NBS1 Functions as a Multifaceted Protein in DNA Damage Repair and Gametogenesis

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NBS1 functioneert als een veelzijdig eiwit tijdens DNA schade herstel en gametogenese

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Chapter 1

Central role of the MRE11-RAD50-NBS1 complex in the DNA damage response

Proper maintenance of the genome is crucial for survival of all organisms. It is of major importance for reproduction and development, that the information encoded in the genome is replicated correctly. However, endogenous and exogenous DNA-damaging agents are constantly threatening the integrity of the genome. When a cell detects damage, it can arrest the cell cycle at specific checkpoints (G1/S, G2/M and intra-S). The activation of cell cycle checkpoints provides time to repair DNA lesions before they can be converted into permanent mutations. Incorrect repair or accumulation of DNA damage results in genome instability, which may lead to impaired functioning of the cell and even to the development of cancer (Figure 1). Therefore, all organisms have a complex network of DNA repair mechanisms, each of which is able to repair a subset of lesions. The biological significance of DNA repair mechanisms is underlined by the large number of repair genes, many of which are conserved from yeast to humans. The importance of the DNA repair pathways is also emphasized by the fact that defects in DNA repair genes often lead to cancer predisposition in humans [1, 2]. When DNA damage cannot be repaired properly, the cell can either go into apoptosis or can contribute to ageing (Figure 1).

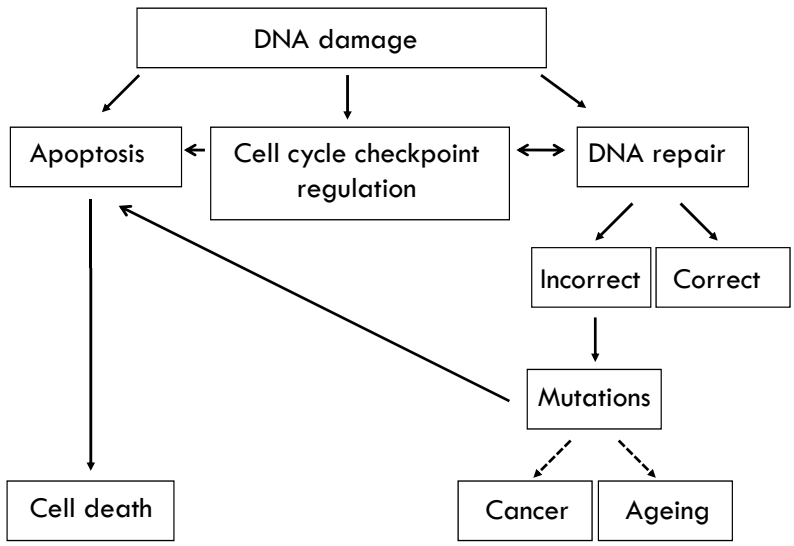

Figure 1: Cellular consequences of DNA damage
DNA damage triggers activation of cell cycle checkpoints. This can lead to cell cycle arrest at G1/S, intra-S or G2/M phases of the cell cycle. During cell cycle arrest, DNA damage can be repaired. Incorrect repair of DNA damage can lead to mutations resulting in genome instability and finally in cancer. When DNA damage cannot be repaired properly, the cell can go into apoptosis.

Activation of cell cycle checkpoints

The first step in the initiation of DNA damage checkpoints is recognition of the DNA damage. For example the MRE11-RAD50-NBS1 (MRN) complex recognizes DNA ends [3]. Once DNA damage is sensed, the cell must transduce this signal to the appropriate effector(s). Members of this group include 53BP1, MDC1 and NBS1 [3, 4], which can activate either ATM or ATR. In mammalian cells, the activation of ATM and/or ATR is essential for proper repair of the DNA damage [5]. Upon activation, ATM and ATR phosphorylate CHK2 and CHK1, which transmit the signal to downstream proteins e.g. the CDC25 family [5]. The cell division cycle (CDC) family of proteins inactivate cyclin-dependent kinase (CDK) complexes, which in turn regulate progression through the cell cycle. CDC25 phosphatases are also components of the checkpoint pathways that become activated after DNA damage (Figure 2). A number of signaling pathways mediate cell cycle arrest or delay of cell cycle progression. Different types of DNA-damaging agents (e.g. ionizing radiation (IR), ultraviolet light (UV) or hydroxyurea (HU)) require different kinases for their repair [6]. IR creates mainly DSBs, UV pyrimidine dimers and HU results in a depletion of the nucleotide pool, which can lead to replication associated DNA strand breaks. IR-induced damage mainly requires ATM whereas UV and HU require ATR, although there is also functional overlap between these two pathways.

G1/S checkpoint

One of the most important players of the G1/S checkpoint is the retinoblastoma protein (RB). The RB tumor suppressor protein mediates cell cycle arrest by antagonizing the transcription factor E2F. This process is regulated in turn by the phosphorylation status of RB family proteins. Only hyperphosphorylated forms of the RB protein family can interact with E2F during the cell cycle [7]. Phosphorylation of RB is caused by activity of the cyclin/cyclin-dependent kinases (CDK) complexes [8]. When phosphorylated RB is bound to E2F, the complex acts as a growth suppressor and prevents progression through the cell cycle [7].

Progression through the cell cycle is regulated by the coordinated activities of cyclin-CDK complexes. The cyclin-CDK complexes are present in phosphorylated form. When CDK activity becomes required for progression into the next cell cycle phase, CDC25 phosphatases can dephosphorylate the cyclin-CDK complex, thereby activating it [9].

The CDC25 phosphatases have been implicated in the control of G1/S and G2/M transitions by regulating the activities of CDK1 and CDK2. CDC25A mainly de-phosphorylates the CDK2-cyclin E and CDK2-cyclin A complexes during the G1/S transition but also has a role in the G2–M transition by activating CDK1- cyclin B1 (Figure 2). Regardless of the initial insult, the net result is an inhibition of CDK-cyclin complexes in order to stop cell cycle progression.

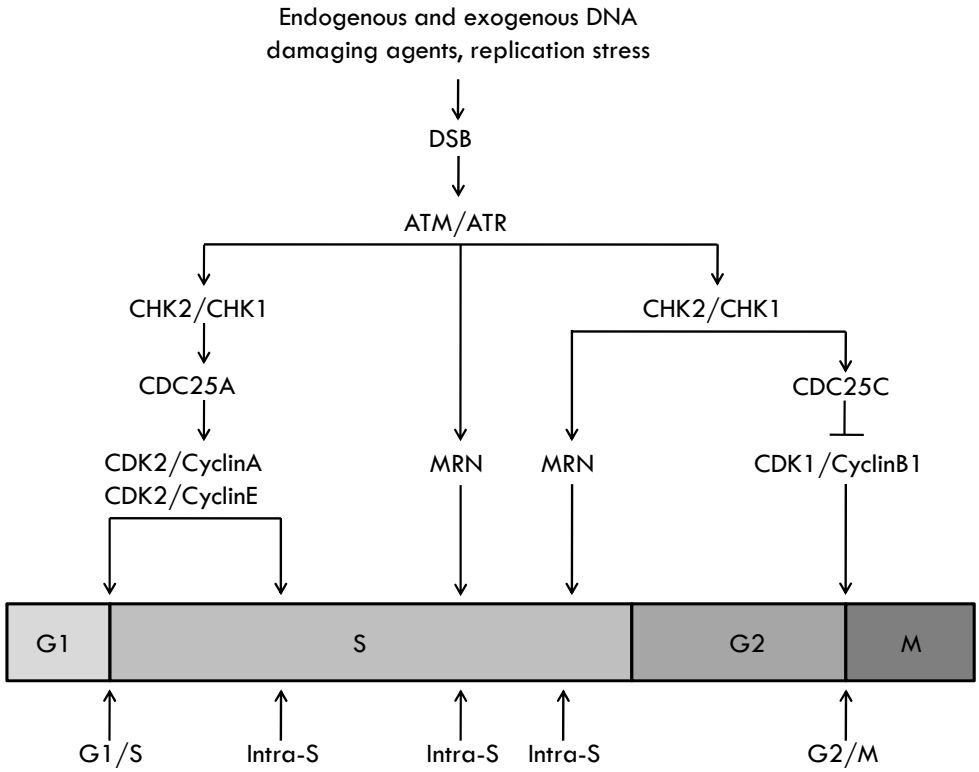

Figure 2: Schematic overview of the cell cycle checkpoints in mammalian cells
The DNA DSB is sensed by ATM or ATR, followed by the activation of CHK1, CHK2 or the MRN complex which, in turn, activate or inactivate other proteins that directly participate in inhibiting the G1/S transition, S-phase progression or the G2/M transition.

Intra-S checkpoint

The intra-S phase checkpoint is activated by genotoxic stress, such as DSBs, which cause a transient delay in S phase progression by inhibiting the firing of new replicons. Defects in the intra-S phase checkpoint response to IR result in the inability of cells to reduce the rate of DNA replication when irradiated, a phenomenon that is known as radioresistant DNA synthesis (RDS). RDS was first reported for cells that were derived from patients with ataxia telangiectasia (AT). ATM triggers two cooperating parallel cascades to inhibit replicative DNA synthesis [10]. ATM in cooperation with MDC1, H2AX and 53BP1 can phosphorylate CHK2 to induce ubiquitin-mediated degradation of CDC25A phosphatase. The degradation locks the S phase-promoting Cyclin E-CDK2 complex in its inactive, phosphorylated form thereby preventing initiation of replication at new origins [10, 11] (Figure 2). ATM also initiates a second pathway to inhibit replicative DNA synthesis by phosphorylating NBS1 as well as SMC1, BRCA1 and FANCD2 [10]. How NBS1 reduces the rate of DNA synthesis is not known [12].

G2/M checkpoint

The G2/M checkpoint is necessary to prevent entry into mitosis with damaged chromosomes. ATM and ATR mediate this pathway by phosphorylating CHK2 and CHK1, respectively, in response to DNA damage [4, 13]. This phosphorylation of CHK2 and CHK1 enhances their kinase activity, which in turn results in the phosphorylation of the CDC25 phosphatases [14]. CDC25B is proposed to be responsible for the initial activation of cyclin B-CDK1 at the centrosome during this transition, which is then followed by a complete activation of cyclin B-CDK1 complexes by CDC25C in the nucleus at the onset of mitosis [10, 15].

Apoptosis

Cells maintain the balance between survival and death by activating cell cycle checkpoints and repair mechanisms in response to DNA damage, and resort to apoptosis when excessive damage levels persist to prevent potential malignant transformation [16, 17].

A diverse range of signals, which may originate either extracellularly or intracellularly, controls apoptosis. After the appropriate stimulus has been received, the cell undergoes the organized degradation of cellular organelles by activated proteolytic caspases. A cell undergoing apoptosis shows a characteristic morphology. The cell shrinks due to the degradation of the cytoskeleton by caspases, which causes compaction of the nucleus, and the cytoplasm appears dense with tightly packed organelles. The chromatin undergoes condensation into compact patches. The nuclear envelope becomes discontinuous and the DNA is fragmented in a process referred to as karyorrhexis. The degradation of the DNA in the nucleus results in several discrete chromatin bodies. The cell membrane shows irregular buds known as blebs. Finally, the cells break apart into several vesicles called apoptotic bodies, which are then phagocytosed [18].

There are several apoptotic pathways; the two main pathways are the intrinsic pathway, which is stimulated by stress stimuli, and the extrinsic pathway, which gets activated by specific ligands and cell surface receptors (Figure 3) [19].

Intrinsic pathway of apoptotic signaling and the BCL2 family

Intracellular apoptotic signaling is initiated by a cell in response to stress. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection and hypoxia are all factors which can lead to the release of intracellular apoptotic signals. In most cases, the exact mechanism of detection remains still unclear.

Within cells there is a mitochondria-dependent apoptosis pathway, in which the BCL2 family members play a key role. Mammals possess an entire family of BCL2-like proteins that includes pro-apoptotic as well as anti-apoptotic members (Table 1). This family may be subdivided into three main groups based on the regions of BCL2 homology (BH) domains and function: multidomain anti-apoptotic, multidomain pro-apoptotic and BH3-only pro-apoptotic. The BCL2 family members play the key role in the regulation of mitochondria-dependent apoptosis.

Two models have been proposed to explain the killing activity of the BH3-only proteins, direct and indirect activation. The indirect activation model proposes that death stimuli activate BH3-only proteins to promote apoptosis by binding to other pro-apoptotic proteins, such as BAK and BAX, which exert their pro-apoptotic activity at the mitochondrial level. The direct activation model suggests that all BH3 only proteins engage only the pro-survival proteins and act by preventing them from inhibiting BAK or BAX activity [20-22].

BH domains	Genes	Pro- or anti-apoptotic
Multidomain	Bcl-2, Bcl-XL, Bcl-w, MCL-1, BFL-1/A1	anti
Multidomain	BAX, BAK	pro
BH3-only	BID, BIM, BAD, BIK, NOXA, PUMA, BMF, HRK	pro

Table 1: Classification of the BCL2 family members according to their BH domains and pro- or anti-apoptotic activity

Extrinsic pathway of apoptotic signaling

Pro-apoptotic signaling may also be mediated by specific ligands and surface receptors, which are capable of delivering a death signal from the (micro)environment. Pro-apoptotic signaling can activate the execution of apoptosis in the cytoplasm and organelles [23]. Extracellular signals may include hormones, growth factors, nitric oxide or cytokines, and therefore must cross the plasma membrane to evoke a response either via receptors or through the membrane. These extracellular signals can activate caspase-8 that cleaves BID, a pro-apoptotic BCL2-family member, to tBID, which connects with the intrinsic pathway (Figure 3). Both the extrinsic and intrinsic pathways of apoptosis come together into a common pathway causing the activation of caspases.

DNA repair

Various DNA damaging agents cause a wide variety of lesions in the DNA. Depending on the type of lesion, a specific mechanism can remove the damage. The first step is to recognize the lesion in a large pool of undamaged DNA. Subsequently, the lesion is processed which will eventually lead to its removal. Consequently, final steps in DNA damage repair are re-incorporation of the missing nucleotides and coupling of DNA ends by a DNA ligase. Depending on the pathway used to repair the lesion, a different set of proteins is required [24]. DNA damage can be categorized into two classes; one class in which only one of the two strands of the DNA double helix is damaged and a second class of lesions that affects both strands of the DNA. When only one strand is damaged, the cell uses the complementary strand as a template to repair this damage. This class of repair pathways encompasses base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). Repair of double-strand breaks

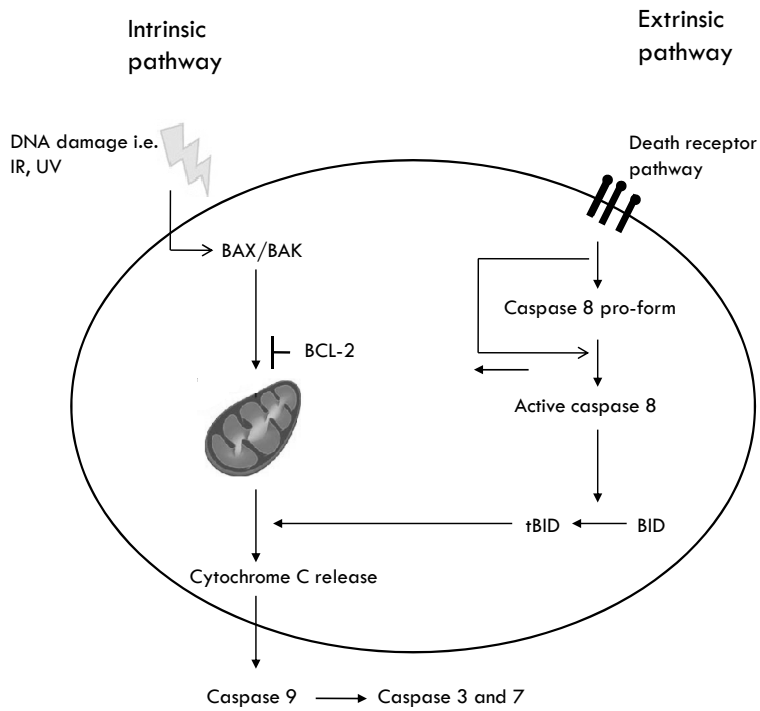

Figure 3: Schematic overview of the major apoptotic pathways and their inhibitors
Apoptosis through the death receptor (extrinsic) pathway involves activation of the initiator caspase 8. The intrinsic pathway is activated by different stimuli and conditions, which leads to release of pro-apoptotic factors from mitochondria. The release of pro-apoptotic factors can be inhibited by the anti-apoptotic members of the BCL2 family of apoptosis regulators.

(DSBs) or interstrand crosslinks (ICL) cannot be accomplished by using the complementary strand as a template. In this case, the cell can use the recombinational repair pathway of homologous recombination (HR), or nonhomologous end-joining (NHEJ), to repair the damage.

Excision repair

Single bases in DNA can be altered, for example by deamination or alkylation, resulting in incorrect base pairing, and consequently this can lead to mutations in the DNA. Upon damage detection by a set of DNA glycosylases the altered base is flipped out of the DNA helix and only that base gets changed. A large family of lesion-specific glycosylases is used to break the β-N glycosidic bond to create an apurinic/apyrimydinic (AP)-site. AP endonuclease recognizes this site and nicks the damaged DNA on the 5' side of the AP site creating a free 3'-OH. DNA polymerase (polβ) fills in the missing base from the free 3'-OH, followed by ligation over the gap by the XRCC1-ligase III complex. BER is also used to repair single-strand breaks (SSBs). Usually, BER is divided into short-patch repair (where a single nucleotide is replaced) or long-patch repair (where 2-10 nucleotides are replaced). Mammalian long-patch repair includes PCNA and polδ and polε for nucleotide resynthesis, FEN1 to cut off the 'flap' including the

abasic site, and LigI. Another protein involved in BER is poly (ADP-ribose) polymerase (PARP), which catalyzes poly (ADP-ribosyl)ation, a covalent post-translational protein modification. PARP-1 can bind to SSBs and stimulate repair [25].

Some forms of colorectal cancer have been shown to be caused by inherited defects in the glycosylases OGG1, MTH1 and MUTYH [26]. The role of BER in the maintenance of genome stability is to counteract oxidative DNA damage, which generates 8-oxoguanine products (8-oxoG), and to counteract methylation products. MUTYH, OGG1 and MTH1 function together to identify and repair 8-oxoG incorporated into DNA, as well as to remove the modified nucleotide from the pool [27].

DNA requires repair due to damage that can occur to bases from a vast variety of sources including bulky chemicals, but also ultraviolet (UV) light from the sun on the skin. NER is an important mechanism for the cell to prevent mutations by removing the vast majority of UV-induced DNA damage. While BER can recognize lesions in the DNA and repairs them with specific glycosylases, NER recognizes distortions in the DNA double helix. Recognition of these distortions is followed by the removal of a short ssDNA segment that includes the lesion, creating a single strand gap in the DNA, which is subsequently filled in by DNA polymerases, which use the undamaged strand as a template [28, 29].

The severe human diseases that result from mutations of NER proteins provide evidence for the importance of this repair mechanism. Xeroderma Pigmentosum (XP), Cockayne's Syndrome (CS) and Trichothiodystrophy (TTD) cause photosensitivity. Moreover, XP patients also suffer from abnormal pigmentation and skin cancer predisposition. These features can be explained by a defect in repair of UV-induced DNA damage. Everything that disrupts DNA base pairing can affect the genetic stability of a cell.

Transcription coupled repair (TCR) operates in tandem with transcription. Genes are copied from DNA to make mRNA to instruct protein synthesis by RNA polymerase II. When RNA polymerase finds a lesion in the DNA, such as those caused by UV light, it stops and the gene is not transcribed. TCR quickly removes these lesions from transcribed genes. Failure of TCR is the cause of CS, an extreme form of accelerated aging that is fatal early in life [30, 31].

Mismatch repair (MMR) is a system for recognizing and repairing insertion, deletion and mis-incorporation of bases that can arise during DNA replication and recombination. Mutations in mismatch repair genes are known to cause hereditary nonpolyposis colorectal cancer (HNPCC) [32].

DNA double-strand break (DSB) repair

There are two main pathways to repair DNA DSBs: HR and NHEJ. HR requires extensive regions of DNA sequence homology and repairs DSBs accurately using information on the undamaged sister chromatid or homologous chromosome. Central to the process of HR are the RAD52 group genes including RAD50, RAD51, RAD52, RAD54, MRE11, and NBS1).

NHEJ uses little or no sequence homology to rejoin broken ends in a manner that need not be error-free.

An NHEJ defect results in severe combined immunodefiency (SCID). V(D)J recombination, a mechanism which randomly selects and assembles Variable (V), Diversity (D) and Joining (J) coding gene segments through a specialized somatic DNA rearrangement mechanism [33]. This site-specific recombination generates a diverse repertoire of T cell receptor (TCR) and immunoglobulin (Ig) genes. The creation of TCR and Ig genes is a stepwise process during which site-specific DSBs are made by the recombination activating proteins RAG1/RAG2, followed by DSB repair by NHEJ [34]. Defects in V(D)J recombination result in SCID characterized by absence of mature B and T cells [34]. Apart from the immune deficiency, a subset of the patients shows hypersensitivity to IR, indicating that they harbor a defect in the DSB repair pathway NHEJ [35]. A large fraction of these patients have mutations in the Artemis gene. Mutations in the DNA-Ligase IV, Cernunnos or XRCC4-like factor (XLF) and DNA-PKcs genes have also been reported [36-40]. The Artemis nuclease is required for hairpin opening and may also be involved in other DNA end- processing events. DSB repair will be discussed in more detail in Chapter 2.

