of DNA. Therefore, we estimated that, by irradiating cells with 80 J/m^2 of UV-C, we induced between 12 and 26 6-4PPs on each DNA fibre analysed. As depicted in Figure 4, around 90% of active replication forks are stable in all cell lines before exposing them to UV-light. However, upon UV-light treatment the initial stability of replication fork was maintained only in wild type and *Rad54*$^{-/-}$ cells. Mild reduction of fork stability to 75% was observed in *Xpa*$^{-/-}$ cells. Strikingly, the percentage of stable forks in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells dropped dramatically to around 35%. This result underlines the importance of HR in maintaining the process of replication in cells in which NER is not functional.

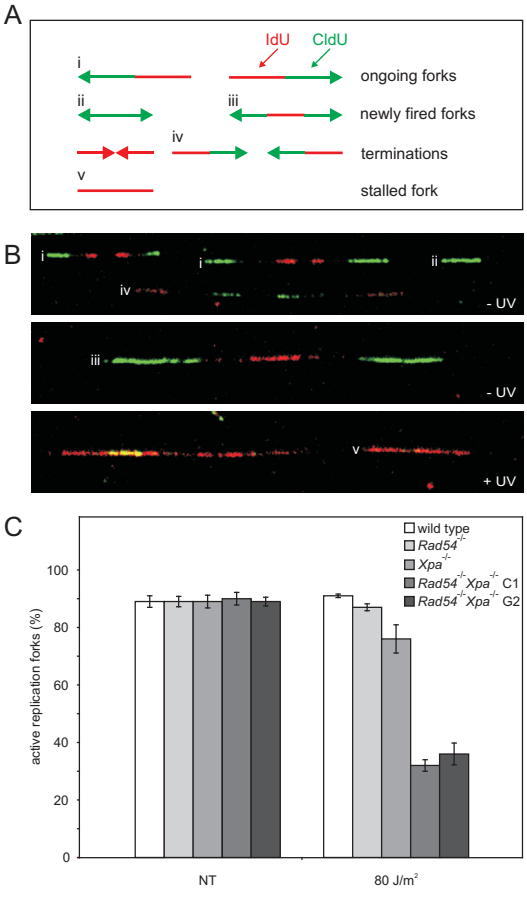

Figure 4. **Recovery of replication in wild type, *Rad54*$^{-/-}$, *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells.** (**A**) Schematic representation of double-labelled replication tracks. Two-colour arrows represent stable replication forks. Events marked in red only correspond to stalled replication forks. (**B**) An example confocal image of fibres with labelled replication events. Stretches green only (ii) represent origins that fired after the IdU was added whilst red and green tracks (iii) represent origins that fired while the IdU was in place. (**C**) Quantification of recovery of replication after UV-light treatment. Shown is percent of stable replication fork before (left panel) and after treating cells with 80 J/m^2 of UV-light (right panel). Genotypes of cell lines are indicated in a box in the upper right corner. All experiments were performed in triplicate. Error bars represent standard error of mean (SEM).

DISCUSSION

The data obtained in Chapter 3 suggest that hypersensitivity of *Xpa*$^{-/-}$ ES cells to UV-light arises, in part, as a consequence of impaired DNA replication. Additionally, we were able to infer that UV-light activates HR in NER-deficient cells (Chapter 3). Furthermore, the DNA damage-induced increase in SCE levels in *Xpa*$^{-/-}$ cells suggests again that activation of HR has occurred following DNA damage [36]. Moreover, bacterial cells inactivated for both NER and HR are unable to restore DNA replication following UV-light treatment [45]. Finally, efficiently targeting at least some of the translesion DNA polymerases to damaged DNA depends on active HR [46-48]. Therefore, we inactivated the *Rad54* gene in *Xpa*$^{-/-}$ ES cells, to directly assess the interplay between NER and HR with respect to the cellular response to UV-light-induced DNA damage.

The importance of HR activity in bypassing UV-light-induced damage in NER-deficient ES cells is underscored by the increased hypersensitivity to UV-light of *Rad54*$^{-/-}$*Xpa*$^{-/-}$ compared to *Xpa*$^{-/-}$ cells (Figure 2). This evidence implies that, under certain circumstances, UV-light-induced DNA damage requires functional HR. Cooperation of HR with other cellular processes that have the potential to remove or overcome UV-light-induced DNA lesion might be crucial for cell survival in those cases where the damage is present at replication forks. It is very likely that UV-light-induced damage, which stalls replication, is present in ssDNA, whilst NER proteins are only able to recognise and subsequently remove DNA damage present in dsDNA. Therefore, one route to repair is to alter the DNA structure of a stalled replication fork site such that the damage is moved to dsDNA to give the NER pathway another opportunity. Some proteins of HR, for example Rad54, have the ability to alter DNA conformation, and thus it is expected that the change of DNA architecture at the site of a stalled replication fork needed for repair can be achieved through the action of the HR pathway [24]. Therefore, it is very likely that cells that are unable to both remove UV-light-induced DNA damage and catalyse modification of DNA structure will experience complications during progression through S phase.

In order to investigate if UV-light causes greater disturbance of S phase in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ than in *Xpa*$^{-/-}$ cells, we analysed cell cycle progression after challenging these cells with the DNA-damaging agent. Surprisingly, after exposing cells to 1 J/m^2 of UV-light, we were unable to detect any significant differences in cell cycle progression between *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells, over the time span analysed (Figure 3A). The *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells showed the same initial delay as well as recovery of S phase progression following UV-light treatment. One possible explanation for the similar progression through S phase of *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells is the activity of specialised translesion DNA polymerases that can synthesise short stretches of DNA through the template tract containing a lesion [49]. However, these polymerases have limited processivity of a lesion bypass. Thus, the increase in the number of DNA lesions with the potential to block more replication forks should result in saturation of the translesion system [50]. Since the number of induced DNA lesions

is directly correlated to the UV-light dose, we increased the dose to 8 J/m^2 [37, 45]. The most striking difference between the cell cycle response to low and high doses is the stage of the cell cycle in which cells were arrested, independently of the mutation carried. While treatment with a low dose of UV-light results in accumulation of cells mainly in mid-S phase, treatment with a high dose causes accumulation of cells much earlier in S phase (Figure 3B). Clearly, numerous stalled replication forks will pose a great problem for a cell already in early S phase. Interestingly, a high dose of UV-light reveals the differences in S phase progression between *Xpa*$^{-/-}$ and *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells. In contrast to *Xpa*$^{-/-}$ cells, *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells are unable to progress through S phase concomitantly with total depletion of the pool of G2 cells. This suggests that UV-light-induced DNA damage, if left unrepaired in DNA undergoing replication, efficiently blocks progression of DNA synthesis in these cells. Additionally, increased accumulation of chromosomal aberrations in *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells compared to *Xpa*$^{-/-}$ cells, which are typical for cells with impaired replication (such as chromatid gaps and chromosomal breaks), emphasises the importance of HR in restoring replication in NER-deficient cells (Table 1). Finally, chromosomal fibre data showed directly the indispensable nature of functional HR in NER-deficient cells in stabilising blocked replication forks (Figure 4). When both pathways were absent from the cells, the level of replication recovery dropped dramatically upon UV-light-induced damage. The observed difference is most likely due to 6-4PPs, because in rodent cells CPDs are not experienced as a major problem even in NER-proficient cells.

In addition to roles of HR in direct lesion bypass and origin-independent restart of replication, as well as in the maintenance of the stalled replication fork structure (discussed in chapter 3), HR may also be involved in bypassing the lesion mediated by translesion DNA synthesis (TLS). Recently, the human translesion polymerase (pol) η has been implicated in HR because it can promote DNA synthesis from a D-loop HR intermediate, which is formed by Rad51 [48]. Moreover, DSB-mediated HR is severely abrogated in DT40 cells depleted of pol η, suggesting that DNA synthesis, which is required for completion of HR process, depends on this translesion polymerase [47]. Although pol η and Rad51 partially colocalise in human fibroblasts upon UV-light treatment it has not yet been directly demonstrated that Rad51 function is important for UV-light-induced TLS in mammalian cells [46]. However, data from *E. coli* does point to an important role of RecA in pol V-mediated TLS [51, 52]. All these data suggest that both processes, at least to some degree, are reciprocally dependent on each other. Thus, in NER-deficient cells with additionally inactivated HR, not only is the capacity to bypass DNA damage by HR and/or stabilise stalled replication forks ablated, but also it is possible that TLS-mediated bypass is impaired, at least to some extend. Therefore, in our murine cells, which might not entirely execute TLS, due to defect in HR, the UV-light-induced damage left unrepaired, that is normally a substrate for pol η, might now be encountered by the replicative polymerase, which is unable to continue DNA synthesis on these damaged templates. Nevertheless, the possibility that other translesion polymerases are still functional in these cells cannot be excluded. Therefore, in order to exceed the capacity of the remaining, putatively still

active, translesion polymerases, high doses of UV-light have to be applied. Additionally, this dose dependent phenotype indicates that HR becomes important in bypassing UV-light-induced lesions at later time points, when other repair processes fail to repair the DNA lesions. For example, filling of UV-light-induced ssDNA gaps still containing lesions might be performed by pol η. It is possible that HR might promote DNA synthesis by providing a DNA substrate from which pol η can replicate over the altered template. This option is in agreement with suggestions that bacterial pol V acts at late time points after induction of UV-damage, when the repair processes failed to remove the lesion [53]. Therefore, HR might act upon UV-light-induced DNA damage at a replication fork in a versatile manner. However, the precise mechanism of action remains to be elucidated.

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CHAPTER 5

Biochemical and genetic analysis of the mammalian Rad54 paralogue Rad54B

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ABSTRACT

Homologous recombination is a versatile DNA damage repair pathway requiring Rad51 and Rad54. Here we show that a mammalian Rad54 paralogue, Rad54B, displays similar biochemical properties compared to Rad54. Furthermore, we show that Rad54B protects mouse embryonic stem (ES) cells from ionising radiation and the interstrand DNA crosslinking agent mitomycin C. Interestingly, at the ES cell level the paralogues do not display an additive or synergic interaction with respect to mitomycin C sensitivity, yet animals lacking both Rad54 and Rad54B are dramatically sensitised to mitomycin C as compared to either single mutant. This suggests that the paralogues possibly function in a tissue-specific manner. Thus, even though the paralogues have similar biochemical properties, genetic analysis in mice uncovered their non-overlapping roles.

INTRODUCTION

DNA double-stranded breaks (DSBs) are among a plethora of lesions that threaten the integrity of the genome. If not properly processed DSBs can lead to cell cycle arrest or illegitimate DNA rearrangements such as translocations, inversions, or deletions. These rearrangements can contribute to cell dysfunction, cell death, or carcinogenesis [1]. DSBs can arise through the action of exogenous DNA-damaging agents, but they also arise from endogenous sources, such as oxidative DNA damage and as a consequence of DNA replication [1, 2]. Homologous recombination (HR) is a major DNA repair pathway by which DSBs are repaired. This pathway is generally a precise way of resolving DSBs because it uses homologous sequence, usually provided on the sister chromatid, as a repair template [3].

HR is a complex process requiring a number of proteins of the *RAD52* epistasis group including Rad51 and Rad54. Rad51 is the key player in this process because it is critical for homology recognition and performs strand exchange between recombining DNA molecules. A pivotal intermediate in these reactions is the Rad51 nucleoprotein filament. This forms when Rad51 polymerises on single-stranded (ss) DNA that results from DNA damage processing [3]. Rad54 is an important accessory factor for Rad51 [4]. A number of biochemical characteristics of Rad54 have been well-defined for different species ranging from yeast to humans [5-16]. Rad54 is a double-stranded (ds) DNA-dependent ATPase that can translocate on DNA thereby affecting DNA topology. Biochemically, Rad54 has been implicated to participate in multiple steps of HR. It can stabilise the Rad51 nucleoprotein filament in an early stage of recombination [17]. At a subsequent stage it can promote chromatin remodelling [18-20] and stimulate Rad51-mediated formation of a joint molecule between the broken DNA and the repair template, referred to as a D-loop [9]. In later stages of the reaction it can displace Rad51 from DNA [21].

Cell biological experiments have revealed that Rad54 accumulates to form dynamic foci at sites of DNA damage that display rapid turn-over of Rad54 [15, 22, 23]. In those foci Rad54 colocalises with and stabilises Rad51 [15, 24]. Chromatin immunoprecipitation experiments using *Saccharomyces cerevisiae* (*S. cerevisiae*) cells underscore the co-operation between Rad51 and Rad54 [25, 26]. In the absence of Rad54, Rad51 is still able to pair homologous sequences, but the joint molecules are qualitatively different from those formed in the presence of Rad54 [25, 27].

Genetic analysis of *RAD54* has been performed in a number of species including yeast and mice. *S. cerevisiae* cells with mutated *RAD54* are DNA damage-sensitive, including ionising radiation, severely defective in gene conversion and exhibit increased chromosome loss [28-30]. Mouse *Rad54* knockout embryonic stem (ES) cells are ionising radiation and mitomycin C-sensitive, show reduced HR efficiency as measured by gene targeting and display aberrant DSB repair [31, 32]. Interestingly, while *Rad54* knockout mice are sensitive to the interstrand DNA cross-linking agent mitomycin C, they are not ionising radiation-sensitive [33]. The contribution of Rad54-mediated HR to repair

of ionising radiation-induced damage in adult animal is revealed when non-homologous DNA end-joining, an alternative and mechanistically distinct DSB repair pathway, is also impaired [33-35]. A possible explanation for this observation is the existence of redundancy in Rad54 function in mammalian cells.

In *S. cerevisiae*, a *RAD54* homologue, *RDH54* (also know as *TID1*), has been identified [36-38]. Its biochemical properties are similar to Rad54, for example, Rdh54 is an ATPase and it stimulates D-loop formation by Rad51 [39]. The phenotypes of *rad54* and *rdh54* mutants are distinct, but do appear to be related to defects in HR. While *rad54* mutants are sensitive to the alkylating agent methyl methanesulfonate, *rdh54* mutants are not or less so [37, 38]. However, in the absence of *RAD54*, the contribution of *RDH54* to cell survival is uncovered because *rad54rdh54* double mutants are more sensitive to methyl methanesulfonate than either single mutant. While *RAD54* affects both intra- and interchromosomal recombination, *RDH54* seems to be more important for the latter only [37, 38, 40]. An interaction between the two genes has been found in meiosis. While sporulation efficiency and spore viability are reduced in the *rad54* and *rdh54* single mutants, these parameters are synergistically reduced in *rad54rdh54* double mutants, likely reflecting partial overlapping functions of *RAD54* and *RDH54* during meiotic recombination [41].

A *RAD54* homologue, named *RAD54B*, has also been identified in human cells [42, 43]. This gene has been labelled the mammalian homologue of yeast *RDH54*. However, this classification is based on amino-acid sequence similarity and not on extensive functional analysis. Here we report the biochemical and genetic characterisation of mammalian *Rad54B*. We show that mammalian Rad54B has biochemical properties similar to Rad54. However, the results of genetic experiments using *Rad54* knockout, *Rad54B* knockout and *Rad54Rad54B* double knockout cells and mice, suggests that mammalian *Rad54B* is unlikely to be the true *S. cerevisiae RDH54* homologue because the genes are not functionally equivalent.

MATERIALS AND METHODS

Isolation of human and mouse *RAD54B* DNA

The *hRAD54B* cDNA was initially cloned as two fragments from a human testis cDNA library by PCR using the following primers (fragment 1: 5'-GCAGGGCCAGTGGTTTCTGTC and 5'-GTGG TCCTGATCAACAGTAAAT; fragment 2: 5'-ATTTACTGTTGATCAGGACCAC and 5'-GAAGAG CAATGGAATGTCAGAA). The two PCR fragments were digested with *Bcl* I, ligated together, and used as a template for amplification of the full length *hRAD54B* cDNA with the following primers: 5'-CGGGATCC*CAT****ATG***AGACGATCTGCAGCACC and 5'-CGGGATCC**CTA**TGTGCCAGTA GCTTGAG (*Bam* H I sites are underlined, *Nde* I site is *italicised*, and start and stop codons are in **bold**). The PCR product was cloned into the *Bam* H I site of pUC18 and sequenced. The mouse *Rad54B* cDNA was isolated using a combination of RT PCR with degenerate primers [44] and cDNA library screening. The resulting 2658 bp cDNA mouse *Rad54B* has the Genebank identification number NM009015.

Purification of mouse Rad54B protein

The cDNA encoding Rad54B tagged with polyhistidine at the amino-terminus and a FLAG tag at a carboxyl-terminus was subcloned into pFastBac1 (BAC-TO-BAC Baculovirus Expression System, Life technologies, Inc.). The resulting plasmid was transformed into DH10Bac *E. coli* cells to obtain a recombinant bacmid. After transfection of the bacmid into Sf21 cells virus stock was produced and amplified as described by the manufacturer. For protein production, 2×10^8 Sf21 cells were infected with the recombinant baculoviruses at a multiplicity of infection of 10. Two days postinfection, cells were collected by low speed centrifugation and washed twice with ice-cold phosphate-buffered saline (PBS). Presence of mRad54B protein in the lysate was confirmed by electrophoresis through SDS-PAGE gels and subsequent blotting to nitrocellulose. The blots were hybridised with anti-FLAG (Sigma) antibodies. The level of expression of the his-Rad54B-flag protein was determined by staining the SDS-PAGE gels with Coomassie Brilliant Blue. For fractionation, the cells were lysed and clarified lysate was neutralised and fractionated as described previously [14]. Briefly, the crude extract was incubated overnight at 4°C with the phosphocellulose column (Whatman P11). Eluted fraction was subsequently loaded on Ni^{2+}-nitrilotriacetate agarose (Qiagen). Aliquots of eluted fractions were stored in –80°C for further analysis. Purity of obtained eluates was verified by running the samples through SDS-PAGE gel followed by Coomassie-staining. Standard loading and molecular mass markers were used.

ATPase assay

Standard reaction mixtures contained 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 100 µg/ml bovine serum albumin, 200 µM ATP, 0.25 µCi of [γ-^{32}P]ATP

(>5000 Ci/mmol), 45 μM DNA (concentration in nucleotides) and 0 or 100 ng of mRad54B protein in a 10 μl volume. Incubations were for 60 min at 37°C. Reactions were initiated by the addition of DNA and $MgCl_2$ to a mixture containing the other components and terminated by the addition of EDTA to 167 mM. Released phosphate was separated from ATP by thin-layer chromatography on polyethyleneimine cellulose using 0.75 M KH_2PO_4 as running buffer. Hydrolysis was quantitated with the use of a Molecular Dynamics PhosphorImager. To determine the optimal pH of the reaction, the following buffers were used: Hepes-KOH (pH 6.0), and Tris-HCl (pH 7.0, 7.5, 8.0, and 8.5) (Figure 2B).

Proteins and DNA-binding reactions

The protein-DNA binding reactions were performed as described previously [11]. Briefly, protein-DNA complexes were prepared by addition of the mRad54B protein to singly nicked 1821-bp DNA plasmid. Reaction mixtures (10 μl final volume) were assembled by mixing mRad54B (up to final concentrations of 0.34 μM) and DNA (76 μM; concentration in nucleotides) in buffer containing 20 mM Hepes-KOH (pH 7.4), 20 mM KCl, 5 mM $MgCl_2$, and 2 mM ATP. After incubation at 30°C for 10 min, glutaraldehyde was added to a final concentration of 0.1%, followed by additional incubation at 30°C for 10 min. Generated protein-DNA complexes were analysed by SFM following standard protocol [11]. Reaction mixtures were diluted 15- to 30-fold in deposition buffer, consisting of 5 mM Hepes-KOH (pH 7.5) and 5 mM $MgCl_2$. Within 15 sec a 10- to 15-μl drop was placed onto freshly cleaved mica. After 30 sec the mica surface was washed with H_2O (glass-distilled, Sigma), followed by drying with a stream of filtered air. The nucleoprotein complexes were imaged in air at room temperature and humidity by using a NanoScope IIIa (Digital Instruments, Santa Barbara, CA) operating in the tapping mode with a type E scanner. Silicon tips (Nanoprobes) were obtained from Digital Instruments.

Generation of a *mRad54B* disruption construct and *mRad54B* knockout mice

A 129 mouse genomic library (Stratagene, Cat# 946308) was screened with a DNA fragment from the *mRad54B* cDNA sequence. A positive clone was picked and the DNA was subsequently subcloned into a pBluescript KS. The restriction map of the genomic DNA was determined using *Eco* R I, *Bgl* II, *Bam* H I and *Xba* I endonucleases; it was also noted that this particular stretch of genomic DNA contained 7 exons. A 1851 bp fragment between *Eco* R I and *Bgl* II sites in the genomic DNA was replaced with 1108 bp *Xho* I/*Hind* III fragment from pMC1neo (Stratagene). This pMC1neo fragment contained the neomycin gene under the control of TK promoter. The neomycin marker gene was flanked by an approximately 9 kb *Eco* R I and a 1097 bp *Bgl* II-*Xba* I fragments of the *mRad54B* locus. When used in gene targeting, this construct was expected to eliminate 28 highly conserved amino acids, in effect knocking out functional *mRad54B*. The targeting construct was electroporated into E14 ES cells, which were then put under G418 selection. Positive clones were

screened by DNA blot analysis using specific probes [45]. One out of 238 clones showed a fragment of a size expected for targeted integration of the construct. This was further confirmed using several restriction enzymes and both upstream and downstream probes. ES cells that contained this targeted event were injected into blastocysts. This gave rise to 17 chimeric males, which were then backcrossed to BDF1 females, in order to get pure knockout mice.

DNA damage sensitivity assays

Cellular clonogenic survival assays have been described previously [32]. Every measurement was performed in triplicate. For ionising radiation survivals assays, cells were exposed to the specified dose of γ-rays. For mitomycin C survival assays, cells were incubated in medium containing the specified concentration of mitomycin C for an hour. The cells were then washed with PBS, and replenished with fresh medium. The cells were allowed to grow for 10 days, after which the colonies were stained and counted. The mitomycin C survival experiments were performed four times. Cloning efficiencies of untreated cells varied between 10-30%.

Ionising radiation and mitomycin C sensitivity of mice was assessed with the use of two to four month old littermates from the various genotypes (wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$, *mRad54*$^{-/-}$*mRad54B*$^{-/-}$). Mice were irradiated with a 7 Gy dose (^{137}Cs source) and monitored for 21 days. Surviving animals were euthanised. Male and female mice were injected with 15, 10, 7.5, 5, 2.5 and 1 mg of mitomycin C per kg bodyweight and monitored for 14 days.

DNA damage processing was indirectly assessed by the micronucleus assay. 100 μl of peripheral blood was collected by orbital puncture, and the micronucleus assay was performed as described [46]. Five hundred polychromatic erythrocytes were scored for the presence of micronuclei using an Axioplan fluorescence microscope.