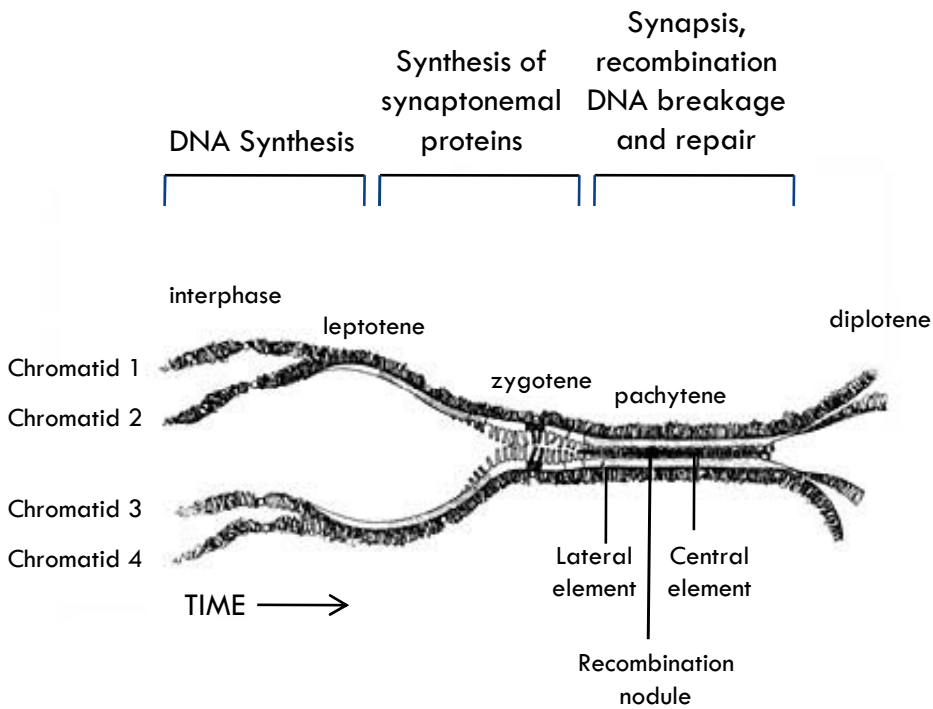

Figure 4: The events occuring during the prophase of the first meiotic division
This schematic representation shows the period of DNA synthesis, the formation of the synaptonemal complex and the processes involved in recombination.

DNA damage repair in meiosis

The role of NBS1 in meiosis will be discussed in chapter 6 of this thesis. As this process also requires DSB repair, mainly via HR, we incude here a short introduction on DNA damage repair in meiosis.

Meiosis is a highly conserved process in eukaryotes, which generates haploid gametes from diploid cells. Meiosis differs from mitosis, since in this process two rounds of cell division, meiosis I and meiosis II, follow a single round of chromosome replication [41]. During the first division, chromosome segregation occurs, resulting in half the number of chromosomes per daughter cell. In the prophase of the first meiotic division, replicated maternal and paternal chromosomes pair, and crossing over occurs. Before crossing over, a pair of replicated chromosomes consist of two homologous chromosomes with four chromatids, with one chromosome coming from each parent [41]. The meiotic prophase I in mammals can be divided in four distinct stages: leptotene, zygotene, pachytene and diplotene (Figure 4). All the events of prophase I occur in association with the synaptonemal complex (SC) [42, 43]. The SC is a protein structure that forms between two homologous chromosomes during meiosis and is thought to mediate chromosome pairing, synapsis, and recombination (crossingover). The synaptonemal complex is a structure consisting of two parallel lateral regions and a central element (Figure 4). Moreover, the SC contains two axial elements that are connected by a central element, which functions as a zipper during synapsis [44]. The central element is composed mainly of the meiosis-specific protein SCP1 (synaptonemal complex protein 1). The axial and lateral elements contain the meiosis-specific proteins SCP2 and SCP3 [45, 46]. SCP3-containing axial elements become closely connected to each other and from that moment on they are referred to as lateral elements. In the leptotene stage, long uncompacted single homologues are visible, unsynapsed axial elements composed of SCP3 start to form, DSBs appear and meiotic recombination is initiated [47]. Shorter and compacted chromosomes that start to synapse characterize zygotene. Mature SCs start to form at this stage, whereas DSBs disappear. The pachytene stage contains compacted and fully synapsed chromosomes and HR intermediates can be detected. In diplotene, SC elements disassemble; still condensed chromosomes are connected only at the sites of recombination, called chiasmata.

In prometaphase, of the first meiotic division, the bivalents become attached to the meiotic spindle and line up at the metaphase plate. In anaphase, the two homologous chromosomes are separated and pulled to opposite poles. In the second meiotic division, not preceded by DNA replication, the sister chromatids are separated, which results in haploid secondary spermatocytes [48].

Double-strand break formation and repair during meiosis

The creation of DSBs in early meiotic prophase I is a conserved feature of meiosis. An evolutionarily conserved meiosis-specific protein, called SPO11, generates meiotic DSBs. SPO11

cuts the DNA through a topoisomerase-like mechanism, in which SPO11 is bound covalently to the DNA [49]. The Mre11-Rad50-Xrs2 (MRX) complex in yeast or MRE11-RAD50-NBS1 (MRN) complex in higher eukaryotes is necessary for the release of SPO11. One of the proteins involved in recombination is the strand exchange protein RAD51 [50]. Already in leptotene RAD51 starts to localize to meiotic chromosomes in the form of foci. The foci persist till pairing of homologous chromosomes occurs and disappear in pachytene. One protein of the RAD51 family, DMC1, is meiosis specific. Localization and expression patterns of DMC1 resemble that of RAD51 in meiotic cells. Both proteins co-localize on SC in zygotene and pachytene in mouse spermatocytes [51]. Mice deficient in *Dmc1* are viable but sterile [52]. Targeted gene disruption in the male mouse showed an arrest of meiosis in germ cells at the early zygotene stage, followed by apoptosis. In female mice lacking the *Dmc1* gene, normal differentiation of oogenesis was aborted in embryos, and germ cells disappeared in the adult ovary [51].

Meiotic recombination differs in several key aspects from homology-directed repair of DSBs in mitotic cells. The choice of repair template is different: meiotic recombination is biased towards use of a homologous chromosome, whereas mitotic recombination is biased toward use of the sister chromatid. The resolution of recombination intermediates is also different: meiotic recombination forms crossovers more readily [53]. Also different proteins are involved.

Despite the conservation of meiotic recombination from yeast to human, a significant level of variability can be observed between different sexes. Although recombination is implemented by nearly identical mechanisms in both sexes, there is consistently more recombination in human and mouse females compared to males [54]. More differences between males and females occur in the timing, synchrony, gamete production, arrest periods, morphology and the aneuploidy rate of the gametes [55]. In females, prophase I is mostly completed during fetal development and the oocytes then enter dictyate arrest [55]. Dictyate arrest is specific to meiosis in females, and potentially lasts for more than a year in mice and more than 40 years in humans. The storage of the oocytes at the dictyate arrest continues until sexual maturation, when a selected number of oocytes are recruited for growth. At each estrous cycle one or a few oocytes reach ovulation, leading to resumption of meiosis. They complete the first meiotic division, extruding the first polar body, and arrest again at metaphase II until fertilization [55]. Male meiosis is an entirely postnatal event. Males start their meiosis at puberty and can remain fertile throughout their adult life [55]. Moreover, both the dictyate arrest in the prophase I and the arrest in metaphase II are absent in male meiosis [55].

Central role of the MRN complex in DNA repair, cell cycle regulation and apoptosis

The MRE11-RAD50-NBS1 (MRN) complex is a central player in various aspects of the cellular response to DSBs, including HR, NHEJ, apoptosis and DNA damage checkpoint activation [56, 57]. The function of the MRN complex is discussed in chapter 2 in this thesis [58]. Biochemical studies showed that MRE11 has nuclease activity [59], while RAD50 has an ATPase activity [60].

An essential biological function of the complex in the context of DSB repair is to tether DNA ends [61]. The core MRE11-RAD50 complex exists as a heterotetrameric assembly, and the morphology of this complex can be divided into modular regions, designated, the head, coil and hook domains. The globular DNA binding head is comprised of two RAD50-ATPase domains and the MRE11 nuclease that binds to the base of the RAD50 coiled coils [62]. Homologs of the MRE11 nuclease and the RAD50 ATPase are found in a large variety of organisms, suggesting that this complex is fundamental for genomic stability [63]. The third component of the MRN complex is termed NBS1 in higher eukaryotes, and Xrs2 in yeast. This third component is less conserved between species than the Mre11 and Rad50 components. Mutations in the components of the MRX complex in *S. cerevisiae* revealed roles in meiotic recombination and in HR between sister chromatids in mitotic cells [64]. The inactivation of components of the yeast MRX complex affects a remarkable number of DNA metabolic processes such as the formation of meiotic DSBs and telomere length maintenance [65]. Diploid strains homozygous for Mre11, Rad50 en Xrs2 deletions fail to form meiosis-specific DSBs and thus are unable to initiate meiotic recombination. Moreover, in meiosis, MRX is required for the resection of meiotic DSBs to generate 3' single strand overhangs. MRX also functions in generating 3' ssDNA at DSBs in cells that are not in meiosis [66]. SsDNA is an important activator of the checkpoint pathways and it functions as an HR intermediate [67]. In higher eukaryotes the MRN complex is essential for viability. Conditional *Mre11* null chicken DT40 cells exhibit a reduced capacity for HR and undergo proliferative arrest associated with high levels of chromosome aberrations [68]. *Nbs1* disruption in DT40 cells reduces gene conversion and sister chromatid exchanges [69].

To analyze the role of the MRN complex in adult mice, mouse models were generated with hypomorphic mutations. Three different hypomorphic alleles of murine *Nbs1*, one in *Rad50* (*Rad50^{S}*) and one in *Mre11* (ATLD) resulted in viable mice [70-74]. Hypomorphic mutations in the *Nbs1* and *Mre11* genes were modeled after mutations occurring in humans that cause the genome instability syndromes Nijmegen breakage syndrome (NBS) and ataxia telangiectasia-like disorder (ATLD), respectively [71, 75-79]. *Mre11$^{ATLD/ATLD}$* mice exhibited impaired ATM function and pronounced chromosomal instability, but these mice are not markedly predisposed to malignancy [73].

A remarkable aspect of the *Rad50^{S}* allele is its dramatically different effect at the cellular versus the organismal level [70]. In yeast, the *Rad50$^{S/S}$* mutant is able to process meiotic DSBs but it is deficient in processing the ends of meiosis-specific DSBs. Therefore, this mutant strain blocks meiotic recombination repair with accumulation of DSBs. However, DSBs are extremely rare in mitosis and, moreover, they have a different repair mechanism. Therefore, *Rad50$^{S/S}$* mutants are able to complete mitosis successfully but they cannot pass through meiosis. The *Rad50$^{S/S}$* mice are cancer predisposed and show haematopoietic failure. At the cellular level, ATM autophosphorylation and 53BP1 phosphorylation were observed [78]. In contrast to the *Nbs1$^{\Delta B}$*, *Nbs1$^{\Delta C}$* and *Mre11^{ATLD}* alleles, which show impaired DNA damage responses, the *Rad50^{S}*

allele exhibits chronic DNA damage signaling [78].

Nbs1 mutant mice are viable and sensitive to IR. *Nbs1* mutant cells are impaired in cellular responses to IR. Cells are IR sensitive, show an intra-S and G2/M checkpoint defect and increased levels of chromosomal aberrations after IR [71, 74]. Recently, *Nbs1*$^{\Delta C/\Delta C}$ mice were created in which the C-terminal ATM interaction domain has been deleted [74]. *Nbs1*$^{\Delta C/\Delta C}$ cells exhibit apoptotic defects, which are comparable to that of *Atm*- or *Chk2*-deficient cells. *Nbs1*$^{\Delta C/\Delta C}$ does not impair the induction of pro-apoptotic genes, but the defects result from impaired phosphorylation of ATM targets [77]. *Mre11*$^{ATLD/ATLD}$ mice exhibit pronounced chromosomal instability, but like the *Nbs1*$^{\Delta B/\Delta B}$ mice they are not markedly predisposed to malignancy [73]. NBS is characterized by microcephaly, growth retardation, immunodeficiency and predisposition to tumors. At the clinical level AT, NBS and ATLD are quite distinct although there are some similarities. Patients from each disorder show a measurable immunodeficiency and both AT and NBS patients have an increased risk of developing lymphoid tumors. B and T cell tumors are seen although the proportions do not appear to be the same for these two disorders. It is not known whether ATLD patients have a predisposition to cancer as too few patients have been described so far [79]. However, *Mre11*$^{ATLD1/ATLD1}$ mice do not develop lymphomas [73].

Scope of this thesis

This thesis addresses several questions concerning the MRN complex as a guardian of the genome with regard to DSB repair, cell cycle regulation, apoptosis and meiosis, and how these processes can be linked.

By using plasmid based transfection assays we have shown the importance of the NHEJ factors DNA-PKcs, KU80 and XRCC4 in the repair of blunt-ended DSBs induced by transposition (Chapter 3). In chapter 4, we have shown functional differences between the KU proteins and DNA-PKcs. Moreover, we have demonstrated that KU70 interacts with the pro-apoptotic BAX protein and the MRN complex in the nucleus, suggesting that it may provide a link between NHEJ, apoptosis and cell cycle checkpoint control.

The MRN complex has multiple functions in the maintenance of chromosomal stability. We investigated how the *Nbs1*$^{\Delta B}$ mutation, which mimics the mutation found in NBS patients, affects genome stability and how this phenotype is influenced by a defect in homologous recombination (*Rad54*$^{-/-}$) (Chapter 5). Moreover, in chapter 6, we describe the meiotic phenotype of the *Nbs1*$^{\Delta B/\Delta B}$, *Rad54*$^{-/-}$ and double mutant mice.

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Chapter 2

Analysis of DNA double-strand break repair pathways

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Abstract

During the last years significant new insights have been gained into the mechanism and biological relevance of DNA double-strand break (DSB) repair in relation to genome stability. DSBs are a highly toxic DNA lesion, because they can lead to chromosome fragmentation, loss and translocations, eventually resulting in cancer. DSBs can be induced by cellular processes such V(D)J recombination or DNA replication. They can also be introduced by exogenous agents DNA damaging agents such as ionizing radiation (IR) or Mitomycin C (MMC). During evolution several pathways have evolved for the repair of these DSBs. The most important DSB repair mechanisms in mammalian cells are nonhomologous end-joining (NHEJ) and homologous recombination (HR). By using an undamaged repair template, HR ensures accurate DSB repair, whereas the untemplated NHEJ pathway does not. Although both pathways are active in mammals, the relative contribution of the two repair pathways to genome stability differs in the different cell types. Given the potential differences in repair fidelity, it is of interest to determine the relative contribution of HR and NHEJ to DSB repair. In this review, we focus on the biological relevance of DSB repair in mammalian cells and the potential overlap between NHEJ and HR in different tissues.

Introduction

The integrity of genetic information encoded in DNA is essential for cell survival. Endogenous and exogenous DNA damaging agents are constantly challenging the stability of DNA inside cells. Because a large variety of lesions occur in DNA, it is not surprising that multiple pathways have developed that each repair a subset of these lesions [1]. Among these lesions, DNA double-strand breaks (DSBs) represent a very toxic lesion. DSBs can be produced by several external factors, for example IR and radiomimetic drugs. In addition, DSBs arise naturally for various reasons. Meiosis and rearrangement of gene segments (V(D)J recombination) during immune cell development are important physiological processes involving DSB intermediates. Non-programmed DSBs occur during a normal cell cycle in S-phase when DNA replication forks stall, for example when the DNA template contains damage. Restarting replication under these conditions can involve generation of a DSB intermediate [2].

The repair of DSBs is critical for maintaining genome stability. If unrepaired, DSBs can lead to cell death. If misrepaired, these DSBs can result in chromosomal rearrangements that can underlie carcinogenesis. Accurate genome duplication is controlled by several checkpoints to prevent cells from initiating DNA replication (G1/S checkpoint), from progressing with replication (intra-S checkpoint) or from going into mitosis (G2/M checkpoint) [3,4]. The P53 protein has been recognized as an important checkpoint protein, functioning mainly through transcriptional control of target genes that influence multiple response pathways. Cells or mice

that are deficient in P53 may fail to undergo cell-cycle arrest or apoptosis in response to conditions that either lead to DNA damage [5,6] or perturb the cell cycle [7]. Therefore, it is noteworthy that several embryonic lethal phenotypes due to defective DSB repair can be rescued in the absence of P53 [8-12].

Molecular analyses have revealed that DSB healing can occur through several distinct mechanisms, HR, NHEJ, single-strand annealing, telomere maintenance or cDNA capture [1,13]. In this review we will focus on the two major DSB repair pathways in mammalian cells, HR and NHEJ. HR requires extensive regions of DNA sequence homology and repairs DSBs accurately using information on the undamaged sister chromatid or homologous chromosome. NHEJ uses no or little sequence homology to rejoin broken ends in a manner that need not to be error-free. Recent experiments, using mice with defined mutations in genes involved in HR and NHEJ have revealed that both pathways are active in mammals and essential for DSB repair. Our main focus will be on the relative importance of the DSB repair pathways and in particular on cell type-specific differences in use of these different pathways.

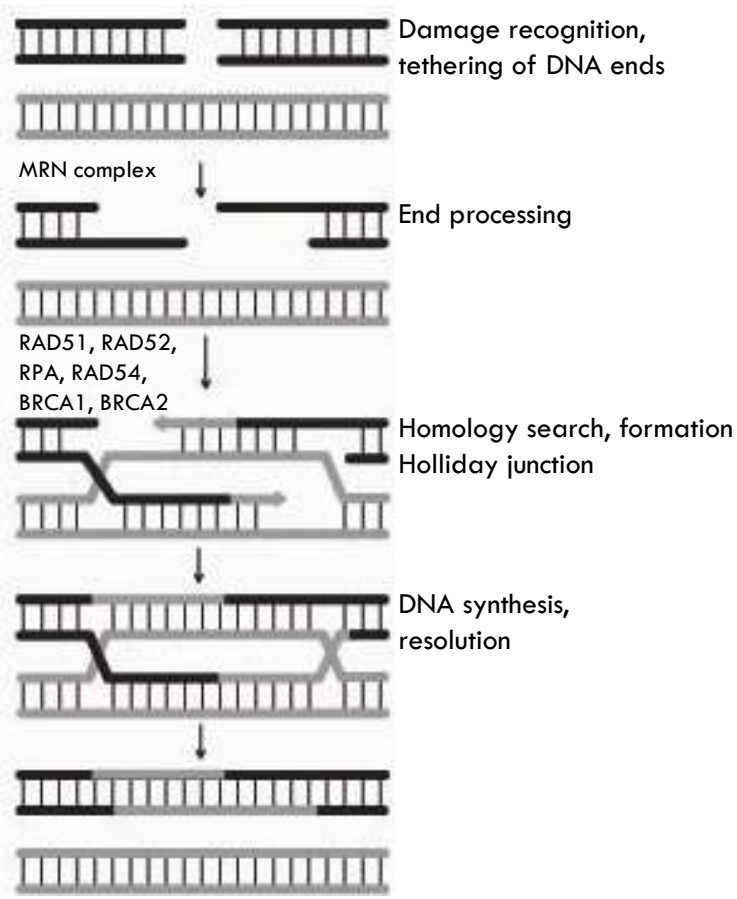

Figure 1: homologous recombination

This is a highly accurate repair mechanism, relying on the presence of a homologous DNA fragment that can be used as template. Free DNA ends that are formed at the site of a DSB are first recognized and processed. After that a nucleoprotein filament is formed which searches for homologous DNA. Subsequently, a joint molecule is formed between the damaged and undamaged DNA molecules. Template guided DNA synthesis then provides the damaged molecule with a copy of the undamaged strand.

Mechanism of homologous recombination

HR can be initiated by processing of DNA ends to produce molecules with 3' single-stranded tails. Following end resection, single-stranded DNA tails are coated with the single-stranded DNA binding protein Replication protein A (RPA). HR mediators, such as RAD52 and BRCA2, help to load RAD51 on this single-stranded DNA tail replacing RPA [14]. The RAD51 coated single-stranded DNA tail, referred to as nucleoprotein filament, can undergo homology-driven invasion of the intact sister chromatid, creating a joint molecule. Capture of the second resected single-stranded DNA tail into the joint molecule is proposed to occur through a number of different mechanisms, including through invasion by the same mechanism as the first end or by DNA synthesis and branch migration also referred to as synthesis dependent strand annealing [15,16]. HR involves a large number of proteins, including the core HR proteins RAD51, the RAD51 paralogs (XRCC2, XRCC3, RAD51B, RAD51C, RAD51D), RAD52, RAD54, RAD54B, the MRN complex and the BRCA proteins [17,18] (Figure 1). Resolution of the joint HR partners requires structure-specific endonucleases. The XRCC3 and RAD51C proteins were shown to be associated with such an activity although its identity is not yet known [19].