RESULTS

Isolation of a cDNA encoding a mammalian Rad54 paralogue

DNA oligonucleotides of degenerate sequence based on conserved amino-acid motifs in the SNF2/SWI2 family of proteins were used to isolate a *Rad54* paralogue, which we named mouse *Rad54B* (*mRad54B*), from mouse cDNA libraries. The *mRad54B* cDNA consisted of a 2658 bp open reading frame with the potential to code for an 886 amino-acid protein with a predicted weight of 103 kDa. Amino-acid sequence comparison of mRad54 and mRad54B revealed 33% sequence identity that extends over the entire length of the proteins. The predicted amino-acid sequence of mRad54B showed 80% sequence identity with human Rad54B (hRad54B) [47]. Further sequence analysis indicated that human and mouse Rad54B displayed 34% sequence identity to both *S. cerevisiae* Rad54 and Rdh54. To obtain insight in the function of mammalian Rad54B, we characterised it biochemically and genetically.

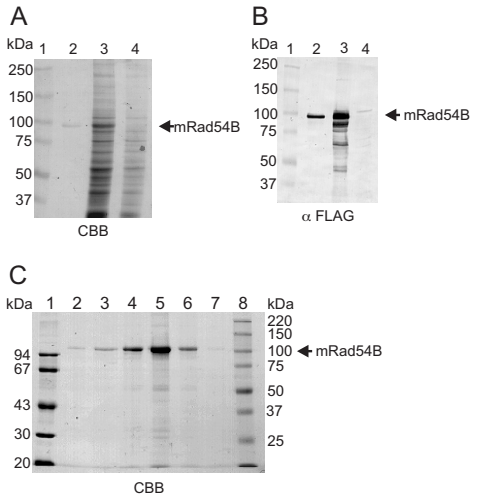


Figure 1. **Expression and purification of the mRad54B protein in Sf21 cells.**

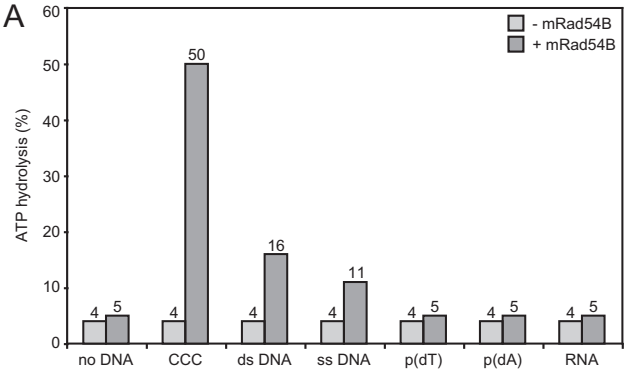
(**A**) Coomassie-stained SDS-PAGE gel containing: in *lane* 2 purified mRad54B (positive control), in *lane* 3 samples of Sf21 cells infected with baculovirus coding for mRad54B, in *lane* 4 sample of Sf21 cells infected with a control virus (hRad54). The size of the protein molecular mass markers in *lane* 1 is indicated in kDa on the left. (**B**) Immunoblot stained with anti-FLAG antibodies to detect mRad54B. The order of the samples is the same as in panel A. (**C**) Coomassie-stained SDS-PAGE gel containing aliquots of fractions of mRad54B protein after fractionation over: Phospocellulose and Ni-Nitrilotriacetate agarose (Ni-NTA) columns. *Lanes* 2-7 contain equal volume of indicated elution fractions from the Ni-NTA column.

Purification of the mouse Rad54B protein.

For the purpose of purification, identification and protein-DNA interactions studies we constructed a cDNA expressing mRad54B containing an amino-terminal polyhistidine tag and a carboxyl-terminal FLAG tag. For protein production we placed the cDNA under transcriptional control of the polyhedrin promotor in a recombinant baculovirus. The virus was used to infect Sf21 cells. A Coomassie-stained SDS-PAGE gel containing crude extract of the cells infected with the mRad54B encoding baculovirus is shown in Figure 1A. Immunoblot analysis with anti-FLAG antibodies demonstrated that the prominent band below 100 kDa of molecular mass marker is the Rad54B

protein (Figure 1B). This band is absent from the extract of Sf21 cells infected with the baculovirus encoding hRad54 protein. The extract was subsequently fractionated over phosphocellulose and Ni^{2+}-nitroacetate agarose. Samples of the Rad54B-containing fractions were analysed by electrophoresis through an SDS-PAGE gel that was stained with Coomassie Blue (Figure 1C). We estimate that the final Rad54B preparation had a purity of approximately 80-90 %.

A



B

Variable	Relative ATPase activity %
Divalent cation	
None	4
Mg^{2+}	87
Mn^{2+}	6
pH	
6.0	73
7.0	89
7.5	89
8.0	97
8.5	100
Temperature	
24 °C	23
30 °C	56
37 °C	94
Nucleid acid cofator	
None	1
Supercoiled DNA	103
Linear dsDNA	53
M13 viral DNA	17
Poly(dT)	1
Poly(dA)	1
RNA	1

Figure 2. **ATPase activity of the mRad54B protein.**

(**A**) Shown is the percentage of ATP hydrolysed by the mRad54B in presence of different nucleic acid cofactors as indicated below the graph (dark grey bars). Bars in light grey show background ATP hydrolysis in the absence of mRad54B protein. (**B**) The ATPase activity of 100 ng of mRad54B was determined under standard conditions. An ATPase activity of 100% corresponds to hydrolysis of 70% of input ATP.

Mouse Rad54B is a dsDNA-dependent ATPase.

Similarly to its paralogue Rad54, Rad54B contains Walker A and B amino-acid sequence motifs that are involved in ATP hydrolysis. Previously we have shown that Rad54 is a dsDNA-dependent ATPase [14].

That prompted us to test whether purified mouse Rad54B could hydrolyse ATP. We varied the parameters of the reaction in order to find out the conditions for the optimal hydrolysis of ATP (Figure 2). Analogously to hRad54, mRad54B is a dsDNA-dependent ATPase (Figure 2A). However, the requirements for the optimal catalytic activity of both proteins are slightly different. In contrast to hRad54 we were able to detect only very modest ATP hydrolysis when we used Mn^{2+} instead of Mg^{2+}. Also mRad54B hydrolysed ATP more efficiently at higher temperature (37°C not 30°C) and at higher pH (8.0 not 7.0) than hRad54. Despite these differences, mRad54B, similarly to hRad54, hydrolysed ATP most efficiently when supercoiled DNA was present, although topologically relaxed dsDNA could also promote the reaction. In the presence of ssDNA or RNA no ATP hydrolysis was detectable. Thus, most likely the stimulation of ATP hydrolysis by M13 viral DNA was due to the presence of secondary DNA structures in the molecule.

Evidence for Rad54B translocation along DNA.

For the Rad54 protein we and others have shown that its ATPase activity is required for translocation along DNA resulting in effective topological changes in the DNA molecule [11]. This has been also demonstrated for hRad54B using topological assay [45]. However, the architecture of the Rad54B-DNA complex leading to those topological changes has not been determined. Therefore scanning force microscopy (SFM) technique was used to analyse complexes formed between purified mRad54B and singly nicked circular dsDNA in the presence of ATP. We observed a Rad54B complex anchoring the junction between relaxed and plectonemically supercoiled domains of the plasmid (Figure 3). This is a similar architecture as found for hRad54-DNA complexes [11]. This highly specific architecture is consistent with a protein complex tracking along the DNA double helix, leaving the DNA domain containing the nick in the relaxed form while simultaneously introducing supercoils into the covalently closed domain.

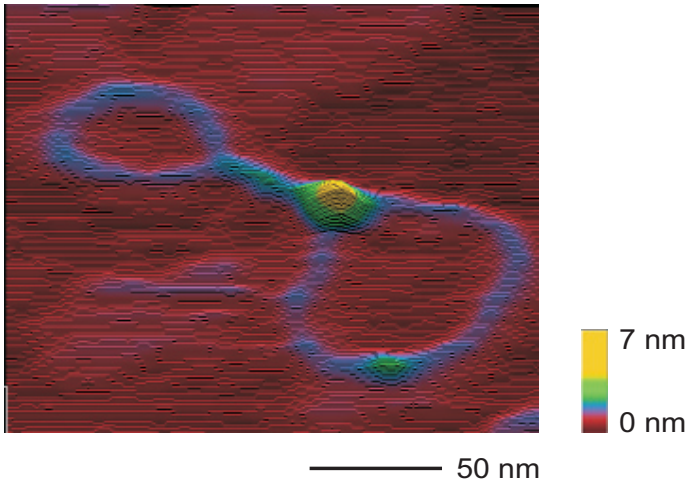


Figure 3. **Evidence for Rad54B translocation along DNA.**
SFM image of a mRad54B-DNA complex formed in the presence of ATP. The image is presented as line plot at a 60° viewing angle to emphasise topography. Height is indicated by colour as shown on the bar at the right. The plasmid has a mRad54B complex bound at the junction of relaxed and supercoiled domains.

Generation of *Rad54B* disrupted mouse ES cells and mice

The biochemical activities of the mammalian Rad54B protein determined above are consistent with a role for the protein in HR. To determine its role *in vivo* and to assess the biological relevance of mammalian Rad54B, we generated *Rad54B* knockout mouse ES cells and mice. A clone spanning the 3' region of *mRad54B* was isolated from a mouse genomic library and subsequently characterised by restriction analysis and intro-exon border mapping and chromosomal localisation [45]. From this clone, a targeting vector was derived that upon homologous integration into the endogenous *mRad54B* locus would eliminate 28 highly conserved amino-acids spanning the last conserved SNF2/SWI2 family member motif. The targeting vector was electroporated into E14 ES cells and after selection, correctly targeted clones were identified by DNA blotting using a unique probe outside the targeting construct [45]. A targeted clone was propagated and injected into blastocysts to generate mice carrying the disrupted *mRad54B* allele. Inactivation of the *mRad54B* gene in *mRad54B*$^{-/-}$ mice was confirmed using RNA blot analysis [45].

Interbreeding of *mRad54B*$^{+/-}$ mice resulted in a Mendelian segregation of the disrupted *mRad54B* allele. Thus, mRad54B disruption did not result in embryonic or neonatal lethality. No statistically significant difference in weight was observed among *mRad54B*$^{-/-}$, *mRad54B*$^{+/-}$ and *mRad54B*$^{+/+}$ littermates. Importantly, the *mRad54B*$^{-/-}$ mice exhibited no macroscopic abnormalities up to at least six months of age. *mRad54B*$^{+/-}$ animals were crossed to obtained blastocyts, which were then used to isolate *mRad54B*$^{-/-}$ ES cells. Two independent *mRad54B*$^{-/-}$ ES cell lines were obtained, one in 129 background, and the other in 129/bl6 background. To obtain cells and animals in which both *mRad54* paralogues were disrupted *mRad54B*$^{+/-}$ mice were crossed with *mRad54*$^{+/-}$ mice. Similar to *mRad54*$^{-/-}$ and *mRad54B*$^{-/-}$ mice, *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice displayed no overt phenotypes and appeared normal. *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$, and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells were isolated from blastocysts obtained from intercrossing mice carrying different combinations of the *mRad54* and *mRad54B* knockout alleles.

Mouse Rad54B deficiency confers ionising radiation and mitomycin C hypersensitivity

We have shown previously that *mRad54* contributes to HR efficiency using gene targeting as an assay [32]. Interestingly, mRad54B did not show a defect in this assay. However, the involvement of *mRad54B* in HR was revealed in absence of *mRad54*. Hardly any homologous integration events were detected in *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells [45]. To determine whether the contribution of *mRad54B* to HR impinges on the ability of the cell to repair DNA damage, we examined the effect of ionising radiation and mitomycin C on the survival of wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells. While *mRad54*$^{-/-}$ ES cells are 2- to 3-fold more sensitive to ionising irradiation than wild type ES cells, *mRad54B*$^{-/-}$ cells were only 1.5-fold more sensitive than wild type ES cells (Figure 4A) [32]. The ionising radiation sensitivity of the double mutant *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells was similar to

that of *mRad54*$^{-/-}$ ES cells. For mitomycin C, *mRad54*$^{-/-}$ and *mRad54B*$^{-/-}$ single mutant ES cells showed a similar hypersensitivity compared to the double mutant *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells (Figure 4B). We conclude that, in addition to mRad54, mRad54B also contributes to repair of ionising radiation and mitomycin C-induced DNA damage.

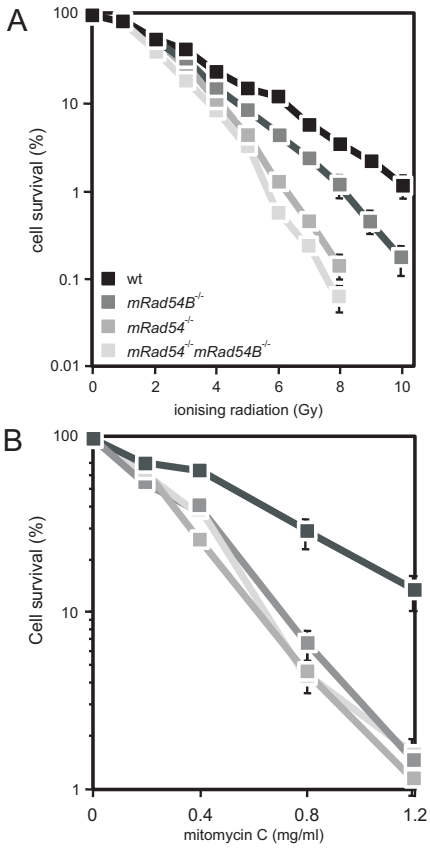


Figure 4. **Effect of ionising radiation and mitomycin C on wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells.**

(**A**) Clonogenic survival of wild type (wt) and mutant ES cells after irradiation with increasing doses of γ-rays. The percentage of surviving cells measured by their colony-forming ability is plotted as function of the γ-ray dose. (**B**) Clonogenic survival of wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells after treatment with mitomycin C. Error bars (some obscured by the symbols) represent standard error of the mean (SEM).

Mice lacking both *Rad54* paralogues are extremely sensitive to mitomycin C

To establish the impact of *mRad54B* on protection from the adverse effects of induced DNA damage in the adult animal wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice were exposed to ionising radiation and mitomycin C. As has been found for m*Rad54*$^{-/-}$ mice neither *mRad54B*$^{-/-}$ nor *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice were sensitive to ionising radiation. All the 2- to 4-months old littermates survived exposure to 7 Gy of ionising radiation (data not shown).

Previously, we showed that *mRad54*$^{-/-}$ mice are hypersensitive to mitomycin C [33]. To reveal whether *mRad54B* also contributes to protection from the mitomycin C-induced DNA damage, wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice were injected peritoneally with

different doses of mitomycin C and monitored for 14 days. As is the case for *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ animals were hypersensitive for mitomycin C (Figure 5). At a dose of 7.5 mg mitomycin C per kg of bodyweight, approximately 60% of the *mRad54*$^{-/-}$ and *mRad54B*$^{-/-}$ mice survived, compared to 100% of the wild type mice. By contrast, none of the *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice survived the treatment. The latency period of death for *mRad54B*$^{-/-}$ mice was comparable to *mRad54*$^{-/-}$ mice. At the lower dose of 5 mg/kg mitomycin C all *mRad54*$^{-/-}$ and *mRad54B*$^{-/-}$ mice survived. In stark contrast, all of the *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice died within 7 days. We conclude that the mammalian Rad54 paralogues function synergistically to protect mice from the deleterious effects of mitomycin C.

The bone marrow is a major target for mitomycin C-inflicted damage *in vivo*. Therefore, we tested whether mitomycin C treatment affected cells in the blood to a different extend in *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ animals versus *mRad54*$^{-/-}$ and *mRad54B*$^{-/-}$ animals using the peripheral blood micronucleus assay. The presence of micronuclei in polychromatic erythrocytes provides a measure of chromosomal aberrations. A single dose of 2.5 mg mitomycin C per kg bodyweight was administered to 6- to 8-week old animals. This treatment resulted in increases in the frequency of micronuclei-containing polychromatic erythrocytes (Figure 5C). Before the mitomycin C treatment the percentage of micronuclei-containing polychromatic erythrocytes was similar in wild type, *mRad54*$^{-/-}$, *mRad54B*$^{-/-}$ and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ animals. Consistent with the mitomycin C hypersensitivity of *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice, the crosslinking agent induced significantly higher levels of micronuclei-containing polychromatic erythrocytes in *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice compared to *mRad54*$^{-/-}$ and *mRad54B*$^{-/-}$ mice (Figure 5C).

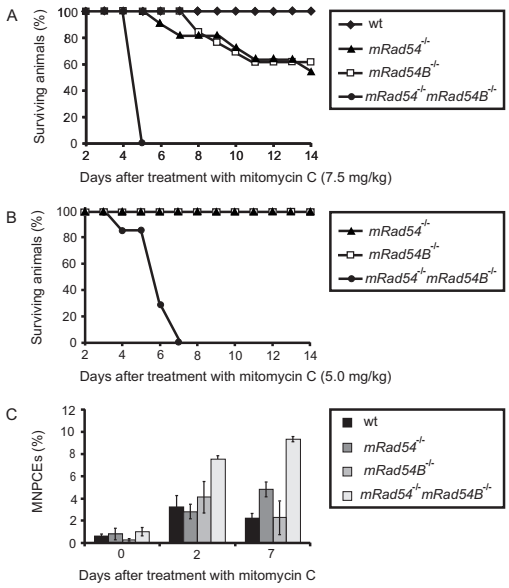


Figure 5. **Impact of *mRad54B* on the mitomycin C sensitivity of mice.**

(**A**) Survival curve of wild type (n=6), *mRad54*$^{-/-}$ (n=11), *mRad54B*$^{-/-}$ (n=13) and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ (n=4) mice after a single intraperitoneal injection of 7.5 mg of mitomycin C per kg bodyweight. (**B**) Survival curve of *mRad54*$^{-/-}$ (n=2), *mRad54B*$^{-/-}$ (n=2), and *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ (n=7) mice after treatment with 5 mg/kg mitomycin C. (**C**) Induction of micronuclei by mitomycin C in polychromatic erythrocytes. Mice of the indicated genotypes were intraperitoneally injected with 2.5 mg/kg bodyweight mitomycin C. Plotted are percentages of micronuclei-containing polychromatic erythrocytes (MNPCEs) per 500 polychromatic erythrocytes at day zero, two and seven after treatment. Data points represent average from three independently treated animals. The standard error of the mean is indicated.

DISCUSSION

HR is a versatile DNA damage repair pathway that is essential for preservation of genome integrity. Among lesions that initiate HR are ssDNA gaps and DSBs. This feature makes HR ideally suited to underpin DNA replication, because ssDNA gaps and DSBs occur at corrupted replication forks that arise either due to spontaneous or induced DNA damage. HR between sister chromatids can rebuild these disabled replication forks *in situ* in the absence of a replication origin [2]. Due to this important function HR is essential for mammalian cell viability.

A key player in HR is Rad51, the homology recognition and DNA strand exchange protein. The crucial role of HR for mammalian cell viability is underscored by the lethality of Rad51 knockout cells and mice [3]. However, not all proteins involved in HR are essential, implying either redundancy in function or the existence of subpathways of recombination that by themselves are not indispensable for cell viability. One example of a HR protein that is not vital for cell viability is the Rad51 accessory factor Rad54 [32, 48]. To address whether there is potential redundancy in Rad54 function or recombination subpathways requiring a specialised Rad54 protein, we biochemically and genetically characterised the mammalian Rad54 paralogue Rad54B.

The mammalian Rad54 paralogues have similar biochemical activities

We performed biochemical analyses of mRad54B to compare and contrast its activities with hRad54. We find that, like hRad54, mRad54B is a dsDNA-dependent ATPase (Figure 2 and [43, 45]). In addition, hRad54B has similar DNA translocase and DNA double-helix opening activities as hRad54 [9, 11, 15, 39]. Here, we show that this is likely due to mRad54B's ability to assemble complexes with DNA that have a similar architecture to hRad54-DNA complexes (Figure 3). Another well-explored activity of hRad54 is its interaction with hRad51 [6, 8, 15, 16]. It has also been shown that hRad54B interacts with hRad54 in a functional manner. Human Rad51 stimulates the ATPase activity, DNA translocase and DNA double helix-opening activities of hRad54B [45]. Conversely, hRad54B stimulates the formation of D-loops, a critical intermediate in HR, generated by hRad51 [45]. We conclude that hRad54B has similar biochemical activities as its paralogue, hRad54. Therefore, hRad54 and hRad54B could provide redundant functions with respect to HR-mediated DNA damage repair or similar functions but in a tissue-specific manner.

Both mammalian Rad54 paralogues contribute to cell survival in response to DNA damage

Because HR is such a versatile mechanism to repair DNA damage compared to repair mechanisms that rely on a specific DNA damage recognition protein to initiate the reaction, it is remarkable that a hRad54B-deficient cancer cell line HCT116 is not hypersensitive to DNA-damaging agents [42]. The disadvantage of this cell line is its genetic instability; it is mismatch repair-deficient,

displays micro-satellite instability and harbours numerous chromosomal aberrations. Given these caveats, we examined DNA damage sensitivity in mouse ES cells instead. Lack of Rad54 in ES cells results in cellular hypersensitivity to ionising radiation and mitomycin C [32]. In contrast to the hRad54B-deficient cancer line, ES cells lacking mRad54B are hypersensitive to ionising radiation and mitomycin C (Figure 4). At the cellular level our results reveal no strong indication for an additive or synergic interaction between the two Rad54 paralogues with respect to the repair of ionising radiation and mitomycin C-induced DNA damage repair.

The Rad54 paralogues synergistically contribute to mitomycin C resistance in mice

The contribution of Rad54 to survival of mice in response to DNA damage differs from that in ES cells. While *mRad54*$^{-/-}$ ES are ionising radiation and mitomycin C hypersensitive, *mRad54*$^{-/-}$ mice are mitomycin C but not ionising radiation hypersensitive [33]. We tested whether the lack of ionising radiation hypersensitivity in mice is due to redundancy in mRad54 function provided by mRad54B. However, this is not the case because *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice are also not overtly ionising radiation-hypersensitive (data not shown). The contribution of HR to repair of ionising radiation-induced DNA damage is revealed in the absence of non-homologous DNA end-joining, an alternative mechanistically distinct DSB repair pathway [33]. Possibly, while HR an important DNA repair pathway for two-ended breaks, such as those induced by ionising radiation, in rapidly dividing ES cells, non-homologous DNA end-joining is much better suited for repair of these lesions in the many non-diving cells of the adult mice. In contrast, mitomycin C-induced DNA interstrand cross-links are processed into single-ended DSBs by DNA replication [49]. Single-ended DSBs cannot be acted upon efficiently by non-homologous DNA end-joining and require HR for repair instead [50]. As is the case for *mRad54*$^{-/-}$ mice, *mRad54B*$^{-/-}$ mice are mitomycin C hypersensitive (Figure 5). Furthermore, *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ double mutant mice are extremely mitomycin C hypersensitive. This synergistic effect of the Rad54 paralogues could be due to their functions in distinct subpathways of interstrand DNA crosslink repair. However, given their biochemical similarities and the lack of a significant difference in mitomycin C hypersensitivity of *mRad54B*$^{-/-}$ versus *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ ES cells this is unlikely. Alternatively, the Rad54 paralogues might have a tissue-specific function by differentially contributing to interstrand DNA crosslink repair in different cell types of mice. This premise predicts that there should be differences in expression of the Rad54 paralogues among different tissues. Although no direct comparison is currently available, the existing data does suggest that this could be the case. For example, while expression of *hRAD54B* is extremely low in the spleen (data not shown) expression of *mRad54* in the spleen is robust [23].