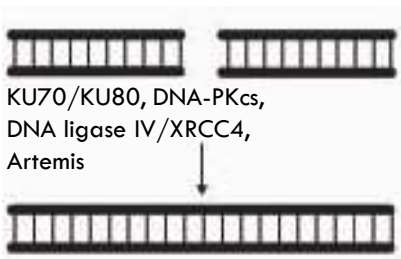

Figure 2: nonhomologous end-joining
This pathway does not require the presence of a homologous template, but mediates repair by directly rejoining DNA strands. NHEJ involves recognition of the DSB, processing of non-complementary or damaged DNA ends and the subsequent ligation of DNA ends.

Mechanism of nonhomologous end-joining

In contrast to HR, NHEJ uses little or no homology at all to repair the break. This pathway is not only used to repair DSBs generated by exogenous DNA damaging agents, such as IR, but is also required to process the DSB intermediates that are generated during V(D)J recombination [17]. V(D)J recombination creates specialized DSBs, whose rejoining generates the exon that encodes the variable domain for immunoglobulins and T-cell receptors. Joining events in pre-B and pre-T cells are the basis of the antigen-specific immune system of vertebrates [20]. Many proteins that are involved in NHEJ are also involved in V(D)J recombination (Figure 2). Humans and mice that have a severely impaired ability to carry out V(D)J recombination develop a severe combined immunodeficiency (scid).

One of the factors, which is involved in NHEJ, is the KU heterodimer, which consists of KU70 and KU80 (also referred to as KU86). The KU proteins show a high affinity for DNA ends. KU bound

to a DNA end attracts the catalytic subunit of DNA dependent protein kinase (DNA-PKcs). *In vitro,* DNA-PKcs can phosphorylate several target proteins such as P53, the KU proteins and itself. A complex that consists of DNA ligase IV and XRCC4 takes care of the ligation step. Another protein involved in the NHEJ process is Artemis, which is required for a subset of NHEJ reactions that require end processing [21,22].

Mouse mutants defective in homologous recombination

The most important proteins involved in this pathway are the core HR proteins, RAD51, RAD52, RAD54, RAD54B, the RAD51 paralogs XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, the BRCA proteins and the MRN complex consisting of MRE11, RAD50 and NBS1. Attempts to assess the biological consequences of defects in HR proteins have been prevented by the embryonic lethality of many mouse knockouts targeting genes involved in HR (Table 1). Below we summarize the most important observations made using HR-defective mice and/or cells derived from them.

RAD51

An important clue to the function of the RAD51 protein came from the observation that *S. cerevisiae* Rad51 showed sequence similarity to the *E. coli* RecA strand exchange protein [23]. Subsequently it was shown that RAD51 is conserved from yeast to humans [24]. Biochemical studies showed that the key reactions in HR, the search for homologous DNA and DNA strand exchange, are performed by remarkably similar mechanisms in bacteria, yeast and human [25]. Conservation of RAD51 from yeast to mammals underscored the importance of this DNA repair pathway and made it possible to investigate HR in vertebrates. Mouse embryos deficient in *Rad51* are lethal, but using inducible knockout chicken DT40 cells this lethality could be studied. Prior to cell death these *Rad51* knockout DT40 cells show chromosomal aberrations and have replication associated problems [26]. As RAD51 is required for cell viability, these mutants cannot be used to investigate IR sensitivity.

RAD52

The RAD52 protein has a number of notable activities that are important in HR. First, RPA is displaced to form an active presynaptic filament. Since RPA is also present in vivo during RAD51 nucleoprotein filament formation, mechanisms must exist to allow filament formation in the presence of RPA. Biochemical studies revealed that the inhibition of RAD51-promoted strand exchange by RPA, is overcome when RAD52 is incubated together with RAD51, RPA and single-stranded DNA, followed by the addition of homologous double-stranded DNA [14,27-30]. Second, RAD52 can bind to single-stranded DNA protecting the ends from nucleolytic degradation and it can form rings interacting with the DNA [31,32].

Genetically there is a central role for RAD52 in HR in yeast. This in contrast to the mammalian

phenotype, where disruption of RAD52 did not result in an aberrant phenotype [33]. The viability of *Rad52*$^{-/-}$ mice and the lack of a detectable aberrant phenotype could be explained by redundancy through the RAD51 paralogs as it was shown in DT40 cells that *Rad52*$^{-/-}$ *XRCC3*$^{-/-}$ double mutant cells are non-viable and exhibit extensive chromosomal breaks, whereas both single mutants are viable [34].

RAD54

Another key component of the HR machinery is the double stranded DNA-dependent ATPase RAD54 [35,36]. Both RAD52 and RAD54 are accessory proteins for RAD51 but they function at different stages. RAD52 functions early to assist RAD51 loading onto resected DNA ends, RAD54 functions later at the point when the intact double-stranded template needs to be or is engaged [32]. In contrast to the embryonic lethality of Rad51 mutants and the lack of an aberrant phenotype of *Rad52*$^{-/-}$ mice, *Rad54*$^{-/-}$ mice are viable and embryonic stem (ES) cells display DNA damage sensitivity.

Rad54$^{-/-}$ mouse ES cells are sensitive to IR and the DNA interstrand crosslinker MMC and display reduced levels of HR. Interestingly, although adult *Rad54*$^{-/-}$ mice were MMC sensitive, they did not show increased sensitivity to IR [37,38].

RAD54B

RAD54B is a mammalian paralog of RAD54, which displays similar biochemical properties as RAD54 with respect to its physical and functional interaction with RAD51 and DNA. RAD54B knockout mice were generated to examine functional redundancy between RAD54 and RAD54B in DNA DSB repair. At the cellular level the paralogs do not show an additive or synergistic effect with respect to MMC sensitivity. Interestingly, animals lacking both *Rad54* paralogs are dramatically sensitized to MMC compared to the single mutants arguing for a possible tissue-specific function of the paralogs [39].

The RAD51 paralogs

Both in yeast and in vertebrates paralogs of RAD51 have been identified. There are two Rad51 paralogs in S. cerevisiae, Rad55 and Rad57. Biochemical studies have shown that they form a heterodimer that interacts with RAD51 and stimulates RAD51-mediated strand exchange and RAD51-mediated pairing. The heterodimer is thought to function early in HR by facilitating assembly of the RAD51 nucleoprotein filament that initiates HR [40]. Consistent with this notion, IR sensitivity of Rad55 and Rad57 mutants can be overcome by over expression of RAD51 or RAD52 [41] or by a mutation in Rad51 that increases its affinity for DNA [42].

Five paralogs have been identified in vertebrate cells; XRCC2, XRCC3, RAD51B, RAD51C and RAD51D. The paralogs are present in two distinct complexes, which have RAD51C as a common component. One complex contains XRCC3 and RAD51C, while the other complex

contains XRCC2, RAD51B, RAD51C and RAD51D [43]. The RAD51B-RAD51C-RAD51D-XRCC2 complex binds single-stranded DNA and single-stranded gaps in duplex DNA, in accord with the proposal that the paralogs play an early role in recombinational repair. Moreover, this complex binds specifically to nicks in duplex DNA [43,44]. The other complex consisting of XRCC3 and RAD51C also has DNA binding activity. Interestingly, in addition to early roles of the RAD51 paralogs in HR, recent experiments also suggest a late role for the RAD51C-XRCC3 complex. This complex has been shown to associate with a Holliday junction resolvase activity [19,44]. To discriminate between the roles of the paralogs, mutants were made in chicken DT40 cells. Disruption of the *Rad51* paralogs showed that all mutants were viable and exhibited very similar phenotypes. Disruption leads to chromosomal instability, moderately increased IR sensitivity, significantly increased sensitivity to MMC and affects HR efficiency [45,46]. In contrast to the results in DT40 cells, disruption of the *Rad51* paralogs in mice leads to embryonic lethality, which is similar to *Rad51* knockout mice [47-49]. Possibly, the *p53*-deficiency of DT40 cells allows viability in the absence of the *Rad51* paralogs. Consistently, the lethality of *Rad51* knockout mutation in mice is delayed by several days in the absence of P53 [11].

The MRE11-RAD50-NBS1 (MRN) complex

The MRN complex, consisting of RAD50, MRE11 and NBS1, is a central player in various aspects of the cellular response to DNA DSBs, including HR, NHEJ, telomere maintenance and DNA damage checkpoint activation [50-55]. Biochemical studies showed that MRE11 has several nuclease activities [53,56-58], while RAD50 has an ATPase activity [59]. An essential biological function of the complex in the context of DSB repair is to tether DNA ends through a DNA-driven conformational change [60-64]. Homologs of the MRE11 nuclease and the RAD50 ATPase are found in different organisms, suggesting that this complex is fundamental for genomic stability [65]. The third component of the MRN complex is termed NBS1 in higher eukaryotes, and Xrs2 in yeast. This third component is less conserved between species than the MRE11 and RAD50 components. Mutations in the components of the MRX complex in *S. cerevisiae* revealed roles in meiotic recombination and in HR between sister chromatids in mitotic cells [66]. In higher eukaryotes the MRN complex is essential for viability. Conditional *Mre11* null chicken DT40 cells exhibit a reduced capacity for HR and undergo proliferative arrest associated with high levels of chromosome aberrations [67]. Also in mammals, the assessment of the role of the MRN complex *in vivo* is complicated by the inviability of *Mre11*, *Rad50* and *Nbs1* null mutants [68-70].

To analyze the role of the MRN complex in adult mice, mouse models were generated with hypomorphic mutations. Two different engineered hypomorphic alleles of murine *Nbs*, one in *Rad50* (*Rad50*^{s}) and one in *Mre11* (*Mre11*^{ATLD}) resulted in viable mice [71-74]. Hypomorphic mutations in the *Nbs1* and *Mre11* genes were modeled after mutations occurring in humans that cause genome instability and cancer predisposition syndromes Nijmegen breakage syndrome

(NBS) and ataxia telangiectasia-like disorder (ATLD), respectively [75-78]. *Nbs1* mutant mice are viable, growth retarded and sensitive to IR. Furthermore, *Nbs1* mutant mice exhibit multiple lymphoid developmental defects and thymic lymphomas. *Nbs1* mutant cells are impaired in cellular responses to IR and defective in cellular proliferation [71,72]. A remarkable aspect of the*Rad50*^{s} allele is its dramatically different effect at the cellular versus the organismal level [73]. *Rad50*$^{s/s}$ mouse embryonic fibroblasts (MEFs) show almost none of the phenotypes that might be expected of perturbed MRN complex function. The cells show no growth defect, no defect in IR-induced subcellular relocalization of the complex, and no hypersensitivity to DNA-damaging agents such as IR and MMC. In addition, although *Nbs1*and ATLD cells are defective in the IR-induced intra-S-phase checkpoint, *Rad50*$^{s/s}$ cells are not. However, even though no overt cellular phenotype of the *Rad50*^{s} allele could be detected, its effect on mice is profound. *Rad50*$^{s/s}$ embryos are susceptible to premature death. Animals that make it through birth are small, and most of them die within three months of severe anemia caused by hematopoietic stem cell depletion, whereas longer-lived animals are predisposed to cancer. *Mre11*$^{ATLD/ATLD}$ mice exhibit several indices of impaired ATM function; they also exhibit pronounced chromosomal instability, but these mice are not markedly predisposed to malignancy [74].

The BRCA proteins

Mutations in the breast cancer tumour susceptibility genes BRCA1 and BRCA2 predispose humans to breast and ovarian cancer. Although BRCA1 and BRCA2 are both involved in HR, BRCA1 appears to be involved in many more DNA metabolic processes as evidenced by a wealth of biochemical data describing multi-protein interactions. BRCA1 co-localizes and interacts with RAD51 and the MRN complex [79-83]. Interactions between BRCA1 and several transcription factors, replication factors and chromatin remodeling protein complexes have been reported [84-87]. BRCA2 co-localizes and interacts with RAD51 and BRCA1 [88,89]. BRCA2 has been reported to interact with at least one chromatin remodeling protein [90]. A possible suggested function for BRCA2 based on biochemical experiments, is for the protein to act preferentially at the interface between double-stranded DNA and single-stranded DNA to displace RPA from the overhang and assist the loading of RAD51 [91]. Following DNA damage and initial DSB processing BRCA2, like RAD54, concentrates in nuclear foci that co-localize with RAD51 foci. An active role of BRCA2 in RAD51 foci formation is suggested, as RAD51 focus formation is impaired in *Brca2*-deficient cells [92,93]. Mouse embryos deficient for *Brca1*, null mutants have been shown to die during early embryogenesis between embryonic day 6 and 13.5 p.c. due to a proliferation defect [94-96]. Furthermore, *Brca1*$^{-/-}$ day 3.5 embryos are sensitive to IR and exhibit chromosomal aberrations [97]. Similar to BRCA1, BRCA2 is indispensable for embryonic development [89]. Notably, *Brca1* and *Brca2* mutant embryos exhibit a similar phenotype. In addition to full knockouts of *Brca1*and *Brca2*, which are embryonic lethal [12,89,94-96,98], mice harboring hypomorphic *Brca1* and *Brca2* alleles have been generated. In most cases, the

Brca1 mutants are associated with a severe developmental delay and a cellular proliferation defect [94,95,99]. Lethality midway in embryonic development was noted in the *Brca1*$^{\Delta 223\text{-}763}$ and *Brca1*$^{\Delta 11}$ embryos, which express a BRCA1 splice product that deletes exon 11 [100,101]. Similar to *Brca1*, *Brca2* deficiency results in early embryonic lethality in the mouse when mutations produce truncations that occur 5' to exon 11 [12,89]. However, when a truncated BRCA2 product retains RAD51 interacting BRC repeat sequences such as*Brca2*^{tm} and *Brca2*^{Tr2014} alleles some mutants are viable and some are embryonic lethal [102,103]. Viable mutant mice succumb from thymic lymphoma with markedly decreased life spans. Both males and females are infertile. *Brca2* mutant mice that lack exon 27, *Brca2*$^{\Delta 27}$, have been reported [104]. These mice have a subtle degree of embryonic lethality, appear developmentally normal, and have a shortened life span. Moreover, a hypomorphic mutant of *Brca2* results in sensitivity to IR and MMC at blastocyst stage.

Mouse mutants defective in NHEJ

KU70, KU80, DNA-PKcs

DNA-PK is a holoenzyme composed of the KU70/80 heterodimer and a catalytic subunit; DNA-PKcs. DNA-PKcs shows serine/threonine protein kinase activity that is enhanced by a simultaneous association with both KU70/80 and DNA. KU was first identified as an autoantigen in the sera of patients with autoimmune disease [105]. The KU heterodimer is the DNA end-binding component of the DNA-PK holoenzyme. Biochemical studies indicate that KU and DNA-PKcs do not associate in the absence of DNA termini [106]. Furthermore, biochemical data indicate that both KU and DNA-PKcs are involved in bridging of DNA ends, binding and activation of processing enzymes and recruitment of the DNA ligase IV/XRCC4 complex [107].

The first NHEJ deficient mouse model that was identified was the naturally occurring scid mutant [108]. This mouse carries a spontaneous mutation that prevents the production of mature B and T cells, due to a defect in joining the DSB intermediates in V(D)J recombination. This phenotype is caused by a mutation in Prkdc, which encodes DNA-PKcs, resulting in a deletion of the 83 carboxy-terminal amino acids of the DNA-PKcs protein [109]. Subsequent experiments have shown that a targeted *DNA-PKcs* mutant gives rise to a similar phenotype [110]. The *DNA-PKcs* mutant mice develop lymphoid tumors of the T-cells and display a scid phenotype, the signal joining is normal but the coding joining is impaired. Moreover, *DNA-PKcs*$^{-/-}$ MEFs are sensitive to IR whereas ES cells are not. This could mean that ES cells may employ a DNA-PKcs-independent DSB repair pathway or a factor redundant with DNA-PKcs for DNA repair that is not principally utilized in other cell types [111].

In addition to the phenotypes described for the *DNA-PKcs* mutant, *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mice show more severe phenotypes, including reduced body weight at birth, slow growth, reduced life span and a marked increase in chromosomal aberrations [112]. Fibroblasts derived from

these mutant mice show early senescence, indicating a general proliferative defect and are sensitive to IR [113,114]. *Ku70* and *Ku80* deficiency affects both signal and coding joint formation. The more severe phenotype of the *Ku70* and *Ku80* knockouts compared to the *DNA-PKcs* mutants might be related to a DNA-PKcs independent function of the KU proteins, for example its function at telomeres [115,116]. It is thought that KU plays a crucial role in protecting telomeres from HR by preventing end-to-end fusion [117].

DNA ligase IV/XRCC4 complex

The DNA ligase IV and XRCC4 form a complex that accomplishes the final ligation step that ligates two juxtaposed ends during NHEJ. Both DNA ligase IV and XRCC4 proteins are of high importance for mammals, since deficiency of either of these proteins leads to embryonic lethality in knockout mice. In *DNA ligase IV* mutant mice lymphopoiesis is blocked and V(D)J recombination does not occur [118]. *XRCC4* deficient embryos display apoptotic death of newly generated post-mitotic neurons throughout the developing nervous system. In addition, progenitor lymphocyte development is arrested due to impaired V(D)J recombination. *XRCC4* deficient embryos are growth retarded and their fibroblasts exhibit decreased proliferation and premature senescence in culture [8].

Artemis

The recently discovered Artemis protein is a DNA processing enzyme that is associated with NHEJ, it is necessary for repair of a subset of breaks [54]. Biochemical studies have shown that Artemis is a 5' to 3' exonuclease [119]. Artemis is essential for successful V(D)J recombination. Moreover, Artemis functions as a genomic caretaker, most notably in prevention of translocations and telomeric fusions. Artemis was discovered as the mutated gene in human radiosensitive T-B-SCID, a disease characterized by lack of B and T lymphocytes and increased radiosensitivity of bone marrow cells and skin fibroblasts [120]. *Artemis* deficient ES cells and *DNA-PKcs* deficient ES cells exhibit similar sensitivities to DNA damaging agents. So, *Artemis* deficient ES cells are not sensitive to IR, indicating that ES cells make less use of NHEJ for the repair of DSBs or alternatively Artemis only plays a role in a subset of the NHEJ events. *Artemis* deficient MEFs on the other hand display sensitivity to IR [121]. A recent study showed that Artemis is required for rejoining of a subset of DSBs, the fraction of unrejoined breaks is dependent upon the nature of the DSB [54].

<u>Overlapping and specialized roles of homologous recombination and nonhomologous end-joining</u>

Rad54$^{-/-}$ mice provided an excellent opportunity to study the biological significance of the mammalian HR pathway. In contrast to homozygous disruption of other genes implicated in DSB repair; such as *Rad51*, *Mre11, Brca1 and Brca2*, which result in an embryonic lethal phenotype,

Rad54$^{-/-}$ mice are viable. *Rad54*$^{-/-}$ mice display no gross abnormalities and have a normal life expectancy. Therefore, *Rad54*$^{-/-}$ mice provide the opportunity to study the biological relevance of HR in mammalian DNA damage repair. Since it was unexpectedly found that adult *Rad54*$^{-/-}$ mice are not IR sensitive, the IR sensitivity of early embryonic stages of Rad54$^{-/-}$ mice was determined. *Rad54*$^{-/-}$ day 3.5 embryos were IR sensitive compared to wild-type embryos [37]. Similar radiation sensitivity assays with *Rad51* and *Brca1* mutant embryos also demonstrated their IR sensitivity [11,97], reflecting an essential role of HR in IR resistance early in development. As *Rad54*$^{-/-}$ mice are not IR sensitive, the hypothesis was postulated that RAD54-mediated HR has an important role in conferring IR resistance early in development in less differentiated cell types. Interestingly, *Rad54*$^{-/-}$ ES cells and mice were found to be sensitive to the crosslinking agent MMC, while cells or mice containing the scid mutation are not MMC sensitive. DSBs are likely intermediates in the repair of these interstrand DNA crosslinks. In theory, both HR and NHEJ should be capable of repairing these DSBs. Given the DNA damage specificity that was observed for *Rad54*$^{-/-}$ and *scid* mice, it appears that the repair of DNA crosslinks is a specialized function of the HR pathway. There are two possible explanations for this observation. Repair of a MMC-induced DNA crosslink could occur through a pathway requiring two DSBs around the lesion. Removal of the crosslink by NHEJ would require that the two DSBs are made more or less simultaneously. If they are not made simultaneously, then the DNA at the undamaged end of the DSB would be joined to the DNA that still contained the crosslink and the repair cycle would be futile. In contrast, HR is very well capable of bypassing a crosslink starting from a single DSB, which is an intermediate that would be generated from a stalled replication fork at an interstrand crosslink [122]. Alternatively, the use of HR instead of NHEJ for the repair of DNA interstrand crosslinks could involve processing of the crosslink. It is possible that the crosslinked region undergoes extensive nuclease digestion. This would result in large single-stranded DNA regions. These large single-stranded DNA regions cannot be joined by NHEJ and most likely require HR for their repair.

There are various explanations for the fact that although *Rad54*$^{-/-}$ ES display a clear phenotype, the observed phenotype of adult *Rad54*$^{-/-}$ mice is relatively mild. A possible explanation for the mild phenotypes of the adult *Rad54*$^{-/-}$ mice observed to date is redundancy of the RAD54 function. This redundancy could occur within the HR pathway itself or between the HR pathway and the NHEJ pathway. Besides redundancy within pathways, redundancy between pathways could possibly account for the mild phenotype of the adult *Rad54*$^{-/-}$ mice. In this regard, it is interesting to compare the similarities and differences in phenotypic consequences of mutations in HR and NHEJ. The IR sensitivity of *Rad54*$^{-/-}$ ES cells is quantitatively similar to that of mouse *Ku70*$^{-/-}$ ES cells [113]. KU70 is one of the components of DNA-PK, which is involved in DSB repair through NHEJ. Thus, both NHEJ and HR can equally contribute to IR resistance in ES cells. However, adult mice defective in either RAD54-dependent HR or DNA-PK-mediated NHEJ differ in IR sensitivity. Rad54$^{-/-}$ mice are not IR sensitive, while a mutation in *DNA-Pkcs* or *Ku80*,

two other components of DNA-PK, confer a 2- to 3-fold IR sensitivity to adult mice [123]. A possible explanation is that accurate repair is crucial for mammalian stem cells and germ cells, whereas inaccurate repair could be more easily tolerated by differentiated somatic cells, given that a large fraction of their genome is no longer functional. Following this hypothesis, the NHEJ pathway could then confer IR resistance to the adult animal. However, in the absence of NHEJ, DNA repair through HR could become an important back-up repair mechanism. The reverse of this hypothesis has clearly been demonstrated in *S. cerevisiae*. The deletion of *S. cerevisiae* genes involved in NHEJ, such as Ku70, Ku80 and DNA ligase IV does not cause sensitivity to IR. However, deletion of HR by deletion of Rad52 together with the deletion of Ku70, Ku80 or DNA ligase IV results in increased IR sensitivity compared to the IR sensitivity of single Rad52 mutants. This hypothesis for mammals was tested with mice, carrying both the scid mutation and *Rad54*-deficiency. These *Rad54 scid* double mutant mice, having defects in both HR and NHEJ, revealed overlapping roles between the two DNA repair systems [123]. The pivotal insight from this study was that in mammals the relative contribution of the two major DSB repair pathways, HR and NHEJ, changes during development of the animal. While HR provides protection against IR induced DNA damage in embryos, its contribution in adults is not detected, unless NHEJ is disabled. There are several possible explanations for the greater contribution of HR to IR resistance early in development. First, in dividing cells there is the availability of a sister chromatid as a repair template in the S and/or G2 phases of the cell cycle [124]. Therefore, HR plays a more prominent role when sister chromatids are available during late S and G2 phase stages of the cell cycle, whereas NHEJ is important during G1 and early S phase stages [125-128]. Second, it is also possible that there is a preference of HR over NHEJ for repair of a DSB with regard to the repair fidelity between these two pathways. HR repairs a DSB more precise compared to NHEJ, which might be important for cells early in development. Imprecise repair could be tolerated more easily by differentiated somatic cells because a large fraction of their genome is no longer functional. Third, separate DSB pathways could have their own specialized function. Although their function can overlap for the processing of DNA damage produced by IR, they can also have pathway-specific functions.

Similar results were obtained by crossing *Rad54* deficiency to mutants in other NHEJ factors like *Ku80* and *DNA ligase IV*. Survival of *Rad54* and *Ku80* double mutant mice is severely compromised compared to *Ku80* and *Rad54* single mutant mice revealing a critical role for RAD54 in supporting *Ku80* mutant mouse viability. Similar to *Rad54* and *DNA-PKcs* double mutant mice, *Rad54* and *Ku80* double mutant mice were also extremely sensitive to IR mutant mice [129]. A synergistic increase in IR sensitivity was previously reported for double mutant *Rad54* and *Ku70* Drosophilia larvae and chicken cells, although in this case a *Rad54* mutation itself also caused IR sensitivity [128,130]. *Ku* mutant cells show a reduced NHEJ capacity if the DSB is produced by V(D)J recombination, but the repair of a DSB produced by an endonuclease and repaired via HR results in an increase [131-134].

Mammalian mutant	Animal viability	Day3.5 embryo blastocysts	IR sensitivity ES cells	IR sensitivity pMEFs	IR sensitivity tMEFs	IR sensitivity mice	References
HR mutants							
Rad51	e. lethal E.8.5	sensitive		lethal			[42]
Rad52	viable		not sensitive	lethal			[33]
Rad54	viable	sensitive	sensitive	not sensitive		not sensitive	[37, 123]
Rad54B	viable		sensitive			not sensitive	[39]
Rad51 paralogs							
XRCC2	e. lethal	sensitive		lethal			[49]
Rad51D	e. lethal (E8.5-E11.5)	not sensitive		lethal			[138]
MRN-complex							
Mre11 $^{ATLD/ATLD}$	viable			sensitive			[75]
Rad50	e. lethal E6.0	sensitive		lethal			[73]
Nbs1 $^{\Delta B/\Delta B}$	viable				sensitive		[72]
Nbs1 $^{m/m}$	viable		sensitive			sensitive	[71]
Brca-proteins							
Brca1	e. lethal	sensitive		lethal			[100, 101]
Brca2	e. lethal	sensitive		lethal			[104]
NHEJ mutants							
DNA-PKcs (null, scid)	viable		not sensitive	sensitive			[108-110]
Ku70	viable		sensitive	sensitive			[113]
Ku80	viable		sensitive	sensitive			[114]
DNA ligase IV	viable			sensitive			[118]
XRCC4	viable		sensitive	sensitive			[111]
Artemis	viable		not sensitive	sensitive			[21]
HR and NHEJ mutants (double mutants)							
Rad54 / scid	viable			hypersensitive		hypersensitive	[123]
Rad54 / DNA ligase IV	viable						[135]
Rad54 / Ku80	viable			hypersensitive		hypersensitive	[129]

Table 1: Viability and IR sensitivity of the murine DSB repair mutants

Empty cell means not determined, e. lethal: embryonic lethal, tMEFs: transformed mouse embryonic fibroblasts, pMEFs: primary mouse embryonic fibroblasts, IR: Ionizing Radiation.

This suggests that HR and NHEJ can be competing pathways for the repair of a DSB in some circumstances. A third study to test the hypothesis that a more critical role for mouse RAD54 would be revealed by the absence of NHEJ was done by Mills et al. [135]. *Rad54* deficient mice were bred onto a DNA ligase IV deficient background. The DNA ligase IV deficient background was selected because this NHEJ factor was assumed the most specific, thus allowing monitoring the role of RAD54 in a background without residual NHEJ activity. As *DNA ligase IV* knockout mice are embryonic lethal, double mutant MEFs were isolated. Survival and chromosomal analysis of these cells showed that RAD54 and DNA ligase IV cooperate to support cellular proliferation and prevent chromosomal aberrations. Besides providing additional evidence for overlapping functions of HR and NHEJ in the repair of DSBs, like in the two previous studies [123,129], the defective proliferation of *Rad54* and DNA ligase IV double mutants also indicates interplay of RAD54 and DNA ligase IV in the repair of post-replication DSBs.

A more specialized role of NHEJ is V(D)J recombination, which generates the exons that encode the variable domain for immunoglobulins and T-cell receptors to obtain an antigen-specific

immune system. Therefore, the advantage of NHEJ being a more inaccurate DNA repair process compared to HR is that it can create diversity during immunoglobulin rearrangement. Disruption of genes involved in HR did not result in a defective immune system [136,137].

In this review we have discussed the cell type specificity of DSB repair pathways in mice. The two major DSB repair pathways, HR and NHEJ, have specialized and overlapping functions concerning their repair of different types of lesions. Clearly, not all DSBs are equal; the context under which a DBS is generated is an important determinant for pathway choice between the two mechanistically distinct DSB repair pathways.

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Chapter 3

End-joining of blunt DNA double-strand breaks in mammalian fibroblasts is precise and requires DNA-PK and XRCC4

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Abstract

DNA double-strand break repair by non-homologous end-joining (NHEJ) is generally considered to be an imprecise repair pathway. In order to study repair of a blunt, 5' phosphorylated break in the DNA of mammalian fibroblasts, we used the *E. coli* cut-and-paste type transposon Tn*5*. We found that the tn*5* transposase can mediate transposon excision in Chinese Hamster cell lines. Interestingly, a blunt 5' phosphorylated break could efficiently be repaired without loss of nucleotides in wild type fibroblasts. *DNA-PKcs* deficiency reduced the efficiency of joining fourfold without reducing precision, whereas both efficiency and accuracy of joining were affected in *Ku80* or *XRCC4* mutant cell lines. These results show that both the DNA-PK and the XRCC4/ligase IV complexes are required for NHEJ and that other, more error-prone, repair processes can not efficiently substitute for joining of blunt breaks produced in living cells. Interestingly, the severity of the end-joining defect differs between the various mutants, which may explain the difference in the severity of the phenotypes, which have been observed in the corresponding mouse models.

Introduction

DNA double-strand breaks (DSBs) are among the most genotoxic lesions that can be caused by ionizing radiation or certain chemicals, but they are also necessary intermediates in essential DNA recombination processes such as V(D)J recombination in the immune system and meiotic recombination in germ cells [1]. They can be repaired by at least two genetically distinct pathways, homologous recombination and non-homologous end-joining (NHEJ) [2, 3]. Homologous recombination makes use of a homologous template (normally the sister chromatid) to carry out precise repair, whereas NHEJ uses little or no homology.

Several features of the NHEJ reaction have emerged recently. The DNA-dependent protein kinase (DNA-PK), which consists of the KU70/KU80 heterodimer and the catalytic subunit of DNA-PK (DNA-PKcs), is probably involved in the early phases of this DSB repair reaction [4]. The KU heterodimer has high affinity for DNA ends, suggesting its involvement in the recognition of DSBs. Subsequently, the DNA-bound KU heterodimer attracts DNA-PKcs, which then acquires protein kinase activity. Together with the recently discovered Artemis protein, DNA-PKcs can stimulate end-processing, which is required to join non-matching ends [5, 6]. Finally, the ligation step is carried out by the XRCC4/ligase IV complex. Mouse knock out models have been developed for all these NHEJ genes [7-17] . The common feature of these knock outs is severe combined immunodeficiency, caused by their inability to carry out V(D)J recombination in their pre-B and pre-T cells. However, they show differences in the severity of their phenotypes. The *DNA-PKcs*$^{-/-}$ and *Artemis*$^{-/-}$ mice show little other defect than the inability to carry out V(D)J recombination, whereas *XRCC4*$^{-/-}$ and *ligaseIV*$^{-/-}$ mice die late in embryogenesis, probably

as a result of severe apoptosis of postmitotic neurons. It is not clear whether the difference in phenotypes reflects differences in the severity of the NHEJ defect or that the XRCC4/ligase IV complex might be involved in other processes, which might confer the embryonic lethal phenotype.

Although the NHEJ genes are essential for V(D)J recombination and normal levels of X-ray sensitivity, they are not essential to rejoin linear plasmids that can be transfected into mammalian cells [18, 19]. However, the joining products obtained in wild type and NHEJ mutant cells are quite different. Transfection of blunt-ended linear DNA into wild type fibroblasts results in mostly precisely rejoined plasmids, whereas joining reactions in cell lines mutated in any of the DNA-PK components or the XRCC4/ligase IV complex are mostly imprecise and use short stretches of homology to align the ends (microhomology-mediated joining).

In the present study we investigate how blunt DSBs in a nuclear episomal plasmid are repaired in mammalian cells. We employed the *E.coli* cut-and-paste type transposon Tn*5*. The Tn*5* transposase produces a blunt DSB, for which it does not require any host factors [20]. As this transposon is derived from an evolutionarily very distant species, we do not expect any specific interactions between the transposase complex and mammalian DSB repair factors. We found that the large majority of junctions after Tn*5* excision did not lose any nucleotides and that the end-joining factors KU80 and XRCC4, and to a lesser extent DNA-PKcs, were required for efficient rejoining. The microhomology-mediated joining pathway could not substitute for the regular NHEJ pathway, showing that blunt DSBs produced in chromatinized DNA depend on the NHEJ pathway for efficient rejoining.

Results

The transposon excision assay

Previously, we investigated the repair of blunt-ended linear DNA in mammalian cells by transfection of a plasmid that had been linearized by restriction enzyme digestion [19]. This assay was set up in such a way that the linear DNA contained a 6 bp direct repeat at the ends, which could be used to align the ends, resulting in deletion of exactly 6 bp. This microhomology-mediated DSB repair was found to happen in 10-30% of all junctions, depending on the method of transfection. However, this is an artificial situation for DSB repair, because the break is made outside the cell and the broken DNA molecule is introduced into the cell as naked DNA. Chromosomal DNA, on the other hand, is packaged in chromatin, which can dramatically influence DNA-protein transactions. We therefore developed an assay to investigate repair of a blunt-ended DSB which is made inside the cell in a chromatinized substrate.

The assay was set up in such a way that the DSB was made by excision of a cut-and-paste type transposon. We chose to use the E.coli transposon Tn*5* to make a blunt DSB, because it does not need any host factors for excision and has been well characterized biochemically [20]. In

addition to the Tn5 transposase, excision only requires two Tn5 transposon ends. Furthermore, Tn5 transposase is not expected to influence the outcome of the subsequent DSB repair reaction, as it is derived from a bacterial species.

In order to reach the highest level of DSB formation, we used a hyperactive mutant Tn5 transposase and so-called mosaic Tn5 transposon ends, which combination results in the highest level of in vitro transposition [20, 21]. The nuclear localization of the transposase was ensured by adding a nuclear localization signal to the C-terminus of the hyperactive mutant transposase. The Tn5 minitransposon is present in an assay construct that contains polyoma sequences for replication in mammalian cells [22]. Transposon excision can be determined by PCR detection using primers that can amplify the region containing the transposon (figure 1A). PCR amplification of substrate pDVH6 (containing the transposon) gives rise to a 563 bp PCR product, while transposon excision and exact repair of the blunt DSB would produce a 213 bp PCR fragment. One of the PCR primers was labeled radioactively in order to simplify detection and quantification after phosphorimaging.

The sequences flanking the Tn5 transposon in pDVH6 have been chosen in such a way that exact joining of the flanking DNA would produce an EcoRV restriction site, whereas use of a 6 bp microhomology that directly flanks the transposon would produce a novel BstXI restriction site (figure 1A). This set up enables detection of the relative efficiencies of these different repair events by digestion of the PCR products with either of these restriction enzymes.

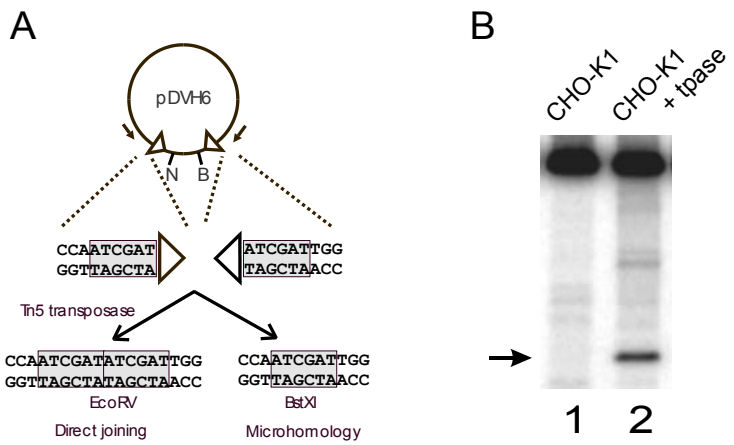

Figure 1: The transposon excision assay

(A) Schematic representation of the transposon excision reaction. Plasmid pDVH6 contains two Tn5 mosaic ends in inverted orientation (depicted as triangles). After cotransfection of this plasmid with a Tn5 transposase expression plasmid, the Tn5 minitransposon is excised. The resulting DSBs can be repaired without loss of sequence, which creates a novel EcoRV site, whereas joining on a 6 bp microhomology at the DNA ends creates a novel BstXI site. N = NheI; B = BamHI; triangles depict Tn5 mosaic ends. (B) Products obtained by PCR over the transposon site in pDVH6. In the absence of the transposase expression construct, only the site containing the transposon is amplified (lane 1). Upon cotransfection with the Tn5 transposase expression construct, a shorter product is formed as well (lane 2). The arrow point to the PCR product resulting from rejoining after transposon excision.

DSB repair in wild type Chinese Hamster cells is precise

We initially used the wild type Chinese hamster cell line CHO-K1 to investigate DSB repair. CHO-K1 cells were transfected with the transposon construct pDVH6 and either with or without the transposase expression construct pDVG148. After 48 hours, the extrachromosomal DNA was isolated and transposon excision was detected by PCR over the transposon site (figure 1B). DNA that had not been replicated in the fibroblasts was removed by digestion with DpnI, which specifically cleaves DNA with the *E.coli* methylation pattern. DNA recovered from transfection reactions in which only the transposon construct had been introduced gave rise to a 563 bp PCR product, indicative of the unrearranged substrate (lane 1). DNA recovered after cotransfection of the transposon and the transposase expression constructs gave rise to a 213 bp PCR product in addition to the unrecombined 563 bp product (lane 2). Approximately 6% of the recovered plasmids contained an empty transposon site, as shown by the relative intensity of the shorter PCR product. This has been confirmed by transformation of the recovered plasmids into E.coli and analysis of DNA from separate colonies: 11 out of 117 recovered plasmids (9%) had a structure consistent with excision of the Tn*5* element followed by end-joining (table 2). We also recovered 2 plasmids, which contained the left Tn5 flank, but had sustained a large deletion of the right flank and had also incorporated two different stretches of unidentified DNA at the junction. The rest of the plasmids were unrecombined substrate molecules. These results confirm that the main route of repair after Tn*5* excision goes through NHEJ. As expected, analysis of DNA recovered from transfection experiments with only the transposon construct yielded exclusively unrecombined substrate molecules (out of a total of *57* plasmids analyzed). Subsequent analysis of a different wild type hamster cell line, CHO9, also showed approximately 6% transposon excision (Figure 3, lane 2).

Sequence	# of clones
Precise joining (EcoRV site at junction)	10
Additional untemplated T	1
Large deletion right flank	2
Unrecombined plasmids	104

Table 2: Plasmids recovered from CHO-K1 cells transfected with the transposon construct pDVH6 and the transposase expression construct pDVG 148

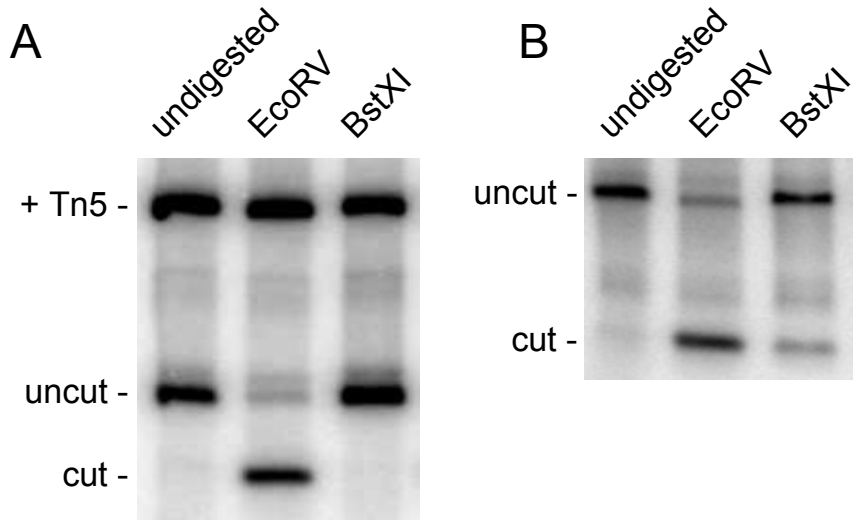

Figure 2: Precision of end-joining in mammalian fibroblasts
(A) CHO-K1 cells were cotransfected with the transposon-containing substrate pDVH6 and the transposase expression construct pDVG148. After 48 hours DNA was recovered and the majority of unrearranged plasmids was removed by digestion with BamHI and NheI. Junctions were amplified and PCR products were digested with EcoRV or BstXI, as indicated. (B) CHO-K1 cells were transfected with pDVG164 linearized with EcoRV. PCR products were digested with EcoRV or BstXI, as indicated. Tn5 = PCR product that contains the Tn5 sequence, uncut = PCR product resulting from transposon excision, cut = PCR product resulting from transposon excision that has been cut with the indicated restriction endonuclease.

Subsequently, we analyzed the types of junctions that were generated after transposon excision. If the junctions are precise, the 213 bp PCR product resulting from transposon excision can be cut by EcoRV, giving rise to 136 and 77 bp fragments. Joining on the 6 bp microhomology results in a 207 bp product, which can be cut by BstXI in 133 and 74 bp fragments. We found that almost all transposon excision products were joined in a precise fashion, without loss of any sequence, as shown by EcoRV digestion (figure 2A, lane 2). BstXI digestion, which would cut all products joined on the 6 bp microhomology, did not digest any of the PCR products, showing that this type of repair is infrequent in wild type cells (figure 2A, lane 3). Consistent with this PCR result, 10 out of 11 plasmids with a structure consistent with excision of the Tn5 element followed by end-joining had gained an EcoRV restriction site at the newly formed junction. The single other rejoining product had incorporated an untemplated T nucleotide at the junction. For comparison, we also transfected CHO-K1 cells with linear DNA containing exactly the ends that are expected after Tn5 excision (figure 2B). In this case, approximately 10% was joined using the microhomology, while the large majority of the junctions are precise. Similar results were obtained with the wild type Chinese Hamster cell line CHO9 (data not shown). Apparently, end-joining after transposon excision is more precise than joining of transfected linear DNA in wild type cells.