Comparison of the yeast and mammalian Rad54 paralogues

Taken together our data lead us to conclude that the premise that mammalian Rad54B is the functional homologue of *S. cerevisiae* Rdh54 is unlikely. While *S. cerevisiae rdh54* mutant cells display no overt DNA damage sensitivities, *mRad54B*$^{-/-}$ ES cells do. Furthermore, both *S. cerevisiae* Rad54 paralogues make significant contributions to meiotic HR. In particular, Rdh54 plays an important role in interhomolog recombination [40]. In contrast, an essential role of the mammalian Rad54 paralogues in meiosis was not detected [45]. It is possible that a yet undiscovered meiosis-specific Rad54 paralogue exists in mammals. Alternatively, rather than being strictly assigned to meiotic DSB repair, this hypothetical Rad54 paralogue might overlap in DSB repair function with Rad54 and Rad54B and take over part of the function of both Rad54 and Rad54B in their absence, which would be consistent with the viability of *mRad54*$^{-/-}$*mRad54B*$^{-/-}$ mice and the essential role of HR for mammalian cell viability. However, when challenged with exogenous DNA-damaging agents such as mitomycin C, the DNA damage load might exceed the threshold of its ability to repair on its own, and this is reflected by the mitomycin C hypersensitivity of Rad54 and Rad54B deficient mice. The DNA damage threshold may also explain the apparent normal viability of the Rad54 and Rad54B-deficient mice, because the level of endogenous DNA damage might be low enough to be effectively handled by a yet unidentified Rad54 paralogue.

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CHAPTER 6

DNA double-strand break repair and chromosome translocations

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DNA double-strand break repair and chromosome translocations

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ABSTRACT

Translocations are genetic aberrations that occur when a broken fragment of a chromosome is erroneously rejoined to another chromosome. The initial event in the creation of a translocation is the formation of a DNA double-strand break (DSB), which can be induced both under physiological situations, such as during the development of the immune system, or by exogenous DNA damaging agents. Two major repair pathways exist in cells that repair DSBs as they arise, namely homologous recombination, and non-homologous end-joining. In some situations these pathways can function inappropriately and rejoin ends incorrectly to produce genomic rearrangements, including translocations. Translocations have been implicated in cancer because of their ability to activate oncogenes. Due to selection at the level of the DNA, the cell, and the tissue certain forms of cancer are associated with specific translocations that can be used as a tool for diagnosis and prognosis of these cancers.

1. Introduction

A chromosomal translocation is a genomic aberration involving the rejoining of a broken chromosome fragment to another chromosome. While there need not be a net change in the amount of genetic material following this event, it can result in the disruption of genes or cause the juxtaposition of elements that disturb normal expression of the gene present at the breakpoint. This becomes critical when such translocations result in rearrangements that create genetic elements with oncogenic characteristics. In this case, it can lead to a selective advantage of cells containing these translocations with the potential for uncontrolled proliferation.

2. Clinical implications

Several documented cases of cancer exist in which translocations play an important role. One of the first described was the Philadelphia chromosome, t(9;22), found in cancer cells of patients suffering from chronic myelogenous leukemia [1]. The proto-oncogene *ABL* (*c-ABL*), a gene encoding a protein tyrosine kinase, was discovered to be located at the breakpoint on chromosome 9 [2]. *c-ABL* is highly regulated in its normal chromosomal environment, but is hyper-activated in the context of the Philadelphia chromosome translocation. Another example is Burkitt's lymphoma, where translocations involving chromosome 8 and chromosomes 2, 14 or 22 have been documented. In this case, the proto-oncogene c-*MYC* and the genes implicated in the production of antibodies (immunoglobulin (*Ig*) light (L) and heavy (*H*) chains) have been found at the breakpoints [3-5]. As a result, *MYC* is misregulated; it is abnormally overexpressed under the influence of the *Ig* gene promoters. *MYC* could increase DNA damage load by increased production of reactive oxygen species, due to its role in mitochondrial gene expression [6]. Other genes have been implicated in cancers involving translocations, for example, deregulation of the homeobox gene *HOX11* by the

t(10;14) translocation is involved in T-cell acute lymphoblastic leukemia [7].

Over the years, many more translocations occurring in leukemias, lymphomas and solid tumors have been identified. While found mostly in hematological cancers, translocations have been implicated in cancers of mesenchymal and epithelial origin as well [8]. An online database that catalogues the occurrence of diseases with specific chromosomal aberration has been compiled (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

The identification of translocations is carried out by classical karyotyping or spectral karyotyping (SKY) analysis [9]. In the clinic, certain types of chromosome translocations are used in classification of primarily haematopoeitic cancers and for the clinical prognosis of patients. For example, in an acute myeloid leukemia patient, a t(8;21) translocation is indicative of a good prognosis, that is, a positive response to treatment and long-term survival, compared to patients with a t(9;22) translocation. This information is then used to identify patients that will benefit from chemotherapy, and to determine the length and dosage of treatment [10].

3. Pathways of DNA double-strand break repair

The mechanism of the formation of translocations has been the focus of intense studies over the years. Chromosome breakage is a first step in the creation of a translocation; in its simplest form, the breakage occurs due to the formation of a DNA double-strand breaks (DSBs) in a chromosome. DSBs can be caused by both exogenous agents, such as ionizing radiation and certain chemicals, as well as by endogenous agents, including the byproducts of cellular metabolism, such as oxygen free radicals [11]. DSBs can also arise spontaneously in each S phase, for example, when a single-strand break in a parental strand is passed by a replication fork, a DSB will result [12]. Besides the pathological DSBs mentioned above, certain cell types undergo processes that require the induction of physiologically important DSBs. For example, nuclease-induced DSBs in germ cells trigger meiotic recombination that results in creation of genetic diversity. In addition, the assembly of active *Ig* and T cell receptor (*TCR*) genes as well as in *IgH* class switch recombination (CSR) occurring in the immune system requires the controlled induction of DSBs.

Repair mechanisms exist in the cell to promote the beneficial effects of the physiologically occurring DSBs and to counteract the deleterious effects of the pathological DSBs. The importance of DSB repair pathways in genome maintenance is underscored by the fact that genomic instability is a characteristic feature of cell lines and animals deficient in DSB repair pathways [13]. Proper functioning of these pathways is important in the deterrence of the illegitimate reattachment of broken chromosomes, preventing the disruption and misregulation of genes in this way. There are two mechanistically distinct methods to rejoin DNA ends, dependent on their requirement for homologous DNA sequences: homology-directed repair and non-homologous end-joining. Both can be divided in a number of subpathways that will be discussed below.

3.1. *Homologous recombination*

Homologous recombination is generally an error-free pathway of homology-directed repair. A DSB is accurately repaired by using the undamaged sister chromatid as a template for the repair of the broken sister chromatid (Fig. 1). Homologous recombination in eukaryotes is carried out by the RAD52 epistasis group of proteins, so called because they were originally identified by the genetic analysis of ionizing radiation hypersensitive *Saccharomyces cerevisae* mutants [14,15]. In human cells, the proteins in this group include the MRN (RAD50/MRE11/NBS1) complex, RAD51, the RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3), RAD54 and RAD54B [16]. The products of the breast cancer susceptibility genes, BRCA1 and BRCA2, are also involved in the modulation of the homologous recombination [17–19].

When a DSB is detected, the initial damage response is mediated through the MRN complex and Ataxia telangiectasia mutated protein (ATM) [20]. The resection of DNA ends is required to generate 3′ single-stranded DNA tails, which are the substrate for homologous recombination, because they are utilized for the nucleation of recombination factors on the DNA. RAD51 is an important protein at the core of homologous recombination. With the help of accessory factors, RAD51 polymerizes on the 3′ tails to create a nucleoprotein filament. After homology search, the nucleoprotein filament invades the target duplex at the site of homology to create a critical intermediate, the D-loop. This joint molecule between the broken sister chromatid and the intact sister chromatid is used as a template for DNA polymerases such that sequence information that was lost in the initial processing of the DSB end is restored. The reaction is concluded with the ligation of DNA strands and the separation of the joint molecules to yield two intact DNA copies (Fig. 1).

One of the proteins involved in the regulation of homologous recombination is the product of the breast cancer susceptibility gene, BRCA2. BRCA2 has been implicated in a mediator-type function involving multiple interactions with RAD51. BRCA2 binds to and sequesters RAD51, presumably preventing the promiscuous binding of RAD51 to DNA, that could instigate illegitimate homologous recombination within highly repetitive DNA content in the genome. However, upon DNA damage induction, RAD51 accumulates at a high local concentration into foci at the sites of damage with the help of BRCA2 [21,22]. Evidence for the involvement of Brca2 in genome stability has been provided by murine cells that express a truncated form of Brca2. These cells display impaired recombination, accumulate DNA breaks, and spontaneous chromosomal abnormalities, including translocations [23].

3.1.1. *Homologous recombination and translocations*

Translocations can occur due to an inappropriate use of recombination mechanisms [8]. In mitotic cells, specifically in the late S/G_2 phase, the template for DSB repair through homologous recombination is preferentially the sister chromatid. This guarantees that the original sequence is restored without any changes [24]. In the human genome however, the presence of highly repetitive sequences can lead to ectopic recombination, resulting in DNA rearrangements including translocations. The highly repetitive *Alu* sequences in the

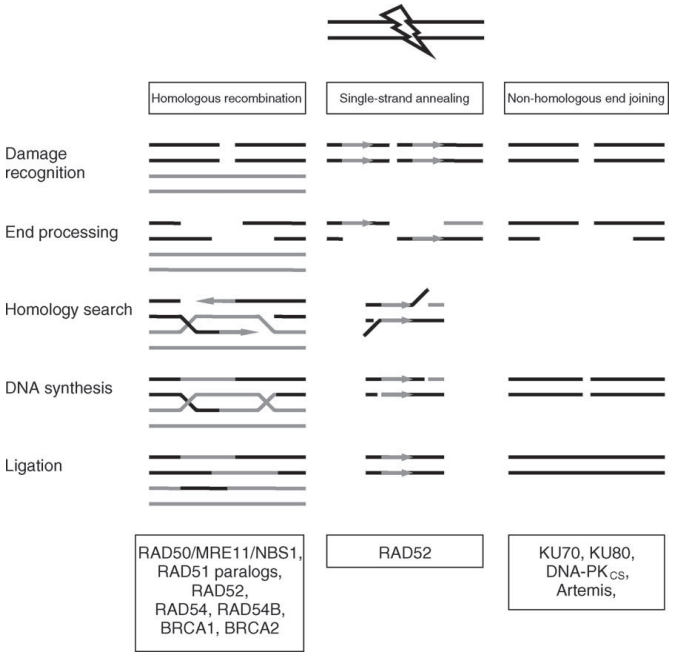

Fig. 1 – Schematic representation of DNA double-strand break repair pathways. The homology-directed DSB repair pathways, homologous recombination and single-strand annealing, are indicated in the left and middle panels, respectively. Non-homologous end-joining is shown in the panel on the right. The broken DNA molecule is indicated by the black double lines. During homologous recombination, the intact, homologous template DNA, indicated in grey, is essential for an accurate repair. DNA replication is portrayed by the arrowhead. Single-strand annealing can repair breaks occurring between or within repeated DNA sequences (indicated by grey arrows). This repair pathway results in deletion of a repeat sequence and the sequences between the original repeats. Homologous DNA is not required for non-homologous end-joining and nucleotides at the break might be added or lost, resulting in inaccurate repair. All three pathways involve damage recognition, resection of broken DNA ends, DNA resynthesis and ligation. Homology search, although in mechanistically distinct manners, takes place during homologous recombination and single-strand annealing. Subsequent stages taking place in the repair pathways are listed on the left. The proteins involved in each pathway, which are discussed in the text, are indicated below each scheme.