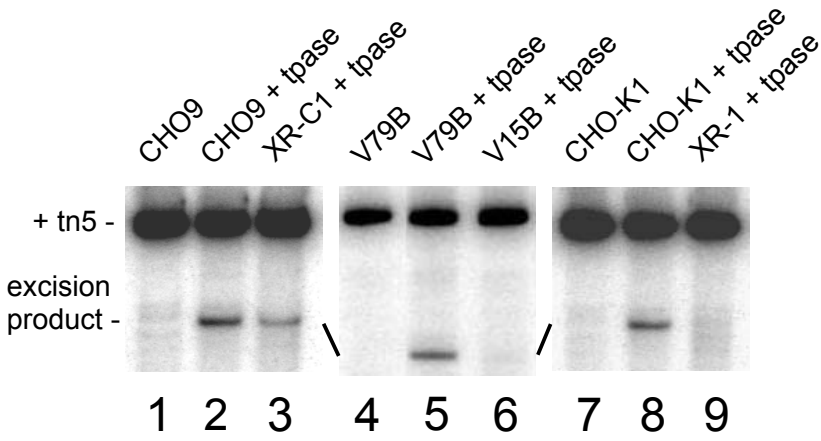

Figure 3: Joining efficiency after Tn5 transposon excision in NHEJ mutant Chinese Hamster cells
The end-joining assay construct pDVH6 was transfected into CHO9 (lanes 1 and 2), XR-C1 (lane 3), V79B (lanes 4 and 5), XR-V15B (lane 6), CHO-K1 (lanes 7 and 8) or XR-1 (lane 9). The transposase expression construct pDVG148 was cotransfected in lanes 2, 3, 5, 6, 8 and 9. After 48 hours, DNA was recovered and digested with DpnI. The region containing the Tn5 transposon was amplified and products were separated on a 6% polyacrylamide gel in TBE buffer.

NHEJ mutants show reduced levels of joining after Tn5 excision

Mutant Hamster fibroblasts defective in the NHEJ components *DNA-PKcs, Ku80* or *XRCC4* show a severe defect in V(D)J recombination, whereas the level of plasmid recircularization is only mildly affected. As shown in figure 3, the level of empty transposon sites is reduced in these three NHEJ mutants. The relative efficiency of rejoining is reduced approximately 4-fold in the *DNA-PKcs* mutant cell line XR-CI (1.5% ± 0.1% compared to 5.8% ± 0.4% in wild type CHO9 cells), whereas rejoining in the *Ku80*-mutant cell line XR-V15B and the *XRCC4*-deficient cell line XR-1 was below the lower limit of detection (approximately 0.2% recombined products), which corresponds to a more than 25-fold reduction compared to wild type cells. When the plasmids that contain the Tn5 transposon were digested with BamHI and NheI, empty transposon sites could be detected much more efficiently. In the case of XR-V15B, we still did not detect any rejoining, while joining after Tn5 excision in XR-1 cells could in some cases be visualized (figure 4C). In XR-V15B cells we could only visualize junctions in a nested PCR approach (figure 4B).

Circularization of linear plasmids showed a dramatic shift towards use of microhomologies at the junctions in NHEJ mutants [19]. We first confirmed that this is also the case with plasmid pDVG164, in which the same blunt ends that can be created by transposon excision, are created by restriction enzyme digestion in vitro. As shown in figure 5, the level of microhomology use was indeed dramatically increased in all end-joining mutants. Subsequently, we investigated the junctions generated after transposon excision in these mutant cell lines by restriction enzyme

digestion. The junctions produced in the *DNA-PKcs* mutant cell line XR-CI were sensitive to EcoRV digestion, showing that they did not lose any sequence before rejoining, similar to the situation in wild type cells (figure 4A). The same phenomenon was also observed in the *DNA-PKcs* deficient V3 cells (data not shown). However, the *Ku80* deficient XR-V15B cells formed a very low level of junctions, that were partially precise (EcoRV sensitive) and partially microhomology-mediated (BstXI sensitive) (figure 4B). The *XRCC4* deficient XR-1 cells showed a complete shift towards use of the 6 bp microhomology at the junction (figure 4C), suggesting that the XRCC4/ ligase IV complex is absolutely required for precise NHEJ.

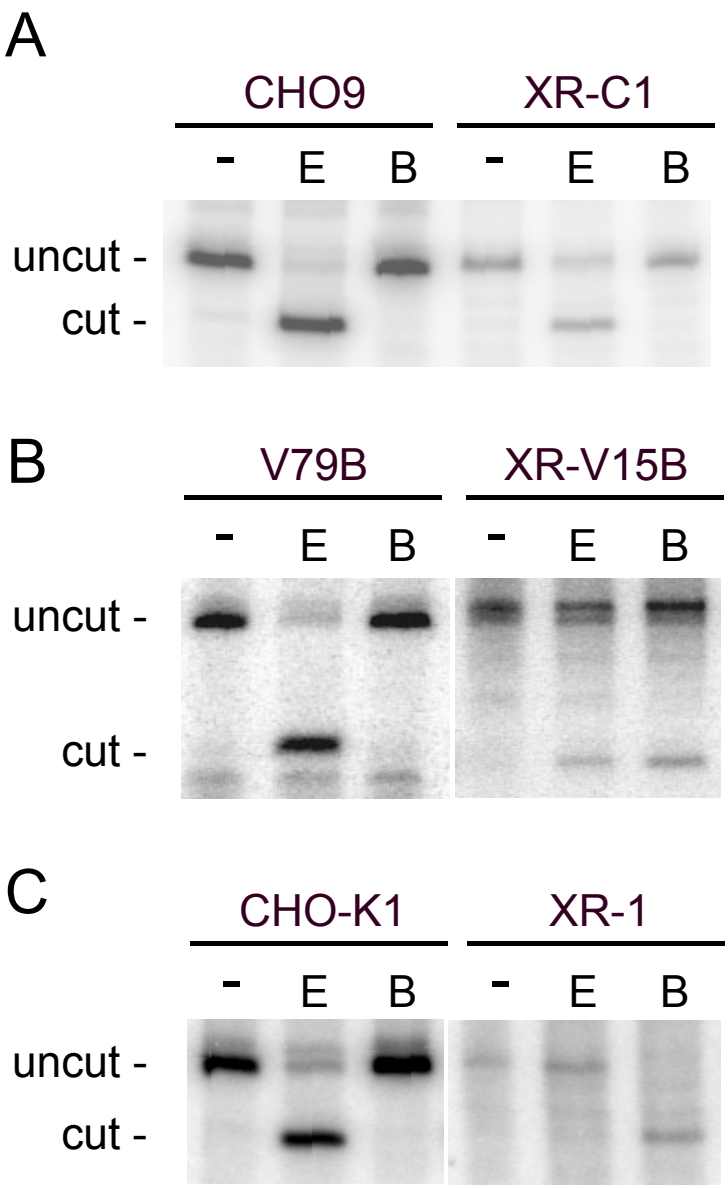

Figure 4: Analysis of joining products after Tn5 transposon excision in NHEJ mutant Chinese Hamster cells

Transposon excision assays were carried out in the DNA-PKcs mutant XR-C1 (A), the Ku80 mutant XR-V15B (B) or the XRCC4 mutant XR-1 (C). For comparison the wild type counterparts CHO9, V79B and CHO-K1 are also shown. The transposon containing plasmids that were recovered from the cells, were first removed by digestion with BamHI and NheI. PCR products shown in (B) are the result of a nested PCR approach (see materials and methods), because XR-V15B did not yield any visible products after the first PCR. Only the PCR products resulting from rejoining after transposon excision are shown. PCR products are shown undigested (-) and after cleavage with EcoRV (E) or BstXI (B), as indicated.

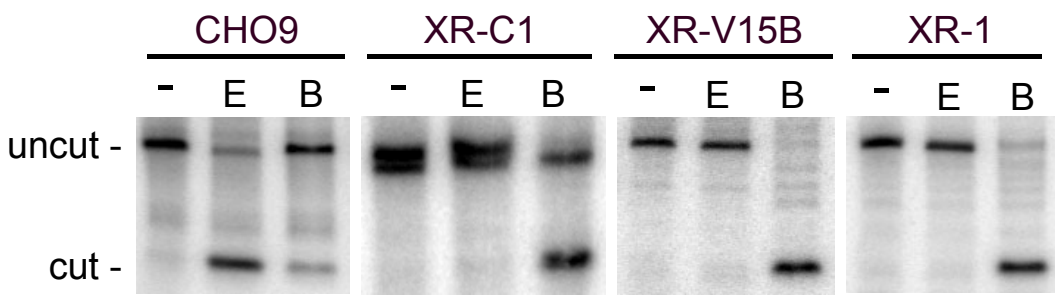

Figure 5: Joining of DNA ends created in vitro by digestion with the restriction enzyme EcoRV
The same cell lines as in figure 4 were transfected with EcoRV linearized pDVG164 DNA. Joining products were detected using the same PCR primers and PCR products were digested as in figure 4.

Discussion

Repair of DSBs in the absence of a homologous sequence is normally done by NHEJ. Here, we show that this is also the case for blunt DSBs, which do not require any processing before joining, and that other repair pathways are very inefficient to substitute for the regular NHEJ pathway. Interestingly, joining after excision of the Tn5 transposon, which leaves behind blunt ends, was almost exclusively carried out without loss of any nucleotides.

Previously, we described that rejoining of linear DNA in NHEJ mutant cells was only slightly less efficient than in wild type cells, while V(D)J recombination was much more strongly reduced [19]. In the present study we investigated the reason for the difference in end-joining efficiency between V(D)J recombination and recircularization of linear plasmids. We considered several possibilities. First, the V(D)J recombinase complex could attract the DNA-PK complex, which would exclude other DSB repair pathways. Second, the difference could be caused by the substrate in which DSBs are made: linear plasmids enter the cell as naked DNA, whereas V(D)J recombinase makes the DSB in a chromatinized template. And third, the DNA ends are different: V(D)J recombination produces DNA hairpins, in which the top strand is covalently coupled to the bottom strand, whereas the linear DNA harbors blunt ends. Finally, we also considered the possibility that the cell cycle phase in which the DSBs are generated might be different: V(D)J recombination mainly takes place in G1, whereas it is unclear when circularization of linear plasmids takes place. The first and third possibilities would result in higher levels of end-joining after Tn5 excision than during V(D)J recombination in NHEJ mutant cells, probably through the microhomology-mediated pathway, whereas the second option would lead to joining levels similar to the V(D)J recombination phenotype. Our results are consistent with the second possibility: if a DSB is made inside the nucleus by transposon excision, repair does require the NHEJ factors KU80 and XRCC4, and to a lesser extend DNA-PKcs, even when the ends do not require processing before ligation. In other words, the microhomology-mediated end-joining

can only substitute for precise NHEJ in the artificial situation of linear DNA transfection, but not for repair of DSBs made in chromatinized DNA inside the nucleus. However, we cannot exclude that Tn*5* excision mainly takes place in e.g. the G1 phase of the cell cycle, which would favor precise end-joining. We do not consider this option to be very attractive, since we would expect the broken DNA molecules to persist into the S and G2 phases of the cell cycle, where a different mode of DSB repair could then have taken over.

Although all NHEJ factors we investigated are required for efficient joining of Tn*5* induced DSBs, the *Ku80* and *XRCC4* mutants are much more deficient than the *DNA-PKcs* mutant, suggesting that DNA-PKcs mainly plays a stimulatory role in ligation of ends that do not require any processing. This difference may explain the severity of the various mouse models deficient in one of the NHEJ genes: the highest level of apoptosis in postmitotic neurons (in the *XRCC4*$^{-/-}$ mouse) correlates with the lowest level of residual precise end-joining capacity [23], while the intermediate phenotype of the *Ku*-deficient mice [24] correlates with the intermediate phenotype we observe in this end-joining assay. Therefore, we hypothesize that mutation of DNA-PKcs leads to a relatively high level of residual end-joining capacity, which is sufficient to repair most of the spontaneous DNA damage in the brain, whereas the (almost) complete inability to carry out NHEJ in the *XRCC4*$^{-/-}$ (and *ligase IV*$^{-/-}$) mouse leads to excessive unrepaired DNA damage in the brain, which triggers apoptosis.

DNA-PKcs has an important role in processing of the covalently closed coding ends in V(D)J recombination, where it stimulates the recently characterized Artemis protein [*5*, *6*]. The involvement of DNA-PKcs in signal joint formation is a matter of debate: most publications describe (somewhat) lower levels of signal joint formation and more imprecise joining in various *DNA-PKcs* mutants, although some reports describe normal levels of signal joint formation [*25*-29]. Here, we describe that a Tn*5*-induced blunt DSB showed a fourfold reduced level of junctions in a *DNA-PKcs* mutant hamster cell line, which is similar to what has been reported for signal joint formation in V(D)J recombination. However, different to what was described for signal joints, we did not find an increased level of imprecise junctions, suggesting that signal ends in V(D)J recombination are handled slightly differently than blunt ends produced by Tn*5* excision, possibly because the RAG proteins that bind to the signal sequences can influence the subsequent joining event. In this respect it should be noticed that signal joint formation in the XR-C1 cell line is much less efficient than DSB repair of blunt ends produced by Tn*5* excision [26]. In conclusion, it is clear that DNA-PKcs has a function in NHEJ which is independent of its role in end processing, although this function is less crucial than the roles of the KU70/80 heterodimer and the XRCC4/ligaseIV complex.

Coding joints in V(D)J recombination and NHEJ products after ionizing radiation treatment of cells often sustain short deletions, suggesting that NHEJ is an imprecise repair process. Unexpectedly, our results show that NHEJ of a blunt DSB is a very precise process in the large majority of joining events. Apparently, the deletions observed in other cases were caused by imprecise

DNA breaks (that needed processing before joining), rather than an inherent imprecision of the joining machinery itself. The DNA hairpin intermediates in V(D)J recombination may therefore be very important to increase the diversity of the variable region of the immunoglobulins, as blunt DSBs would be repaired precisely, limiting the diversity to the number of possible combinations of V, D and J segments.

The precision of NHEJ observed in these experiments should also lead to careful reassessment of experiments carried out using the I-SceI restriction enzyme to make DSBs [30]. The efficiency of NHEJ relative to the frequency of homologous recombination was determined by measuring the loss of the I-SceI site. If most NHEJ events of the DNA ends (which contain 5' cohesive ends) are precise, as we observed for blunt ends in this study, this calculation would lead to an enormous underestimation of the frequency of NHEJ. Liang et al. [31] reported that approximately 30-50% of DSB repair was carried out by homologous recombination between directly repeated sequences in fibroblasts. If we assume that the relative level of precise rejoining is more than 95% (as we generally observe for blunt DSBs), these numbers should be changed in more than 98% NHEJ and less than 2% homologous recombination. However, we do not want to imply that homologous recombination is a minor DSB repair pathway in mammalian cells. Most probably, the major DSB repair pathway after DNA replication in the S phase of the cell cycle is gene conversion between sister chromatids. On the other hand, we expect that homologous recombination between directly repeated sequences in the genome is a minor DSB repair pathway, since this would result in unacceptable levels of genomic instability. Indeed, increased levels of chromosomal instability have been observed in *Ku80* deficient fibroblasts, suggesting that prevention of recombination between repetitive sequences may depend on efficient NHEJ. It will be of great interest to perform DSB repair assays with chromosomal substrates using a transposon excision strategy similar to the I-SceI assays described before.

Materials and Methods

DNA constructs

The Tn*5* transposase expression construct pDVG148 was assembled from the BspHI/BglII fragment of pGRTEMP2 containing the transposase open reading frame [20], the mammalian expression vector pCMV/Myc/Nuc (Invitrogen) digested with NcoI and NotI and the adapter oligonucleotide DG191/192.

The Tn*5* transposon vector pDVH6 was constructed as follows. First, the EcoRI/NotI fragment of pCMV/Myc/Mito/GFP (Invitrogen) containing the GFP open reading frame was cloned into the EcoRI/NotI sites of pCMV/Myc/Nuc (=pDVG153). Subsequently, the oligonucleotides DG195/DG196 and DG197/DG198 were annealed and ligated into the PmlI/BssHII sites of pDVG153, thereby creating pDVG154. The GFP sequence was replaced by the EGFP sequence derived from pEGFP-C3 (Clontech) by exchanging the AgeI/Bsp120I fragment of

Oligoname	Sequence
DG181	5'-ATCCCTTAAGCTCGACCAGGATGGGCACCACCCC
DG191	5'-GATCTACGCGTC
DG192	5'-GGCCGACGCGTA
DG195	5'-CGCGTACGAGATCTGCTAGCCCCGCAGCAGCAGCGGCGTCAG GACGGACATGGTCAC
DG196	5'-GTGACCATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTAG CAGATCTCGTA
DG197	5'-CGCGTCCCGGGGCTTGACAGGCTCGGCCCGGCGGCTCCCAG TGCCG
DG198	5'-CGCGCGGCACTGGGAGCCGCCGGGCCGAGCCTGTCAAGCCC CGGGA
DG201	5'-CCCAAGCTTCACATTGATTATTGAGTAGTTATT
DG202	5'-CGGGATCCCGGCGGCGGTCACGAACTC
DG203	5'-CTAGCTAGCAGATGTGTATAAGAGACAGCAGCAGCGGCGTCAG GACGGACATGGTC
DG204	5'-ACGACGCGTAGATGTGTATAAGAGACAGGGCTTGACAGGCTCG GCCCGGCGGCTCC
DG205	5'-CTAGCATCCAATCGATATC
DG206	5'-GATCGATATCGATTGGATG
DG207	5'-CGCGTGATATCGATTGGGCCCGGCGGCTCCCAGTGGCCG
DG208	5'-CGCGCGGCCACTGGGAGCCGCCGGGCCCAATCGATATCA
DG241	5'-GGTGGGAGGTCTATATAAGCAGAGC
DG242	5'-CAGATGAACTTCAGGGTCAGCTTGC
DH1	5'-ACCACTAGTCCAATCGATCTGTCTCTTATACACATCTGCTAG
DH2	5'-TTGGCGCGCCCAATCGATCTGTCTCTTATACACATCTACGCG
LB2fwd	5'-CTCATCAATGTATCTTAACGCG
163.1.1	5'-CTAGAGAACCCACTGCTTACTGGCA
163.1.2	5'-GTGGCCGTTTACGTCGCCGTCCAGC

Table 1: List of oligonucleotides

pEGFP-C3 for the PstI/NotI fragment of pDVG154 and the BglII/MluI fragment of pEGFP-C1 containing the polyA signal was inserted into the BglII/MluI sites, creating pDVG156. The Tn5 mosaic ends [21] were introduced by cloning PCR products resulting from amplification of pDVG156 with primers DG202 and DG204 into the NcoI/MluI sites followed by insertion of the PCR product resulting from amplification of pDVG156 with primers DG201 and DG203 into the NheI/NdeI sites, resulting in pDVH2. The sequences containing Tn5 in pDVH2 were PCR amplified using primers DH1 and DH2 and cloned into the NheI/BssHII sites of pDVG164 (see below), giving rise to pDVH6.

The vector containing the exact same blunt DNA ends after EcoRV digestion as would result after transposon excision from pDVH6, was created as follows. First, oligonucleotides DG207 and DG208 were annealed and cloned into the MluI/BssHII sites of pDVG156, followed by insertion of the annealed DG205 and DG206 into the NheI/BamHI sites. Subsequently the AvrII/SspI fragment was cloned into the HinCII/SpeI sites of pDVG158 (which is similar to pDVG94 [19]; exact sequence available upon request), creating vector pDVG164.

Recircularization of linear DNA

Plasmid substrate pDVG164 was digested with EcoRV, BamHI and NheI, which creates a linear vector with blunt ends. Transfection of Chinese hamster cells and recovery of extrachromosomal DNA was carried out as described [19]. Joining was analyzed by PCR amplification of junctions using primers 163.1 and 163.2, followed by digestion of the PCR products with EcoRV or BstXI as indicated.

Transposon excision assay

The transposon containing substrate pDVH6 was cotransfected with the transposase expression construct pDVG148 (1 µg of each plasmid per 3 cm dish) into Chinese hamster cell lines using Superfect (Qiagen) as described [19]. After 48 hours the extrachromosomal DNA was recovered and analyzed by PCR amplification using primers 163.1 and 163.2. PCR conditions were chosen such that amplification was in the linear range and was consistent with data obtained from experiment in which individual plasmids were analyzed (see below). PCR products were digested with EcoRV or BstXI as indicated and analyzed by gel electrophoresis and phosphorimaging [19]. In order to analyze transposon excision products in NHEJ mutant cell lines XR-CI, XR-V15B or XRCC4, the DNA recovered from the cells was digested with BamHI and NheI prior to PCR amplification in order to remove plasmids that did not undergo transposon excision. For comparison, the same treatment was given to the DNA recovered from the corresponding wild type cells prior to amplification in these experiments. Joints from the V79B and XR-V15B cell lines were analyzed using a nested PCR approach, in which the DNA was first amplified using primers DG241 and DG242, followed by digestion with BamHI and NheI (to remove any amplified products that still contain the transposon) and amplification

using primers 163.1 and 163.2.

Analysis of transposon excision products by transformation of recovered DNA to *E.coli* was done as follows: DNA was digested with DpnI (which removes all non-replicated plasmids) and transformed to *E. coli* strain DH5α. Colonies were streaked onto three replica plates and hybridized with the oligonucleotides 163.1, 163.2 and LB2fwd, which recognize the 5' flanking DNA, the 3' flanking DNA and the transposon itself, respectively. Colonies that hybridized to oligonucleotides 163.1 and 163.2 or only one of these were investigated further. Plasmid DNA was isolated and digested in order to investigate whether a novel EcoRV restriction site was present at the junction. Other plasmids were sequenced using primer 163.1.

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Chapter 4

Functional and physical interactions in nonhomologous end-joining

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Abstract

DNA damage responses include DNA repair and signal transduction pathways that affect cell cycle checkpoint arrest and/or apoptosis. The KU70/KU80 heterodimer and DNA-PKcs are central components of the nonhomologous end-joining (NHEJ) pathway of DNA double strand break (DSB) repair.

NHEJ is not expected to have a function in repair of replication-associated DSBs. However, when we increased the number of single strand breaks by PARP inhibition, which is believed to cause single-strand breaks that can be converted to double-strand breaks during replication, we observed hypersensitivity of *DNA-PKcs*$^{-/-}$ ES cells. *Ku70*$^{-/-}$ ES cells, on the other hand, did not show PARP inhibitor sensitivity. This could in principle be explained by a KU-independent function of DNA-PKcs or an additional KU70 function that might counteract the increased sensitivity by NHEJ inhibition. Interestingly, we found an interaction between nuclear KU70 and the pro-apoptotic BAX protein, suggesting that KU70 may link DSBs to apoptotic pathways via BAX. We also observed an interaction between KU70 and the MRE11-RAD50-NBS1 (MRN) complex, which is involved in sensing DSBs independently of KU70/80. Taken all these data together, we hypothesize that KU70 might be the link between DSBs and apoptosis.

Introduction

DNA double strand breaks (DSBs) are repaired either by homologous recombination (HR) or by nonhomologous end-joining (NHEJ). HR appears to be the predominant mechanism of replication-associated DSB repair. NHEJ, which simply pieces together the broken DNA ends, can function in all phases of the cell cycle. Therefore, NHEJ is the major pathway in mammalian cells for repairing DSBs [1, 2]. When left unrepaired, DSBs are among the most dangerous of DNA lesions and can lead to cell death and genomic rearrangements [3].

The NHEJ process is initiated by the binding of the KU70/80 heterodimer to both ends of the broken DNA molecule. The association of a DNA end with the KU70/80 heterodimer creates a scaffold for the assembly of the other NHEJ factors. At an early stage of NHEJ-mediated DSB repair, the DNA-KU70/80 complex attracts the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the DSB. Biochemical studies indicate that KU70/80 and DNA-PKcs do not associate in the absence of DNA termini [4]. Furthermore, it has been shown that both KU proteins and DNA-PKcs are involved in bridging DNA ends and binding and activation of processing enzymes and the ligase [5-10]. Ligation of the processed DNA ends is carried out by the ligase IV/XRCC4 complex, which can be stimulated by the recently discovered XLF/Cernunnos protein [11, 12]. Up till now, many targets for the DNA-PKcs kinase have been found in vitro, but the biological relevance of these observations is unclear in most cases. It is very well established that DNA-PKcs has the ability to autophosphorylate itself [13].

Autophosphorylation leads to alteration of the protein's affinity for DNA and to the inactivation of its kinase activity. Such phosphorylation-induced alterations are important during DSB repair in vivo because mutations in the phosphorylation cluster cause severely increased IR-sensitivity and decreased DNA repair [14-16]. Simultaneously with NHEJ, the ATM cascade is initiated by the MRN complex assembly at DSBs [17, 18]. The MRN complex recruits ATM to the break where it is activated. ATM subsequently phosphorylates members of the MRN complex and a variety of other proteins that function in cell cycle checkpoint activation and apoptosis. The NBS1 C-terminus is required for apoptosis but not for ATM activation per se [19].

Deficiency in both *Atm* and *DNA-PKcs* is synthetically lethal in developing mouse embryos. In contrast, simultaneous *Nbs1*$^{\Delta B/\Delta B}$ and *DNA-PKcs* deficiency are viable. Cell cultures from *Nbs1*$^{\Delta B/\Delta B}$ *Prkdc*$^{scid/scid}$ embryos displayed severe defects, including premature senescence, mitotic aberrations, sensitivity to IR, altered checkpoint responses, and increased chromosome instability [20].

Interestingly, KU70 can also be linked to apotosis. It has been reported that KU70 can bind to BAX, which is a pro-apoptotic member of the Bcl2 family [21-23]. The cytosolic BAX-KU70 interaction suppresses apoptosis induction [21, 22]. Also for the MRN complex an apoptotic function has been shown [19, 24, 25]. It is not yet clear how IR and other types of DNA damage exactly trigger the apoptotic machinery.

In this chapter, we show that KU70 interacts with the pro-apoptotic BAX protein and the MRN complex in the nucleus, suggesting that it may be the link between NHEJ, apoptosis and cell cycle checkpoint control.

Results

Analysis of DNA-PKcs and KU70 in various types of double strand break repair

DSB repair is the most important factor for survival after induction of DSBs. Therefore, we first investigated the relative importance of the NHEJ factors DNA-PKcs and Ku70 in mouse Embryonic Stem (ES) cells. Previous reports claimed that primary *DNA-PKcs*$^{-/-}$ MEFs were sensitive to ionizing radiation (IR), but *DNA-PKcs*$^{-/-}$ ES cells were not [26]. However, we found that both *Ku70*$^{-/-}$ and *DNA-PKcs*$^{-/-}$ ES cells were IR sensitive (Figure 1A). As expected, *Ku70*$^{-/-}$ MEFs were also IR sensitive, showing that this defect is not cell type specific (Figure 1B) [27].

As the effect of a KU70 defect resulted in a more pronounced IR sensitivity than the DNA-PKcs defect, we chose to investigate the functional and physical interactions of KU70 with other components of the DNA damage response in more detail. We first addressed the relative importance of KU70 in NHEJ. HR has been found previously to function as a backup for NHEJ. Cells deficient in *Rad54* in addition to a *DNA-PKcs, Ku80* or *DNA Ligase IV* defect were IR hypersensitive [28-30]. Similarly, we found that the *Ku70*$^{-/-}$ *Rad54*$^{-/-}$ double mutant ES cells were extremely IR sensitive (Figure 1A), showing that NHEJ deficiency caused by *Ku70* deletion is similar to the previously reported NHEJ mutants.

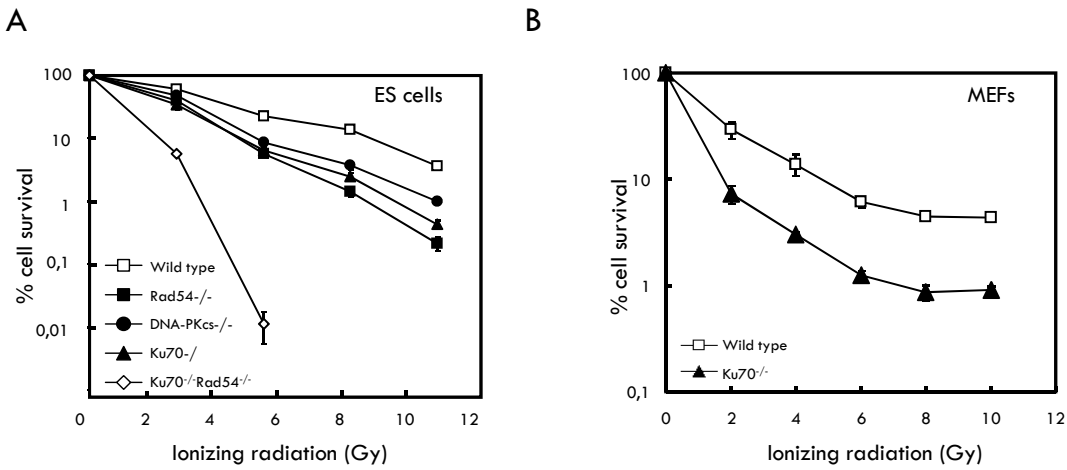
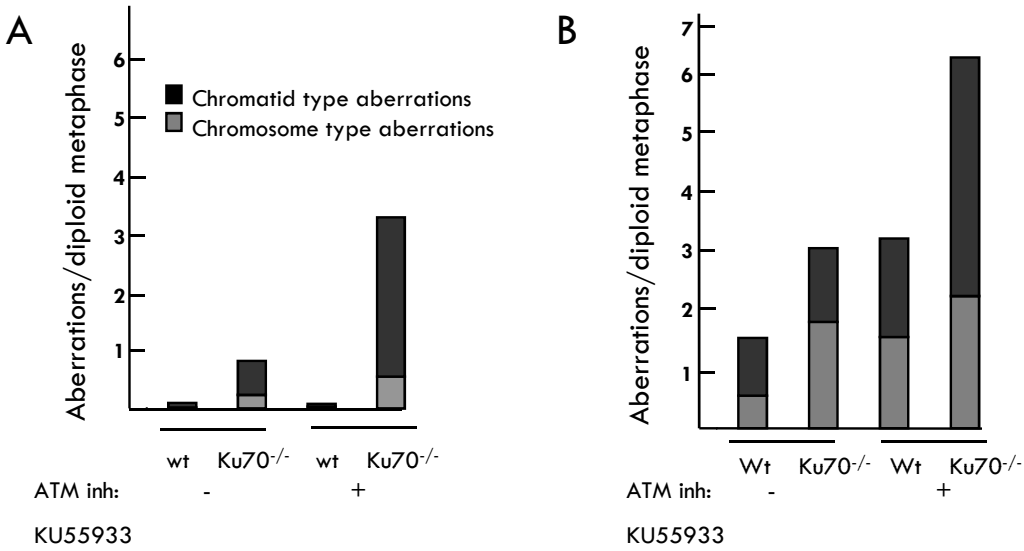


Figure 1: IR-sensitivity of NHEJ mutants

(A) Colony survival assay after IR treatment of *Ku70*$^{-/-}$, *Rad54*$^{-/-}$, *DNA-PKcs*$^{-/-}$, *Ku70*$^{-/-}$ *Rad54*$^{-/-}$ and wild type ES cells. Two different ES cell lines were used for all genotypes except for DNA-PKcs$^{-/-}$, where only one cell line was used. No variation between cell lines of the same genotype has been observed (B) Colony survival assay after IR treatment of *Ku70*$^{-/-}$ and wild type MEFs. The gamma-ray doses are displayed on the X-axis on a linear scale, while the colony survival is displayed on the Y-axis on a logarithmic scale. Error bars show the standard error of the mean for at least three experiments.

The IR sensitivity observed in *Ku70*$^{-/-}$ ES cells prompted us to investigate the effect of this mutation on chromosomal stability. In contrast to wild type MEFs, *Ku70*$^{-/-}$ MEFs displayed chromosomal instability even in an unchallenged situation (Figure 2A). This instability phenotype was exacerbated when ATM was inhibited, whereas in wild type cells no chromosomal aberrations were present even after ATM inhibition. This shows that the combined effect of *Ku70* deletion and ATM inhibition results in severe chromosomal instability, probably as a result of the combined defects in NHEJ and the G2/M checkpoint. Subsequently, we exposed these ES cells to IR (1 Gy) and investigated the effect of the DSB repair defect. After IR, the number of chromosomal aberrations increased both in the *Ku70*$^{-/-}$ and in the wild type cells (Figure 2B). ATM inhibition again increased the number of chromosomal aberrations. Both chromatid type and chromosome type aberrations increased after ATM inhibition. However, the chromatid type aberrations increased more than chromosome type aberrations in *Ku70*$^{-/-}$ ES cells, indicating that most metaphases resulted from cells that had been irradiated during or after replication.

NHEJ is generally believed to have a major function in repair of DSBs throughout the cell cycle, but this repair pathway has not been implicated in repair of replication-associated DSBs. To test whether NHEJ is indeed not involved in repair of this type of DSBs, we investigated the response of *DNA-PKcs*- and *Ku70*-deficient ES cells to the PARP inhibitor KU58948. PARP-1 is involved in single strand break (SSB) repair and its inhibition is believed to cause SSBs that



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Wild type	Chromatid	Chromatid (%)	Chromosome	Chromosome (%)
-IR, -ATM	0.08	80	0.02	20
-IR, +ATM	0.04	50	0.04	50
+IR, -ATM	0.96	63	0.56	37
+IR, +ATM	1.66	52	1.54	48
Ku70$^{-/-}$	Chromatid	Chromatid (%)	Chromosome	Chromosome (%)
-IR, -ATM	0.58	71	0.24	29
-IR, +ATM	2.76	83	0.56	17
+IR, -ATM	1.24	41	1.80	59
+IR, +ATM	4.02	64	2.24	36

Figure 2: Quantification of chromosomal aberrations in MEFs

(A) Chromosomal aberrations in wild type and *Ku70*$^{-/-}$ MEFs in the presence or absence of the ATM inhibitor KU55933 (10μM). (B) MEFs subjected to 1 Gy IR. (C) Table of the chromatid and chromosome type aberrations per metaphase in wild type and *Ku70*$^{-/-}$ MEFs in the presence or absence of the ATM inhibitor KU55933.

can be converted to DSBs during replication [31]. To our surprise, we observed a difference between *DNA-PKcs* and *Ku70* deficiency in ES cells. The *DNA-PKcs*$^{-/-}$ ES cells were hypersensitive whereas the *Ku70*$^{-/-}$ ES were not (Figure 3). We considered two possible explanations for this phenomenon. First, DNA-PKcs may not require KU70 for its activation by SSBs or replication-associated DSBs. Alternatively, KU70 could be required for DNA-PKcs activation (which has a pro-survival effect), as well as for another process in the DNA damage response (which may promote cell death). As a possible mechanism for promotion of cell death we first explored the possibility that KU70 has a function in apoptosis.

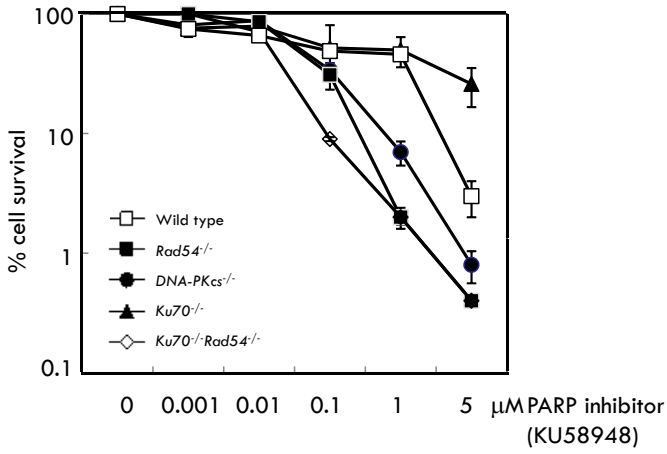

Figure 3: Sensitivity of DSB repair mutants to PARP inhibitor
Colony survival assay after treatment with the PARP inhibitor KU58948 in ES cells. Two different ES cell lines were used for all genotypes except for *DNA-PKcs*$^{-/-}$, where only one cell line was used. No variation between cell lines of the same genotype has been observed. The PARP inhibitor dose is displayed on the X-axis on a logarithmic scale, while the colony survival is displayed on the Y-axis on a logarithmic scale. Error bars show the standard error of the mean for at least three experiments.

KU70 and a possible role in apoptosis

We first investigated the interaction between KU70 and the pro-apoptotic protein BAX. These proteins have previously been shown to interact in the cytosol [21, 22]. Interestingly, immunoblot analysis and immunofluorescence studies demonstrated that the nuclear localization of BAX was severely diminished in *Ku70*$^{-/-}$ MEFs (Figure 4A and B), suggesting that BAX is retained in the nucleus by interaction with KU70.

KU70 can accumulate on damaged DNA in the absence of KU80

KU70 functions together with KU80 as a heterodimer that stimulates DNA-PK activity. The (GFP-)KU70/80 heterodimer accumulates at DSBs that can be induced by laser micro-irradiation [32]. This complex accumulates in the damaged area within minutes and remains visible for more than an hour. If the KU70 function in apoptosis is independent of the KU/DNA-PK complex, it might accumulate in the damaged area in the nucleus to transmit the signal, even in the absence of KU80. Therefore, we introduced YFP-KU70 into *Ku70*$^{-/-}$ *Ku80*$^{-/-}$ MEFs and investigated its accumulation on DNA damage. We observed a low level of transient YFP-KU70 accumulation in the damaged area within one minute after laser micro-irradiation (Figure 5A and B). This accumulation disappeared within a few minutes. We conclude that, although most KU70 accumulation is KU80 dependent, a KU80-independent mode of accumulation exists as well. This accumulation exists only for a few minutes after damage induction, suggesting that it may have a function early in the DNA damage response. Our data suggest that KU70 needs another factor to monitor the DNA damage.

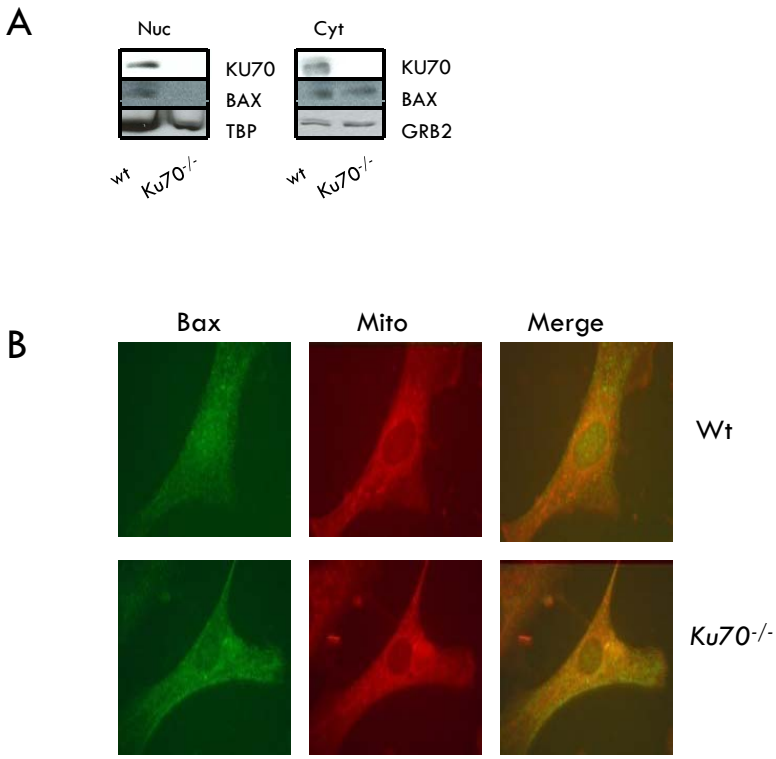

Figure 4: BAX nuclear localization depends on KU70 interaction
(A) Subcellular fractionation and Western blot analysis in wild type and *Ku70*$^{-/-}$ MEFs. Western blot analysis of BAX and KU70
(B) Immunofluorescence analysis of BAX in wild type and *Ku70*$^{-/-}$ MEFs. Mitotracker (Invitrogen) was used as a marker for mitochondria.

Interaction between KU70 and the MRN complex

Subsequently, we investigated how a signal might be transmitted from DNA damage to the apoptotic machinery. More specifically, we asked whether the known KU70-BAX interaction might provide a possible route for apoptosis induction. The KU70-BAX interaction in the cytosol has been shown previously [33] to be independent of KU80. Therefore, we investigated whether KU70 can bind to the MRN complex in the absence of KU80. We produced KU70 and MRN complexes in insect cells and purified them to near homogeneity. The MRN complex was indeed co-immunoprecipitated with free KU70 (Figure 5C). We propose that the interaction with the MRN complex, which accumulates independently of KU70/KU80 at DBSs, may attract KU70 to the damaged DNA.