genome can undergo homology-promoted replication slippage or homology-mediated illegitimate DSB repair within and between sister chromatids, homologous or heterologous chromosomes. The acute lymphoblastic leukemia 1 (*ALL1*) gene presents an example of how mistakes in homologous recombination can result in oncogenic translocations [25]. *ALL1* is unique among leukemia genes because it can be involved in fusions with a large number of different partner genes located on various chromosomes. Furthermore, *ALL1* can itself be rearranged or duplicated internally by tandem duplication of a portion of the gene [26–28]. In one case of leukemia with a normal karyotype, parts of the duplicated *ALL1* gene have been found fused with *Alu* sequences, such that a chimeric, full-length *Alu* sequence is recreated [29]. This evidence points to homologous recombination between two *Alu* sequences as a molecular mechanism for the partial duplication of *ALL1*.

3.2. *Single-strand annealing*

Single-strand annealing is another subpathway of homology-directed repair, but in contrast to homologous recombination, it is non-conservative, because it results in loss of genetic material (Fig. 1). Single-strand annealing is limited to operating between directly repeated sequences. The action of an exonuclease or helicase at a DSB between such direct repeats results in the exposure of complementary single strands. In a RAD51 independent fashion, complementary sequences (ranging between 30 and 100 base pairs flanking the breaks) anneal to form an intermediate, facilitated by the DNA binding and annealing properties of RAD52 [30]. Single-stranded non-complementary overhangs are removed by nucleases. The result is the loss of one repeat unit and the intervening sequence, demonstrating the imprecise nature of this mechanism.

3.2.1. *Single-strand annealing and translocations*

Single-strand annealing within a chromosome can lead to deletions, and between two chromosomes, it can result in translocations. This was demonstrated in an experimental set-up in which the site for the rare cutting endonuclease I-Sce I was incorporated in the context of homologous sequences placed in chromosomes 14 and 17 in mouse embryonic stem cells [31]. Following the induction of breakages of the chromosomes by transient expression of I-Sce I, the status of exchanges between chromosomes was determined. Translocations were only detected when the I-Sce I sites were present on both chromosomes. Sequence analysis revealed that none of the recombinants could have arisen by gene conversion accompanied by crossing-over, and were therefore the products of single-strand annealing between homologous sequences [32].

3.3. *Non-homologous end-joining*

Conceptually, non-homologous end-joining is the simplest way of repairing DSBs: the straightforward religation of ends without the requirement for a template (Fig. 1). Non-homologous end-joining plays a major role in the elimination of DSBs during G_1 phases of the cell cycle since homologous recombination is not efficient in this phase due to the lack of sister chromatids. In addition, non-homologous end-joining is the mechanism by which the DSBs that initiate V(D)J-recombination and *Ig* CSR are processed in the immune system.

After DSB formation, the KU70/80 heterodimer binds the DNA ends. This facilitates the recruitment of DNA dependent protein kinase catalytic subunit (DNA-PKcs) to the DSB. This sequential binding of the proteins activates the phosphorylation function of DNA-PKcs, phosphorylating itself, the KU heterodimer, and other proteins involved in cell cycle regulation [33]. It has been speculated that KU70/80 might function as an alignment factor that binds DSB ends, creating easy access for and greatly stimulating the function of the DNA ligase IV-XRCC4 complex, increasing the efficiency and accuracy of non-homologous end-joining [34–36]. The Ligase IV-XRCC4 complex then ligates the juxtaposed DNA ends.

A subpathway of non-homologous end-joining requires the nuclease Artemis. Based on pulsed-field gel electrophoresis and phosporylation of the histone H2 variant H2AX as measures of DSBs, approximately 10% of radiation-induced breaks were shown to require processing by Artemis. This process also requires various other players like NBS1, MRE11 and DNA-PK, as well as mediator proteins like H2AX and p53BP1. This highly error-prone pathway repairs DSBs with slower kinetics compared to the Artemis independent pathway [37,38].

Besides the repair of DNA damage induced pathological DSBs, non-homologous end-joining is essential for processing of programmed DSBs in the lymphocyte, a cell-type specific for Ig and TCR production for a functional immune system. Ig and TCR diversity is created during early B and T cell development. In these cells, the programmed induction of DSBs occurs at specific sites, named recombination signal sequences, in the germline variable (V), diversity (D) and joining (J) gene segments. These DSBs are introduced by recombination activating gene (RAG) 1 and 2 proteins. One of each region (V, D and J) is brought together and ligated via non-homologous end-joining, which completes V(D)J recombination and leads to the production of functional Igs and TCRs [33]. The programmed generation of DSBs is restricted to the G_1 phase of developing lymphocytes, by the cell cycle dependent expression of the RAG2 protein [39–41].

Mature B cells undergo clonal expansion in response to an antigen. In order to accommodate this, these cells undergo a process called CSR. Here, the preassembled VDJ_H exon is attached to different constant (C_H) region, changing the antibody effector function. DNA lesions are introduced by activation induced deaminase (AID) in the large repetitive switch (S) region, upstream of the C_H genes; these lesions are thought to be processed by mismatch repair or base excision repair proteins. This creates staggered single-strand breaks on both DNA strands, ultimately resulting in DSBs within the S regions. The repair mechanism of these DSBs is still unclear, although it is thought to involve elements of both non-homologous end-joining and homologous recombination, including KU70/80, DNA-PK_{CS}, H2AX, and p53BP1 [41,42].

3.3.1. *Non-homologous end-joining and translocations*

The process of breakage and rejoining in V(D)J recombination that is utilized in the physiological process of mature receptor generation can be misused to create aberrations. The consequence of this might be the joining of a proto-oncogene locus with the elements of the antigen receptor locus, bringing the oncogene under an active promoter. An example of this is the activation of c-*MYC* in Burkitt's lymphoma [43,44].

Translocations can arise when non-homologous end-joining is not functioning properly. The first non-homologous end-joining defective mouse discovered was the severe combined immunodeficiency (SCID) mouse. The cells from this mouse were found to be defective in DNA-PK_{CS}, and therefore is defective in joining the DSB intermediate in V(D)J recombination [45,46]. As a result, these mice cannot develop a functional immune system, and eventually succumb to lymphoid tumors [13]. The cancer phenotype is exacerbated in mice deficient for both non-homologous end-joining and p53, which develop pro-B cell lymphomas much earlier compared to *scid* mice. A possible explanation for this is that there is reduced apoptosis in cells from $p53^{-/-}$ mice, and cells that accumulate genetic aberrations are not efficiently eliminated [47,48]. The RAG-dependent lymphomas in p53-deficient *scid* mice often contain a characteristic translocation between chromosomes 12, containing the *IgH*, and 15 containing c-*MYC* [41,47,49]. This is also seen in humans, where the *IgH*/c-*MYC* translocation is frequently seen in lymphomas [41,47,50].

Theoretically, the RAG proteins might make a cut at one true recombination sequence which is at the V(D)J region, and another cryptic recombination sequence at the other chromosome involved, resulting in the aberrant rejoining of ends. Thus, the chromosomal loci containing such sequences might be more likely to participate in translocations. For example, the major breakpoint region of the *BCL2* locus, which forms unusual structures that render them RAG targets (see below). It is conceivable that RAG post-cleavage synaptic complexes capture non-specific chromosome ends and join these together with ends generated during V(D)J recombination. It has also been shown that many B lineage tumors, such as spo-

radic forms of Burkitt's lymphoma, harbor translocations that map within the C region sequences, suggesting aberrant repair of a DSB that was generated through AID during CSR [41,51].

4. Aberrant DNA structures and translocations

As has been discussed above, translocations can be a consequence of inappropriate action of DSB repair pathways. However, another important aspect with regard to the occurrence of translocations is the stability of the genome itself. The genome is not uniformly stable and contains fragile sites, so called because they have been implicated in chromosome breakage and DSB formation [52–54]. Many fragile sites are therefore also recombination "hot spots", i.e., regions where recombination has a higher probability of occurring compared to the overall genome [55]. Fragile sites have been shown to contain expanded repeated sequences [52], specifically purine and pyrimidine repeat regions.

Other aberrant DNA structures might also trigger the DSB repair pathways to act. For example, for the translocation t(14;18), implicated in follicular lymphomas, it was found that the major breakpoint region, about 150 bps on chromosome 18, assumes a non-B-DNA structure with single-stranded characteristics, which appears to be cleaved by the RAG complex [56]. A similar non-B-DNA type region was discovered in the human *PKD1* gene, where breakages were seen in a 2.5 kbp poly(purine/pyrimidine) stretch [57]. Furthermore, DNA with mirror repeat symmetry can form an intra-molecular triplex structure called H-DNA, which was seen to be clustered in *c-MYC*, is also hypothesized to be mutagenic [58]. Another abnormal DNA structure appears in palindronic AT-rich repeats that have been shown to be involved in DSB formation (at the center of the palindrome) and subsequent translocations, such as t(11;22) [59–61]. The in vitro tertiary structure of this palindrome was investigated and revealed to form a cruciform structure under physiological conditions. It was hypothesized that this unstable conformation might be a cause for translocations observed involving this region [62]. These observations indicate that while the translocations are a direct consequence of the dysfunction of DSB repair mechanisms, the DNA itself may exist in abnormal conformations, which are then erroneously recognized as substrates by specific DSB repair proteins. Therefore, DNA itself, instead of exogenous DNA damage, can be the causative agent of aberrations.

5. Perspective

The factors that can exacerbate the formation of translocations can be classified into *cis*- and *trans*-acting factors. An example of a *cis*-acting factor is DNA structure. It has been shown in various studies that non-cononical DNA structures can be prone to breakage. Since the central intermediate in the generation of a chromosome translocation is a DSB, loci which contain hotspots for DSB formation have a higher probability of being involved in a translocation. The fact that some of these fragile sites have been shown to be at the breakpoints of documented oncogenic translocations is consistent with this. Exogenous agents that cause DNA damage and/or impede DNA replication can uncover fragile sites [63]. In this regard, an interesting correlation between an increase in the expression of fragile sites involved in breakage resulting in cancer and smoking has been documented [64]. On the other hand, *trans*-acting factors are the proteins involved in the different types of DSB repair pathways. Cells defective in a DSB repair protein contain a higher frequency of chromosome aberrations compared to cells in which DSB repair pathways function optimally.