Conclusion and discussion

We observed that *Ku70*$^{-/-}$ and *DNA-PKcs*$^{-/-}$ ES cells behaved similarly to the DNA DSB causing agent ionizing radiation (IR). However, their response to an increase in single strand breaks resulting from PARP inhibition was quite different. *DNA-PKcs*$^{-/-}$ ES cells were hypersensitive to PARP inhibition, while *Ku70*$^{-/-}$ ES cells were not, suggesting that this phenomenon does not depend on an intact KU-DNA-PKcs complex. We provide some preliminary indications that KU70 may be involved in transmitting DNA damage signals to the apoptotic machinery via

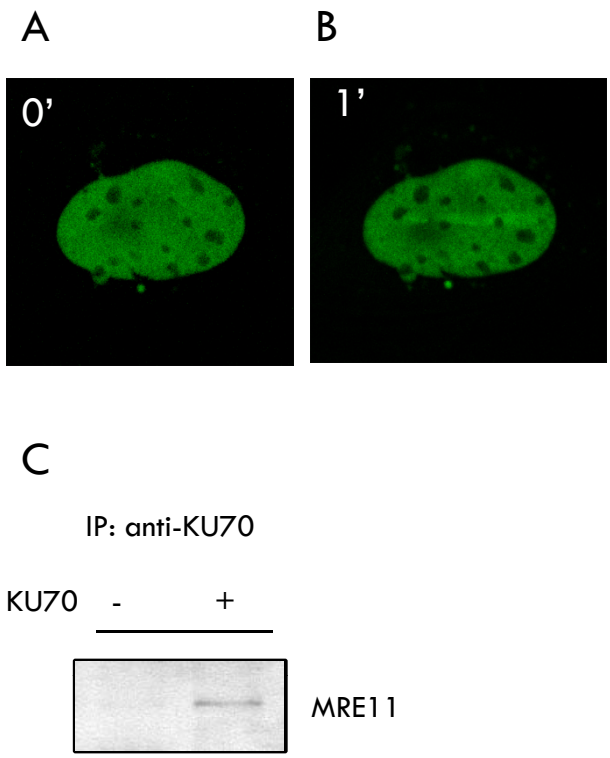

Figure 5: KU70 accumulation on laser-induced DNA damage and interaction with the MRN complex
YFP-KU70 accumulation in Ku70$^{-/-}$ Ku80$^{-/-}$ MEFs before (A) and 1 minute after (B) laser micro-irradiation. (C) Purified MRN complex with or without purified KU70 was immunoprecipitated with anti-KU70 and analyzed by Western blotting using MRE11 antisera.

the pro-apoptotic BAX protein, independently of KU80 or DNA-PKcs.
PARP inhibition is believed to cause SSBs, which can be converted to DSBs during replication. Such DNA breaks are not expected to require NHEJ for their repair, since only one DNA end is generated, which requires HR to restart the collapsed replication fork [2]. Interestingly, DNA-PK phosphorylates RPA2 after camptothecin treatment [34], which results in DNA damage critically linked to DNA replication [35]. However, DSBs in the lagging strand may not result in replication fork collapse and might be repaired by NHEJ. It is also possible that a (low number of) direct DSBs is formed upon PARP inhibition, for example by two closely opposing SSBs. Such lesions may require NHEJ for their repair, which could explain the (low level of) sensitivity of *DNA-PKcs*-deficient cells.
In this scenario, KU70 is necessary for DNA-PKcs activation, but possibly also for other functions (such as apoptosis), which may counteract the reduced survival caused by loss of NHEJ. This possibility is consistent with our finding that KU70 interacts with the pro-apoptotic BAX protein. Although the KU70/BAX interaction in the cytosol appears to be associated with inhibition of apoptosis induction, it is possible that nuclear BAX plays a role in transmitting the DNA damage signal to the apoptotic machinery. However, the sensitivity of *DNA-PKcs*$^{-/-}$ ES cells to PARP inhibition could also be explained by the lower amount of ATM in these cells [36], which can result in a decrease of HR. This decrease of ATM expression is independent of the DNA-PK

kinase activity, which is in line with the difference observed between *Ku70* and *DNA-PKcs* knock outs. Moreover, ATM deficiency also results in PARP inhibitor sensitivity [37].

We also found an interaction between KU70 and the MRN complex. As MRN accumulates at DNA ends independently of KU70/80, this direct interaction might attract KU70 (and BAX) to DSBs. It is possible that BAX is activated in an ATM-dependent fashion when it is attracted to the DNA damage. We conclude that KU70 might form the link between DSB repair and apoptosis after IR (Figure 6).

Alternatively, DNA-PKcs might function outside NHEJ and might be activated by other mechanisms than via KU70/80 stimulation. In this context, it is interesting that the C1D protein has been shown to interact with DNA-PKcs and to activate DNA-PKcs is a way that does not require DNA ends [38]. If this is the case, the KU80 or Ligase IV/XRCC4 function should not influence PARP inhibitor sensitivity, either.

The observation that both chromatid and chromosome type aberrations were increased in *Ku70* deficient cells, implies that KU70 (and therefore NHEJ) is important for DSB repair in both G1 and G2.

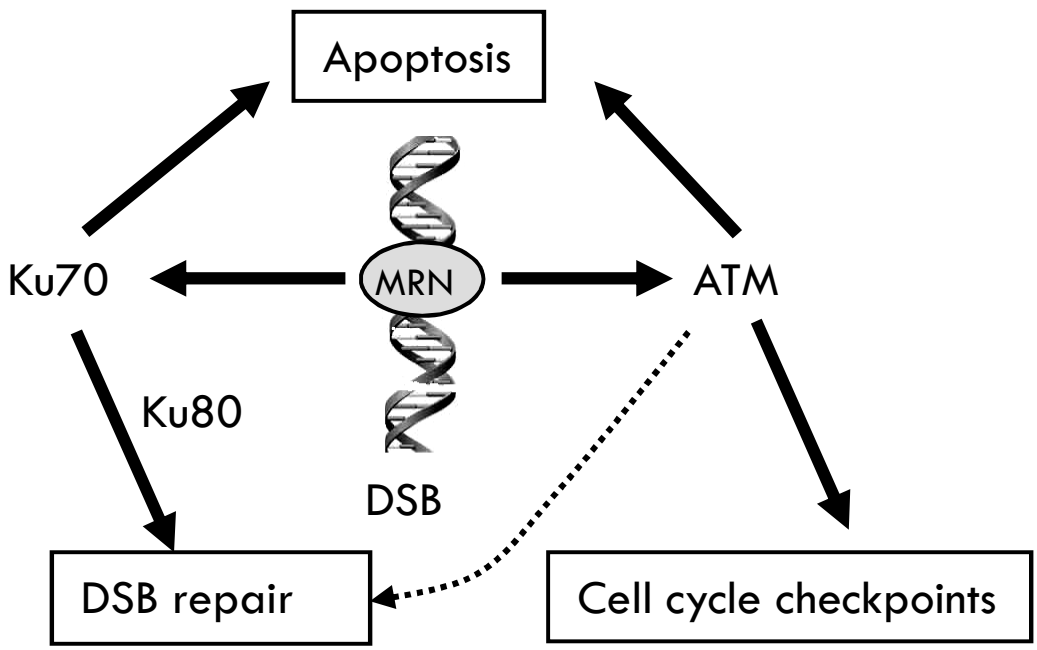

Figure 6: Proposed model for KU70 functioning in DSB repair and in apoptosis

Proposed model in which KU70 functions as a central player in DSB repair and apoptosis.

Materials and Methods

Survival analysis

Cellular clonogenic survival assays were performed in triplicate as described previously [39]. Cells were exposed to the specified dose of gamma rays from a 137Cs source at 0.8 Gy/min. For PARP inhibition survival assays, cells were incubated in medium containing the specified concentration of the KU58948 PARP inhibitor [40], ES cells were exposed continuously, and after 3 days the medium was replaced by fresh medium with the inhibitor. For all survival assays, the cells were allowed to grow for 10 days, stained and colonies containing more than 50 cells were counted. MEFs were cultures at 3% oxygen, ES cells were cultured at 20% oxygen.

Immunofluorescence assays

Cells were grown on glass coverslips, washed twice with PBS containing 0.05% Triton X-100, and fixed with 2% paraformaldehyde/0.025% Triton X-100 at 37°C. Coverslips were washed four times with PBS containing 0.1% Triton X-100 and once with PBS+ (PBS containing 0.15% glycine and 0.5% BSA). Cells were incubated at room temperature with primary antibodies to Bax (P-19, Santa Cruz Biotech) in PBS+ for 90 min at room temperature. After two washes with PBS with 0.1% Triton X-100 and one with PBS+, coverslips were incubated for 1 h with secondary antibodies in PBS+. Excess antibody was washed away by two 10-min washes with PBS/0.1% Triton X-100, and cells were mounted with Vectashield including DAPI (Vector Laboratories, Burlingame, CA). When indicated, cells were incubated for 1 h with mitotracker red prior to fixation and staining. Stainings were visualized using a LSM 510 NLO microscope (Carl Zeiss, Jena, Germany).

Life cell imaging

The YFP-KU70 expression construct was transfected into *Ku70*$^{-/-}$ *Ku80*$^{-/-}$ MEFs. DSBs were introduced by a pulsed near infrared laser system and YFP fluorescence was monitored in living cells, as described previously [32].

Protein purification and analysis

KU70 was expressed in a Baculovirus expression system and purified as previously described for the KU70/80 heterodimer [41]. The MRN complex was produced in insect cells by co-infection with Baculovirus vectors for the three components and the complex containing MRE11, RAD50 and NBS1 was purified essentially as described [42].

Immunoblot and immunoprecipitation assays were performed using polyclonal antibodies that specifically recognized KU70 (M-19) (Santa Cruz Biotech), BAX (P-19) (Santa Cruz Biotech), MRE11 [43], TBP (Santa Cruz Biotech) and a monoclonal antibody against GRB2 (BD Transduction Laboratories) was used as described previously [32]. Staining of mitochondria

was carried out with an antiserum directed against the mitochondrial F1-ATPase alfa-subunit (F1alfa, Molecular Probes).

Subcellular fractionation

Approximately 107 cells were homogenized with 0.2 ml of ice-cold homogenization buffer (250 mM sucrose, 20mM Hepes, pH7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA and 0.1 mM PMSF) and subcellular fractionation was performed as reported previously [44].

Metaphase spreads

MEFs were subjected to IR (1 Gy) and analyzed after 16 hr. Cells were fixed and metaphase spreads prepared by standard methods [45]. Colcemid is added 2 hours after irradiation. Fifty metaphases were scored for chromosomal aberrations. Chromatid type aberrations: aberrations restricted to a single chromatid, including gaps, breaks and radial structures. Chromosome type aberrations: aberrations involving both sister chromatids, including gaps and breaks.

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Chapter 5

NBS1 cooperates with homologous recombination during replication

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Manuscript in press DNA repair

Abstract

Nijmegen breakage syndrome (NBS) is characterized by genome instability and cancer predisposition. NBS patients contain a mutation in the NBS1 gene, which encodes the NBS1 component of the DNA double strand break (DSB) response complex MRE11/RAD50/NBS1. To investigate the NBS phenotype in more detail, we combined the mouse mimic of the most common patient mutation (*Nbs1*$^{\Delta B/\Delta B}$) with a *Rad54* null mutation, which diminishes homologous recombination. Double mutant cells were particularly sensitive to treatments that cause single strand breaks (SSBs), presumably because these SSBs can be converted into detrimental DSBs upon passage of a replication fork. The persistent presence of nuclear RAD51 foci and increased levels of chromatid type breaks in metaphase spreads indicated that replication associated DSBs are repaired inefficiently in the double mutant cells. Since neither homologous recombination nor cell cycle checkpoint activation is more defective in the double mutant than either single mutant, we suggest that NBS1 and RAD54 function cooperatively, but in separate pathways to counteract chromosomal instability as a result of replication problems.

Introduction

DNA repair is essential for the successful maintenance and propagation of genetic information. Endogenous and exogenous DNA damaging agents are constantly challenging the stability of DNA inside cells. Because a large variety of lesions occur in DNA, it is not surprising that multiple pathways have developed that each repair a subset of these lesions [1]. DNA double-strand breaks (DSBs) form a very genotoxic class of lesions [2,3]. Unrepaired DSBs can lead to cell death or loss of heterozygosity, whereas misrepaired DSBs may result in chromosomal rearrangements that contribute to carcinogenesis. Effective DSB repair is critical for maintaining genome stability. In eukaryotes, two main DSB repair pathways have been identified that differ in their requirements for DNA homology. Non-homologous end-joining (NHEJ) uses little or no sequence homology to rejoin broken ends in a manner that need not be error-free. Homologous recombination (HR) requires extensive regions of DNA sequence homology and repairs DSBs accurately using information on the undamaged sister chromatid.

HR involves a large number of proteins, including RAD51, RAD52 and RAD54 [4]. Upon DNA damage induction these proteins accumulate into nuclear foci which can be detected by immunofluorescence microscopy [5]. The importance of the HR proteins is underscored by the lethality imposed by disruption of *Rad51* [6,7]. However, *Rad54*$^{-/-}$ mice are viable and therefore provide a suitable model system to study the biological significance of a defect in the mammalian HR pathway [8]. *Rad54*$^{-/-}$ ES cells are sensitive to ionizing radiation (IR) and the interstrand crosslinking agent Mitomycin C (MMC) whereas the knockout mice are only MMC sensitive [8]. Furthermore, the *Rad54* deletion dramatically aggravates the IR sensitivity

of NHEJ mutant mice [9,10], showing that HR can function as a backup pathway in complete absence of NHEJ.

DSBs can occur in all phases of the cell cycle. Progression of the cell cycle is controlled by several checkpoints that prevent cell cycle progression when DNA damage has not been repaired [11,12]. DNA damage can prevent initiation of DNA replication (G1/S checkpoint), slow down S phase progression (intra-S checkpoint) or delay mitosis (G2/M checkpoint). The MRE11/ RAD50/NBS1 (MRN) complex is a central player in various aspects of the cellular response to DSBs, including HR, NHEJ and DNA damage checkpoint activation [12-15]. Mutations in NBS1 cause Nijmegen Breakage Syndrome, a human disorder characterized by microcephaly, IR hypersensitivity and predisposition to haematopoietic malignancy. An important function of NBS1 is the maintenance of the intra-S phase checkpoint, which is also a hallmark of Ataxia-telangiectasia (AT) cells. Wild type cells inhibit firing of new replication origins to prevent replication forks from running into DNA damage. AT and NBS cells inhibit DNA synthesis less efficiently after DNA damage, which can be observed as radioresistant DNA synthesis (RDS) [16]. The MRN complex activates the AT-mutated (ATM) kinase, which explains the similarity in cellular phenotypes of both syndromes [14,17]. In addition to the activation of ATM kinase, NBS1 also facilitates ATR-dependent phosphorylation [18]. ATM and ATR phosphorylate CHK2 and CHK1 respectively, leading to activation of the intra-S, G1/S and G2/M DNA damage induced checkpoints.

The role of Nbs1 in mammalian cells has been investigated in more detail using mice, which mimic the mutation that is found in most NBS patients [19]. The $Nbs1^{\Delta B/\Delta B}$ mice are IR sensitive and cells derived from these mice are sensitive to various DNA damaging agents, show increased levels of chromosomal aberrations after IR treatment and display cell cycle checkpoint defects [19]. We combined the *Rad54*$^{-}$ and $Nbs1^{\Delta B}$ mutations to get more insight into the genetic interactions of HR and the various functions of NBS1. We found that defective HR aggravates the sensitivity of $Nbs1^{\Delta B/\Delta B}$ cells to agents that can induce replication associated DSBs.

Cross	Expected	Born
$Nbs1^{\Delta B/+}Rad54^{+/-}$ X $Nbs1^{\Delta B/+}Rad54^{+/-}$	1/16 (6.25%)	1/61 (1.6%)
$Nbs1^{\Delta B/+}Rad54^{+/-}$ X $Nbs1^{\Delta B/+}Rad54^{-/-}$	1/8 (12.5%)	6/78 (7.7%)
$Nbs1^{\Delta B/\Delta B}Rad54^{+/-}$ X $Nbs1^{\Delta B/+}Rad54^{+/-}$	1/4 (25%)	3/46 (6.5%)
$Nbs1^{\Delta B/\Delta B}Rad54^{+/-}$ X $Nbs1^{\Delta B/+}Rad54^{-/-}$	1/4 (25%)	3/75 (4%)
overall	11%	5%

Table 1: Crosses to generate double mutant mice
The expected Mendelian frequencies and numbers of born pups are plotted for each cross.

Results

Generation of double mutant cells

NBS1 has been reported to have many different functions in the DNA damage response. We investigated the effect of combining the hypomorphic *Nbs1*$^{\Delta B}$ mutation with a knockout *Rad54* mutation. Both single mutants have no overt defects. The double mutant mice are present at the expected Mendelian frequency up to day 18 p.c. However, they were born at sub Mendelian frequencies (Table 1) and the mice that survived perinatal death had a reduced body weight (by approximately 15%). However, no abnormalities were detected in the gastrointestinal tract, lungs and skeleton and the animals developed without obvious defects, although they remained smaller than their wild type and single mutant littermates.

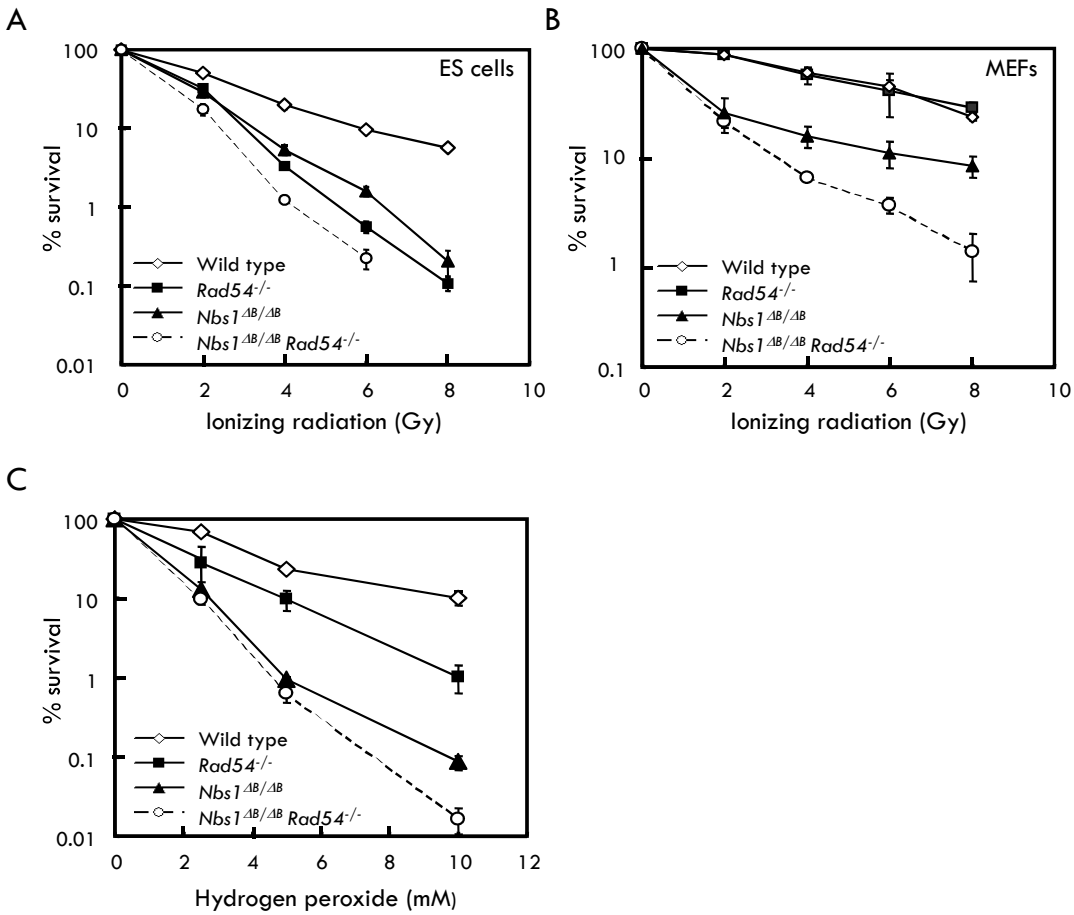

Figure 1: Sensitivities of double- and single mutants to DSB-inducing agents

(A) ES cell and (B) MEF colony survival assay for IR and (C) ES cells colony survival assay for H_2O_2. The doses of various genotoxic agents are displayed on the X-axis on a linear scale, while the percentage of surviving colonies is displayed on the Y-axis on a logarithmic scale. Error bars show the standard error of the mean for triplicate experiments.