A crucial question that still remains to be answered is: what is the actual frequency with which chromosomal translocations occur in normal DSB repair proficient cells in the body? This issue is not an easy one to address due to complications at various levels. First of all, there is the consideration that the genome is not uniformly stable. Because certain DNA structures are more likely to lead to breakage of the DNA backbone, loci containing such structures have a higher probability of being involved in a translocation over loci lacking such sites. Second, there is selection at the cellular level. A critical consideration here is that not all translocations provide the cells with a proliferative advantage. In fact, cells containing gross DNA aberrations are more likely to undergo apoptosis. Third, the effects of aberrations might be difficult to document at the tissue level, because translocations that are oncogenic in the context of one tissue may not be oncogenic in the context of others.

Although the factors mentioned above conspire against determining the exact frequency with which translocations occur, they do provide an advantage in the context of cancer therapy. The bias at the level of the DNA, the cell, and the tissue eventually result in the occurrence of specific chromosomal translocations in certain cancers, such that they can be used for the diagnosis and prognosis of these cancers.

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ABBREVIATIONS

aa	amino-acid
ACS	autonomously replicating sequence consensus sequence
ARS	autonomously replicating sequence
ATP	adenosine 5'-triphospahte
BLM	Bloom
BIR	break-induced replication
cDNA	complementary DNA
CDK	cyclin-dependent kinase
D-loop	displacement loop
DDK	Dbf-dependent kinase
χ	*Chi* sequence
CHO	Chinese hamster ovary
CPD	cyclobutane pyrimidine dimmer
Da	Dalton
DNA	deoxyribonucleic acid
DSB	double-stranded break
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
ES	embryonic stem
FACS	fluorescence-activated cell sorting
FL1	green fluorescence
FL2	red fluorescence
FSC-H	forward scatter plot
GFP	green fluorescent protein
Gy	Gray
γ-H2AX	phosphorylated H2AX
GINS	go, ichi, ni, san
h	human
HJ	Holliday junction
HR	homologous recombination
J	Joule
m	mouse
Mcm	minichromosome maintenance complex
NER	nucleotide excision repair
ORC	origin recognition complex
oriC	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
pol	DNA polymerase
polyA	polyadenylation
pre-RC	pre-replicative complex
RFC	replication factor C
RNA	ribonucleic acid
RPA	replication protein A
SCE	sister chromatid exchange
S. cerevisiae	*Saccharomyces cerevisiae*
SFM	scanning force microscopy
SSB	ssDNA binding protein
ssDNA	single-stranded DNA
ter	replication termination DNA sequence
TLS	translesion DNA synthesis
UV-light	ultraviolet light
WRN	Werner
XPV	xeroderma pigmentosium variant
6-4 PP	pyrimidine-(6,4)-pyrimidone adduct

SUMMARY

Genome stability is of primary importance for the survival and proper functioning of all organisms. The diversity and large quantity of DNA lesions cause a constant threat for genome stability. Various types of lesions can arise due to either endogenous or exogenous factors. Normal cellular metabolism is the endogenous source of damages. For example, reactive oxygen species, which form as a natural byproduct of the normal metabolism of oxygen, can result in oxidative modifications of DNA leading subsequently to disintegration of the DNA molecule. Ionising radiation, UV-light or various chemical agents are examples of a danger from the external cellular environment. In order to survive, cells have evolved various mechanisms to protect the stability of their genome. For instance, nucleotide excision repair (NER) deals with the wide class of helix-distorting lesions, whilst two major pathways repair DNA double-stranded breaks (DSBs): homologous recombination (HR) and non-homologous end-joining (NHEJ).

Accurate, coordinated, replication of total genomic DNA that happens only once during each cell cycle is also a challenge for the integrity of the genome. Any damage left unrepaired at the site of a progressing replication fork can stall the DNA replicative polymerase. Initiation of inappropriate DNA rearrangements caused by blocked replication becomes a potential cause of losing chromosomal integrity. Lastly, improper DNA distribution to daughter cells can result in cell death or transformation into a tumour.

For normal growth, survival and maintenance of genome integrity rescue of stalled replication forks due to a block in the template DNA is necessary. Numerous sites in the mammalian genome at which replication is fired during each cell cycle are potential targets for DNA-damaging agents. When DNA polymerase encounters a DNA lesion, replication discontinues until the damage is successfully removed from the template by DNA repair proteins or bypassed. It is then essential to restart the stalled replication fork. Since DNA lesions occur randomly in a genome it is very likely that replication has to be restored independently from an origin of replication. This suggests involvement of additional proteins to those engaged in replication initiated at a site of origin of replication. The possible roles of HR in dealing with DNA damage with a potential to disrupt replication were investigated in this thesis. In addition, the role of mouse *Rad54* paralogue, *Rad54B* in DSBs repair, analysed by biochemical and genetic approaches, is incorporated. Finally, the examples of consequences of improper repair of DSBs by either HR or NHEJ are presented.

In **Chapter 1** the potential roles of HR in supporting DNA replication in both prokaryotic and eukaryotic cells are discussed. Replication of genomic DNA, which is crucial for amplification of most cells is a complicated and highly regulated process. Duplication of a single copy of a bacterial genome seems relatively easy when compared to replication of eukaryotic chromosomes. The small size of bacterial genome together with only one origin of replication facilitates controlling

mechanisms preventing re-replication of a genome. In contrast, the vast size of the eukaryotic genome and the abundance of origins of replication pose the cell with a challenge to ensure unique duplication of its genome. In both cases prevention of re-replication is assured at the stage of its initiation. Additionally, replication might be prematurely disrupted by a variety of factors. The most deleterious for the cell are DNA-damaging agents. Existing evidence shows that bacteria employ HR to support replication. Subsequently, replication can be re-initiated in an origin-independent manner by the PriA protein, which is a helicase that binds to DNA in a structure-specific manner and has the potential to reload replicative polymerases onto DNA. The analogous mechanism to restart replication in eukaryotic cells is unknown. However, some similarities between other DNA repair processes and replication restart can be predicted based on the mode of these repair mechanisms. The proteins of HR have the potential to recognise and modify DNA structure at sites of stalled replication forks. For example, it is very likely that UV-light DNA lesions induced at replication sites are present in single-stranded (ss) DNA. However, proteins of NER need double-stranded (ds) DNA to perform repair. Therefore, proteins of HR might be involved in translocation of the lesion from ssDNA into dsDNA. However, it is not know how the replisome is reconstituted after damage removal.

Chapter 2 describes the generation of a Rad54 knockin system in mouse embryonic stem (ES) cells either proficient (*Xpa*$^{+/+}$) or deficient (*Xpa*$^{-/-}$) in NER. *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cells were generated by transfecting wild type and *Xpa*$^{-/-}$ ES cells with Rad54 cDNA fused to green fluorescent protein (GFP). In order to exclude the possibility that the tagged protein might not be functional a set of control experiments was performed. The presence of endogenous levels of expression of the Rad54-GFP protein was confirmed by a protein blot using anti-Rad54 antibodies. Additionally, there was no difference in the level of the Rad54-GFP protein expression between both generated cell lines. The similar ability to form colonies upon induction of specific types of DNA damage by *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ compared to control cells confirmed that the GFP tag did not interfere with the Rad54 protein activity in both wild type and *Xpa*$^{-/-}$ ES cells. Also fluorescence activated cell sorting (FACS) analysis confirmed similar levels of expression of Rad54-GFP protein in both cell lines and that tagged protein is homogeneously expressed in the respective cell populations. Additionally, ionising radiation induced Rad54-GFP foci detected by GFP were identical to the foci detected by anti-Rad54 antibodies. Therefore, we conclude that Rad54-GFP knockin construct is functional and that generated *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cell lines can be used for further analysis.

The differences between induction and distribution of Rad54 foci induced by either ionising radiation or UV-light are discussed in **Chapter 3**. The results show that upon ionising radiation Rad54 protein redistributed into bright foci in both *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cell lines. However,

after exposing cells to UV-light more prominent response in Rad54 foci formation was observed in NER-deficient cells. Additionally, depending on the treatment the foci appearance and distribution pattern differed. Ionising radiation treatment caused large foci evenly distributed within the nucleus, whilst UV-light-induced relatively smaller foci rather at the peripheral areas of nucleus in a pattern resembling replication marker, proliferation cell nuclear antigen (PCNA). This indicated that Rad54 foci induced by UV-light represent sites of stalled replication forks. Moreover, immunofluorescence analysis showed that Rad54 colocalised with another HR protein Rad51 and γ-H2AX, which is used as the DNA damage marker for ssDNA gaps and DSBs. This suggests that UV-light-induced DNA damage present at stalled replication fork requires activation of HR. Cell cycle analysis of *Rad54*$^{+/GFP}$ and *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ cells after exposure to UV-light revealed that cells deficient for NER are delayed in exit from S phase when compared to NER-proficient cells.

The effect of inactivation of the Rad54 protein in *Xpa*$^{-/-}$ ES cells on cellular metabolism is investigated in **Chapter 4**. Targeting of *Rad54* in *Xpa*$^{-/-}$ ES cells with *Rad54* knockout constructs resulted in total inactivation of the *Rad54* gene in these cells. The ability to form colonies upon UV-light treatment showed that *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cells were more hypersensitive to the treatment than *Xpa*$^{-/-}$ cells. Cell cycle analysis revealed that in contrast to control cells *Rad54*$^{-/-}$*Xpa*$^{-/-}$ cells are unable to restore the G2 cell population after exposure to UV-light. Further, the number of structural chromosomal aberrations typical for cells with replication problems was the highest in these cells. Finally, chromosomal fibre data showed that efficiency of replication restoration after UV-light treatment was the lowest in these cells.

Chapter 5 describes biochemical and genetic analysis of mouse *Rad54* paralogue, *Rad54B*. Isolation of *Rad54B* cDNA and precise sequence analysis is presented. Purification of the recombinant Rad54B protein from insect cells allowed investigation its biochemical properties. Similarly to Rad54, Rad54B is a dsDNA ATPase with the ability to translocate along DNA molecules. The hypersensitivity of *Rad54B*$^{-/-}$ ES to ionising radiation and mytomycin C showed contribution of Rad54B to HR-mediated DNA damage repair. Additionally, mice lacking both *Rad54* and *Rad54B* were more hypersensitive to mitomycin C treatment than any of the single mutant. Consequently, the level of chromosomal aberrations in a bone marrow cells, which are the target for mitomycin C was the highest in cells isolated from *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ mice.

Chapter 6 gives an overview on an effect of DSB repair on chromosomal translocations. Improper repair of DSBs by either HR or NHEJ might result in chromosomal translocations, which might have a detrimental effect because of their ability to activate oncogenes. Well-documented cases involving the Philadelphia chromosome translocation or Burkitt's lymphoma are just two of many examples of tumours resulting from chromosomal translocation. Precise mapping of a translocation might

be used for diagnostic and prognostic reasons. Therefore, for therapeutic purposes it is of crucial importance to understand the potential causes of the translocations as well as develop methods for their precise localisation.

SAMENVATTING

De stabiliteit van het genoom is uitermate belangrijk voor de overleving het functioneren van alle organismes. De stabiliteit van het genoom wordt constant bedreigd door een grote hoeveelheid, verschillende soorten beschadigingen. Deze beschadigingen kunnen ontstaan door zowel endogene als exogene factoren. Endogene factoren komen voort uit het normale cel metabolisme. Zo kunnen bijvoorbeeld zuurstof radicalen, die ontstaan tijdens de normale citroenzuurcyclus, oxidatieve schade toebrengen aan het DNA. Ioniserende straling, UV-licht en verschillende soorten chemische stoffen zijn voorbeelden van exogene factoren die het DNA bedreigen. Om te kunnen overleven zijn in de evolutie verschillende mechanismen ontstaan, die het genoom beschermen tegen DNA schades en instabiliteit. Zo kan bijvoorbeeld een groot aantal schades die de helix structuur van het DNA verstoren gerepareerd worden door nucleotide excisie reparatie (NER). Andere vormen van DNA beschadigingen, zoals dubbelstrengs breuken (DSBs), worden weer verholpen door mechanismen die gebruikmaken van homologe recombinatie (HR) of het ligeren van de niet homologe breuk eindes (NHEJ).

De stabiliteit van het genoom wordt ook op de proef gesteld tijdens de celcyclus. Tijdens de cel cyclus wordt het totale genoom gerepliceerd en bij de celdeling doorgegeven aan de dochtercellen. Precieze en goed gecoördineerde DNA replicatie uitermate belangrijk is voor de integriteit van het genoom. Bijna iedere ongerepareerde beschadiging van het DNA kan leiden tot het stoppen van de DNA replicatie vork, doordat het enzymen die de replicatie uitvoeren, DNA polymerases, vastlopen. Het spreekt voor zich dat verkeerde keuzes tijdens het verhelpen van de replicatie vork blokkade kan leiden tot het verlies van DNA . Maar ook niet evenredige verdeling van het DNA tussen de dochtercellen kan desastreuze gevolgen hebben, zoals celdood of het ontstaan van een tumor.

Het verhelpen van vastgelopen DNA replicatie vorken is nodig voor de stabiliteit van het genoom en overleving. Veel gebieden in het zoogdier genoom, waar tijdens de celcyclus DNA replicatie wordt gestart (origine), vormen een bedreiging voor de replicatie doordat ze schades hebben opgelopen door DNA beschadigende stoffen. Maar ook wanneer een DNA polymerase tijdens DNA replicatie een beschadiging in het DNA tegenkomt, zal de replicatie worden gestopt. Deze stop duurt totdat de schade is hersteld door DNA reparatie enzymen of totdat andere minder specifieke DNA polymerases, die minder last hebben van de DNA schades, de schade hebben omzeild. In beide gevallen is het essentieel dat DNA replicatie weer wordt herstart. Daar DNA beschadigingen willekeurig omtreden in het genoom is het zeer aannemelijk dat replicatie wordt hersteld onafhankelijk van de replicatie start gebieden.

In dit proefschrift worden de mogelijke functies van HR in relatie tot DNA schades, die mogelijk van invloed zijn op DNA replicatie, onderzocht. Ook wordt de rol van de muizen *Rad54* paraloog, *Rad54B* in DNA DSB reparatie onderzocht met behulp van biochemische en genetische

analyse technieken. Als laatste worden voorbeelden van de consequenties behandeld ontstaan door onjuiste reparatie van DNA DSB door HR of NHEJ.

In **Hoofdstuk 1** worden mogelijke functies van HR in het ondersteunen van DNA replicatie van zowel prokaryote en eukaryote cellen behandeld. De replicatie van het DNA is een gecompliceerd en zeer geordend proces en cruciaal voor de vermeerdering van de meeste cellen. De duplicatie van een eenvoudig kopie van bacteriële genen lijkt relatief gemakkelijk vergleken met de verdubbeling van eukaryote chromosomen. De aanwezigheid van een relatief klein bacterieel genoom met maar één origine van replicatie maakt het controle mechanisme, die meerdere ronden van replicatie moeten verhinderen, gemakkelijk. Dit in tegenstelling tot het relatief veel grotere genoom van eukaryoten, waarin zich ook nog meerder origines van DNA replicatie bevinden. In beide gevallen wordt de DNA her-replicatie voorkomen bij de start van de replicatie. Door toedoen van talrijke factoren kan DNA replicatie ook vroegtijdig gestopt worden. De meest schadelijke voor de cel zijn die factoren die het DNA beschadigen. Er is wetenschappelijk bewijs dat laat zien dat in bacteriën HR wordt gebruikt ter ondersteuning van accurate DNA replicatie. Daardoor kan de herstart van DNA replicatie plaatsvinden op een manier die onafhankelijk is van de aanwezigheid van een origine van replicatie.Hierbij speelt het PriA helicase eiwit een rol. Dit eiwit bindt op een structuur-specifieke manier aan het DNA en kan DNA polymerases herladen op het DNA. Of het analoge DNA replicatie herstart mechanisme ook in eukaryote cellen functioneert is niet bekend. Echter, enkele overeenkomsten tussen DNA reparatie mechanismen en DNA replicatie herstart kunnen worden voorspeld op bases van de werking van de reparatie mechanismen. De eiwitten betrokken bij HR kunnen in principe bij een vastgelopen replicatie vork DNA structuren herkennen en veranderen. Het is bijvoorbeeld zeer aannemelijk dat UV-licht geïnduceerde DNA schade door de replicatie verandert in enkelstrengs DNA. Echter het NER mechanisme kan alleen dubbelstrengs DNA repareren. In dit geval zouden eiwitten van het HR mechanismen betrokken kunnen zijn in de translocatie van enkelstrengs DNA naar dubbelstrengs DNA. Echter, het is niet bekend hoe de replisome wordt opgebouwd na het verwijderen van de schade.

Hoofdstuk 2 beschrijft de generatie van een '*Rad54* knockin' systeem in embryonale stamcellen (ES) van de muis, waarin een Xpa, een DNA reparatie enzym betrokken bij NER, wel of niet aanwezig is. *Rad54*$^{+/GFP}$ en *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cellen werden gemaakt door normale en *Xpa*$^{-/-}$ ES cellen te transfecteren met *Rad54* cDNA gefuseerd aan groen fluorescerend eiwit (GFP). Om de mogelijkheid dat het Rad54-GFP eiwit niet functioneel zou zijn uit te sluiten, zijn controle experimenten gedaan. De aanwezigheid van het Rad54-GFP eiwit werd bewezen door een eiwit blot gehybridiseerd met een Rad54 antilichaam. Er kon geen verschil in expressie niveaus van Rad54-GFP ontdekt worden tussen beide gemaakte cellijnen. Het vermogen om uit te groeien tot kolonies na aanbrengen van specifieke DNA schades van zowel *Rad54*$^{+/GFP}$ en *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$

ES ellen was verglijkbaar met controle cellen. Dit leidde tot de conclusie dat het GFP-label geen invloed heeft op de enzymatische activiteit van Rad54 in cellijnen. Uit 'Fluorenscence-activiated cell sorting' (FACS) experimenten bleek dat beide cellijnen Rad54-GFP vergelijkbare expressie niveaus hadden. Bovendien bleek uit deze experimenten ook dat in beide cellijnen de hoeveelheid gemerkt eiwit in alle cellen gelijke was. Ook bleek dat Rad54-GFP spots, geïnduceerd door ioniserende straling, identiek waren, onafhankelijk van de detectie manier; in dit geval GFP of Rad54 antilichamen. We concluderen daarom dat het '*Rad54-GFP* knock-in' construct functioneel is en dat de gemaakte *Rad54*$^{+/GFP}$ en *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cellijnen bruikbaar zijn voor toekomstige studies.

De verschillen tussen de inductie en distributie van Rad54 spots, ontstaan na behandeling van cellen met ioniserende straling of UV-licht wordt behandeld in **Hoofdstuk 3**. De resultaten laten zien dat na ioniserende bestraling het Rad54 eiwit aanwezig is in heldere spots in zowel *Rad54*$^{+/GFP}$ en *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cellen. Echter, na UV-licht behandeling, waren de Rad54 spots meer prominent aanwezig in de Xpa positieve cellen. De vorm van de spots was verschillend, afhankelijk van de behandelingsmethode. De behandeling met ioniserende straling veroorzaakte spots die evenredig verdeeld waren over de celkern, terwijl UV-licht behandeling resulteerde in kleinere spots, die bovendien in de perifere gebieden van de kern tevinden waren. Deze verdeling leek sterk op die van een ander eiwit, proliferation cell nuclear antigen (PCNA), tijdens de celcyclus. Uit deze resultaten konden we afleiden dat rad54 spots, ontstaan door UV-licht, in werkleijkheid vastgelopen DNA replicatie vorken zijn. Ook immunofluorescentie analyses toonden aan dat Rad54 sport samengingen met die van een ander HR eiwitten, Rad51 en γ-H2AX. De laatste wotrdt ook wel gebruikt als 'marker' voor enkelstrengs DNA en DSBs. Deze resultaten doen sterk vermoeden dat UV-licht geïnduceerde DNA schade, aanwezig in een vastgelopen DNA replicatie vork, activiteit van het HR mechanisme nodig heeft voor herstel. Celcyclus experimenten lieten zien dat vergeleken met *Rad54*$^{+/GFP}$, *Rad54*$^{+/GFP}$*Xpa*$^{-/-}$ ES cellen langzamer uit de S phase kwamen, na behandeling met UV-licht.

Het effect van Rad54 inactivatie in *Xpa*$^{-/-}$ ES cellen op het cellulaire metabolisme wordt onderzocht in **Hoofdstuk 4**. Het gericht inactiveren van het *Rad54* gen in *Xpa*$^{-/-}$ ES cellen door '*Rad54* knockout' constructen resulteerde in totale inactivatie van Rad54 in deze cellen. Uit proeven die het vermogen om uit te groeien tot kolonies na UV-licht behandelingen onderzochten bleek dat *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cellen veel gevoeliger waren voor UV-licht dan *Xpa*$^{-/-}$ ES cellen. Uit celcyclus analyses bleek dat in tegenstelling tot controle cellen, *Rad54*$^{-/-}$*Xpa*$^{-/-}$ ES cellen niet in staat waren hun G2 celpopulatie te herstellen na UV-licht behandeling. Ook was het aantal structurele chromosomale afwijkingen, kenmerkend voor cellen met replicatie problemen, het hoogste in deze cellen. Tenslotte toonde 'chromosomal fibre data' aan dat het vermogen om DNA replicatie te herstellen het laagste was in deze cellen.

Hoofdstuk 5 beschrijft biochemische en genetische analyses van de muizen *Rad54* paraloog, *Rad54B*. De isolatie van *Rad54B* cDNA en de precieze DNA sequentie analyse wordt beschreven. Door opzuiveren van het recombinant Rad54B eiwit uit insecten cellen konden we de biochemische eigenschappen onderzoeken. Rad54B is ,net zoals Rad54, een dsDNA ATPase met het vermogen om over het DNA te bewegen. De overgevoeligheid van *Rad54B*$^{-/-}$ ES cellen voor ioniserende straling en Mitomycine C laten een bijdrage van Rad54B zien aan het DNA herstel door HR. Muizen die geen Rad54 en Rad54B hebben zijn nog meer overgevoelig voor Mitomycine C behandeling dan de muizen die slechts 1 van de 2 eiwitten moeten missen. Dientengevolge was het aantal gevonden chromosomale afwijkingen in beenmerg cellen het hoogst in cellen geïsoleerd uit *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ muizen.

Hoofdstuk 6 geeft een overzicht van het effect van DNA DSB op chromosomale translocaties. Foutieve reparatie van DNA DSB door HR of NHEJ kan resulteren in chromosomale translocaties. Deze kunnen desastreuze gevolgen hebben wanneer ze oncogenen activeren. Goed gedocumenteerde cases met het Philadelphia chromosome en Burkitt's lymphoma zijn slechts twee uit vele voorbeelden die laten zien dat translocaties kunnen resulteren in tumorgenese. Het vinden van de precieze locatie van een translocatie kan gebruikt worden voor diagnostiek en prognose. Het is daarom van therapeutisch belang om te begrijpen wat de mogelijke oorzaken van translocaties zijn en nieuwe methode the ontwikkelen voor het vinden van de precieze locatie

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