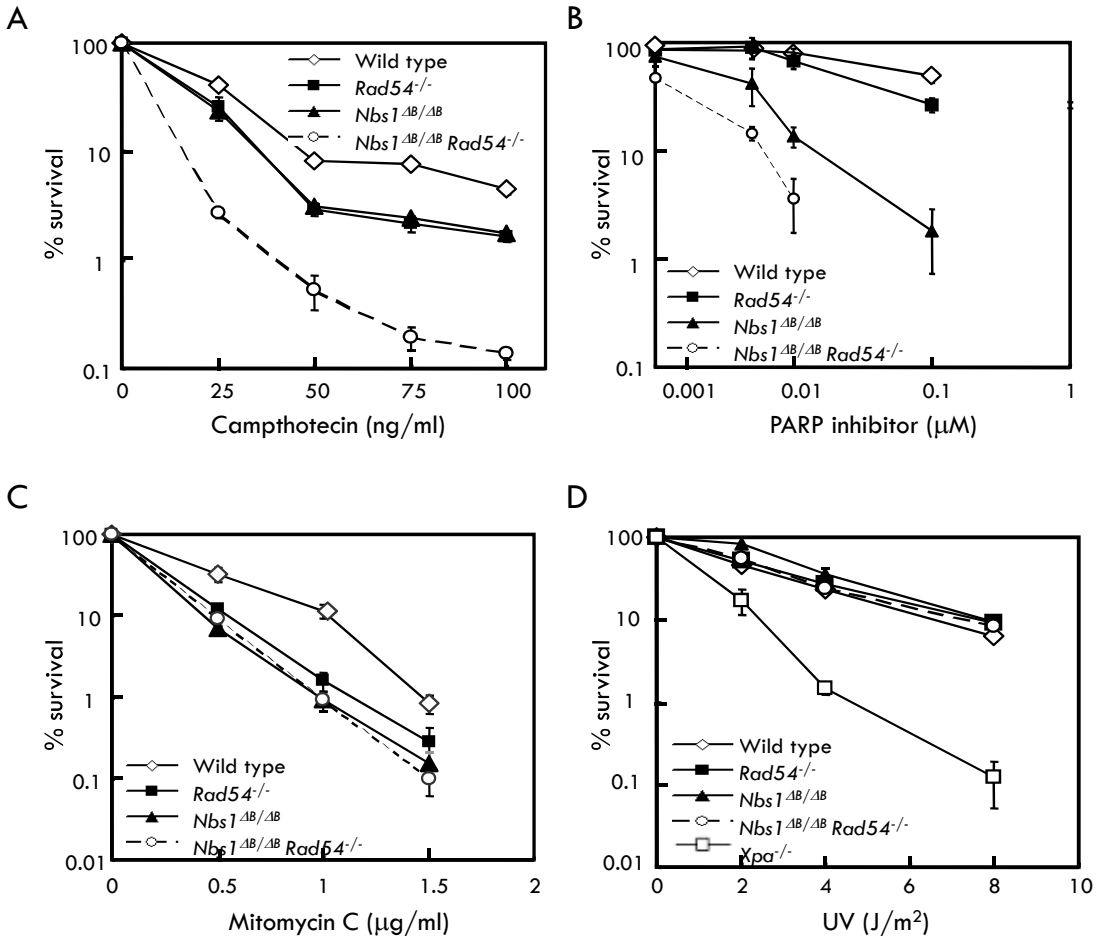

Figure 2: Sensitivities of single- and double mutants to various DNA damaging agents

Colony survival assays after treatment of ES cells with (A) CPT, (B) PARP inhibitor (KU58948) (C) MMC and (D) Ultra Violet (UV-C) irradiation in ES cells. The doses of various genotoxic agents are displayed on the X-axis on a linear scale, except for the PARP inhibitor, while the percentage of surviving colonies is displayed on the Y-axis on a logarithmic scale. Error bars show the standard error of the mean for triplicate experiments.

Mouse embryonic fibroblasts (MEFs) and embryonic stem (ES) cells were isolated and genotyped. The single and double mutant MEFs and ES cells showed normal cell viability, proliferation and morphology.

Analysis of sensitivities to DNA damaging agents

To get more insight into the genetic interactions of RAD54 and NBS1, we exposed the ES cells to various DNA damaging agents. We first tested treatments that can directly create DSBs: IR and hydrogen peroxide (H_2O_2). The *Nbs1*$^{\Delta B/\Delta B}$ and *Rad54*$^{-/-}$ single mutant ES cells both showed increased levels of IR sensitivity (Figure 1A). Double mutant ES cells were slightly more IR sensitive than the single mutants, suggesting that NBS1 and RAD54 function in different

pathways to counteract the deleterious effects of IR. To test whether the sensitivity found in the double mutant ES cells is cell type specific we also analyzed MEFs. *Rad54*$^{-/-}$ MEFs were not IR sensitive, whereas *Nbs1*$^{\Delta B/\Delta B}$ were [19]. The double mutant MEFs were also hypersensitive to IR, showing that the additive effect of *Nbs1* and *Rad54* deficiency is not ES cell specific (Figure 1B).

Rad54$^{-/-}$ and *Nbs1*$^{\Delta B/\Delta B}$ ES cells also showed increased sensitivity to H_2O_2 (Figure 1C). Interestingly, the *Nbs1*$^{\Delta B/\Delta B}$ cells were more sensitive than the *Rad54*$^{-/-}$ cells, suggesting that NBS1 is especially important to counteract H_2O_2 induced DNA lesions. H_2O_2 causes mainly SSBs, which can be converted to DSBs during replication [20]. Apparently, NBS1 is required to counteract the negative effect of these lesions. The double mutant cells were only slightly more sensitive to H_2O_2 than the *Nbs1* single mutant, indicating that NBS1 has a more important role in counteracting this type of SSBs than RAD54.

To investigate the effect of S phase specific DSBs in more detail, we used camptothecin (CPT), which acts as an inhibitor of topoisomerase 1 and results in stable covalent DNA – topoisomerase complexes. CPT-induced breaks can be converted to DSBs by advancing DNA polymerases during replication [21]. CPT treatment was given for 24 hours to act on all cells in S phase. Clonogenic survival experiments indicated that *Nbs1*$^{\Delta B/\Delta B}$ and *Rad54*$^{-/-}$ ES cells were equally sensitive, whereas the double mutant cells were much more sensitive than the single mutants (Figure 2A). The double mutant cells showed a much more pronounced hypersensitivity to CPT than to IR treatment, suggesting that double mutant cells are especially deficient in repairing S phase specific DSBs.

As an alternative approach to increase the number of replication associated DSBs, we used the PARP inhibitor KU58948, which causes increased levels of SSBs [17,22]. The PARP inhibitor creates a SSB or a single-strand interruption in the template DNA. When the replication fork approaches this SSB it collapsed and another replication fork has to reach this remaining single-strand interruption from opposite direction. After the second replication fork has reached the interruption and collapsed, this results in a DSB in one of the daughter chromosomes [23].

The *Nbs1* mutant ES cells were much more sensitive to this treatment than *Rad54* deficient cells, suggesting that replication associated DSBs may pose a particularly severe problem to cells with diminished function of the MRN complex. Interestingly, the PARP inhibitor caused an additive sensitivity in the double mutant cells, suggesting that RAD54 and NBS1 function in different pathways that counteract replication associated DSBs (Figure 2B).

Subsequently, ES cells were exposed to the interstrand crosslinking (ICL) agent MMC, because HR is involved in the repair of these types of lesions. The *Nbs1*$^{\Delta B/\Delta B}$, *Rad54*$^{-/-}$ and double mutant cells showed similar levels of sensitivity (Figure 2C), suggesting that both genes function in the same pathway of ICL repair.

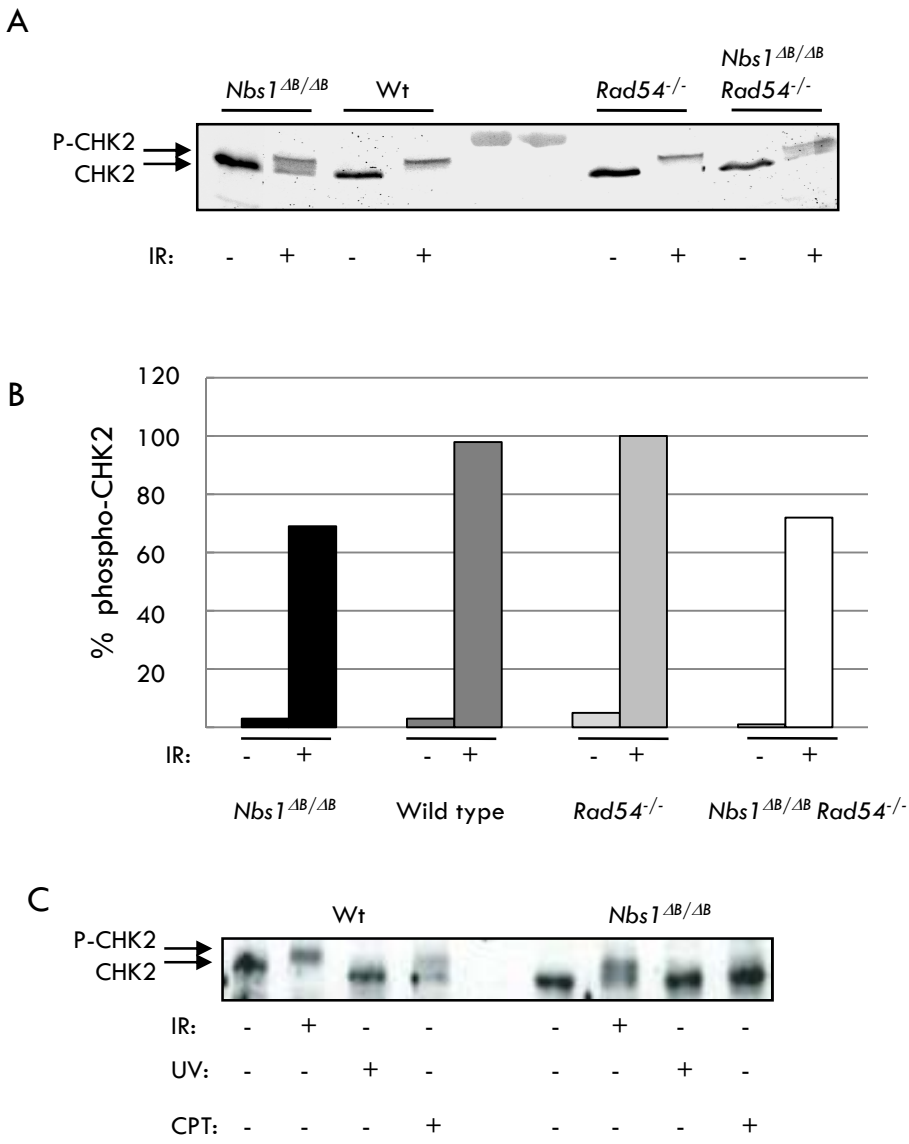

Figure 3: CHK2 phosphorylation after DSB induction depends on NBS1
Western blot analysis with CHK2 antibodies (A) 1 hour after gamma irradiation with 12 Gy and (B) 1 hour after gamma irradiation with 12 Gy, after 24 hour CPT treatment (10 μM) or 1 hour after UVC irradiation (20 J/m^2).

Repair of intrastrand crosslinks was assayed by ultraviolet (UV) irradiation, which causes pyrimidine dimers that can be repaired via nucleotide excision repair. The *Nbs1*, *Rad54* and double mutant cells did not show hypersensitivity to UV-light (Figure 2D).

CHK2 phosphorylation depends on NBS1 after IR and CPT treatment

NBS1 is important for cell cycle checkpoint control through activation of ATM. Therefore, we

studied CHK2 phosphorylation by ATM, which is thought to be NBS1-dependent after IR treatment [24,25]. All CHK2 was hyperphosphorylated in IR treated wild type and *Rad54*$^{-/-}$ cells. However, CHK2 phosphorylation was markedly reduced in the *Nbs1*$^{\Delta B/\Delta B}$ cells and double mutant cells (Figure 3A).

CPT also induced CHK2 phosphorylation, suggesting that DSBs activate ATM after this treatment (Figure 3B). In *Nbs1*$^{\Delta B/\Delta B}$ cells, we did not observe any CHK2 phosphorylation after CPT treatment, showing that signalling from replication associated DSBs via CHK2 is fully dependent on NBS1 function. *The Rad54*$^{-/-}$ cells showed wild type levels of CHK2 phosphorylation after CPT treatment whereas the double mutant cells showed the same pattern as the *Nbs1*$^{\Delta B/\Delta B}$ cells (data not shown). We conclude that RAD54 does not influence ATM dependent signalling.

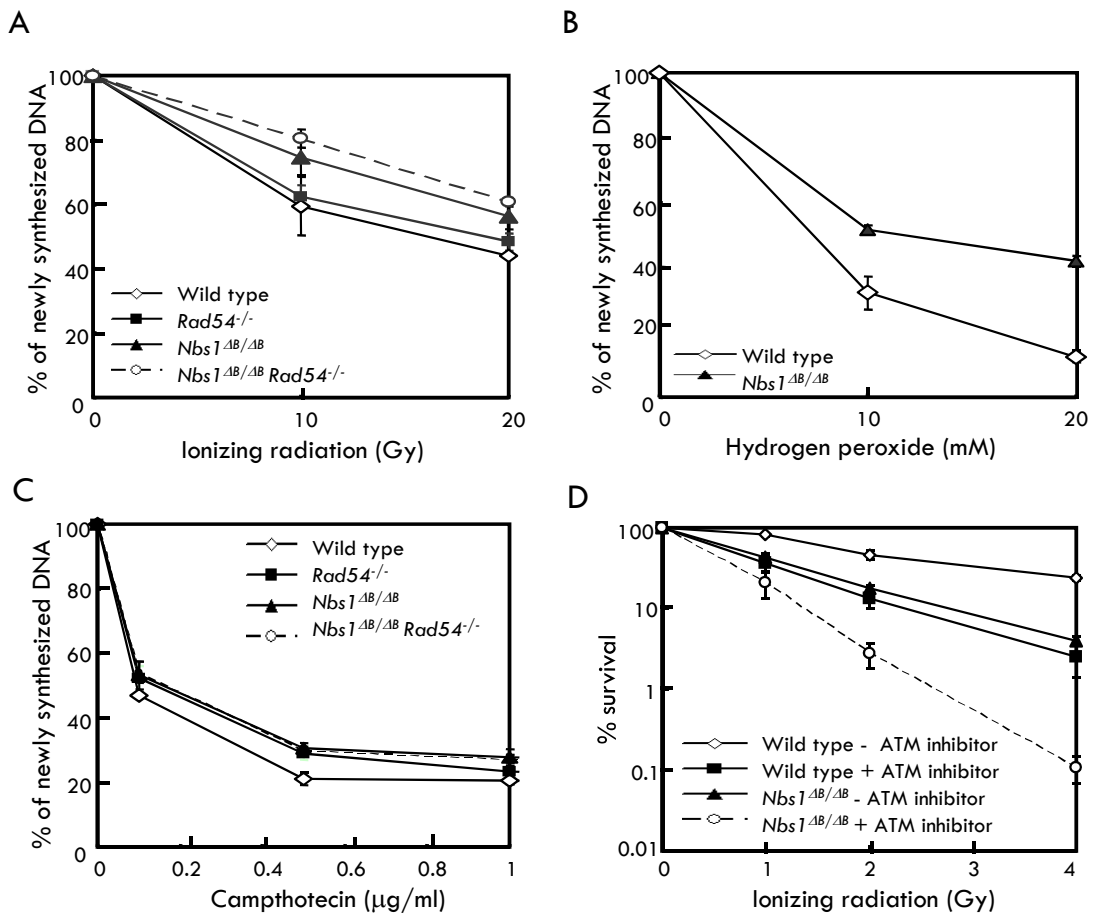

Figure 4: Inhibition of DNA synthesis after DNA damage

The relative level of 3H-Thymidine incorporation was measured after (A) γ-irradiation, (B) H_2O_2 treatment and (C) 1 hr of CPT treatment (D) ES cells colony survival assay for IR after ATM inhibition. The doses of IR are displayed on the X-axis on a linear scale, while the percentage of surviving colonies is displayed on the Y-axis on a logarithmic scale. Error bars show the standard error of the mean for triplicate experiments.

Therefore, we investigated whether the intra-S phase checkpoint defects in *Nbs1*$^{\Delta B/\Delta B}$ cells might explain the additive effects, observed in the survival assays with *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ cells.

Intra-S phase checkpoint

NBS1 dependent phosphorylation events are important to establish the IR-induced intra-S phase checkpoint. We confirmed that wild type and *Rad54* mutant cells down regulated DNA synthesis after IR treatment, whereas *Nbs1*$^{\Delta B/\Delta B}$ and double mutant cells showed a less pronounced decrease of DNA synthesis under these conditions (Figure 4A). We observed a similar difference between wild type and N*bs1*$^{\Delta B/\Delta B}$ cells after H_2O_2 exposure (Figure 4B). However, 1 hr CPT treatment, showed little or no difference in DNA synthesis rate between wild type and Nbs1 mutant cells (Figure 4C). The induced radiosensitivity can partially explain the intra-S phase problem in *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ cells, but the CPT sensitivity cannot. Therefore, the various sensitivities to DNA damaging agents cannot exclusively be explained by the defect in the intra-S phase checkpoint. For that reason, we sought for an additional defect and we investigated whether the NBS1 defect was epistatic with ATM activity. *Nbs1*$^{\Delta B/\Delta B}$ cells and wild type cells were treated with the ATM inhibitor KU55933 and colony survival after IR treatment was determined [26,27]. Interestingly, both the Nbs1$^{\Delta B/\Delta B}$ and wild type ES cells became more IR-sensitive after treatment with the ATM inhibitor (Figure 4D). *Nbs1*$^{\Delta B/\Delta B}$ cells were more sensitized to the ATM inhibitor compared to wild type cells, indicating an additional ATM-independent function of NBS1. The ATM inhibitor did not cause hypersensitivity of AT fibroblasts (data not shown) excluding off target effects of the inhibitor.

Homologous targeting efficiency is not affected in *Nbs1* mutant cells

Subsequently, we investigated whether a decreased HR capacity could explain the excess residual DSBs in the double mutant cells. We determined the HR levels in ES cells with a homologous gene-targeting assay, using a promoterless RAD54 GFP knock-in construct [28]. GFP is only expressed upon correct targeting into the Rad54 locus and not after random integration. *Nbs1*$^{\Delta B/\Delta B}$ and wild type ES cells showed a similar targeting efficiency (Figure 5), indicating that HR was not affected by the *Nbs1*$^{\Delta B}$ mutation. The targeting efficiency in R*ad54*$^{-/-}$ and double mutant cells was reduced to similar levels (5% and 8%, respectively), suggesting that the repair defect in double mutant cells could not be explained by a general defect in HR.

Persistent DSBs in double mutant cells

The sensitivity of the double mutant cells after IR and H_2O_2 treatment could in principle be explained by a combination of a HR defect, caused by RAD54 deletion and an intra-S phase checkpoint defect. However, the extreme sensitization by ATM inhibition in *Nbs1*$^{\Delta B/\Delta B}$ cells compared to wild type cells suggested that NBS1 has additional, ATM-independent functions

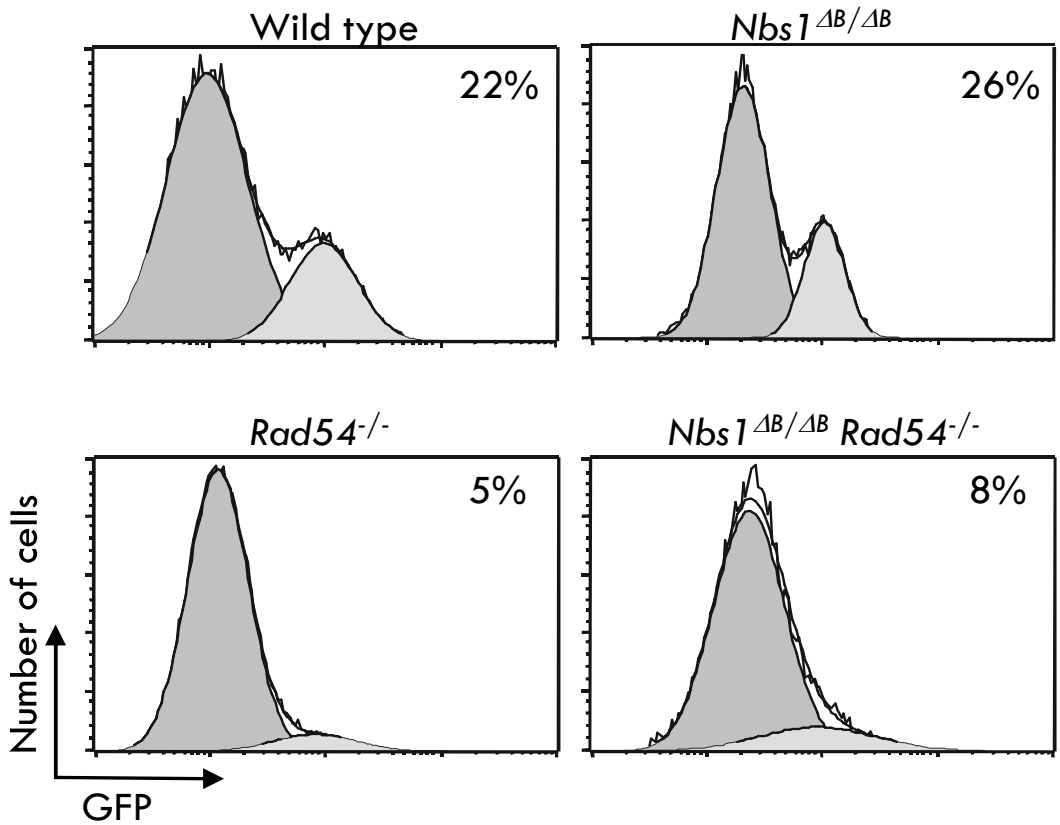

Figure 5: Homologous gene targeting does not depend on NBS1

Cells of the indicated genotypes were electroporated with the mRAD54-GFP knock-in construct that results in GFP expression after homologous gene targeting, but not after random integration. GFP expression was analyzed by flow cytometry after seven days of antibiotic selection. The calculated targeting efficiency is displayed for each experiment.

in the DNA damage response. Therefore, we investigated the accumulation of RAD51, which accumulates in subnuclear structures (foci) as a marker for sites of HR. Interestingly, untreated double mutant MEFs showed a 5-fold higher level of RAD51 foci positive cells than the wild type or the single mutant MEFs, indicating that endogenous DNA damage levels were increased in these cells (Figure 6A). γ-irradiation induced RAD51 foci in wild type and mutant MEFs. The highest percentage of foci positive cells was observed 2 hours after irradiation and then decreased over the next 24 hours to the low level observed before irradiation (Figure 6B). However, the RAD51 foci in double mutant MEFs persisted for a longer period of time and even after 24 hours the number of RAD51 foci had not returned to pre-irradiation levels. Spontaneous RAD51 foci were also increased in double mutant ES cells, indicating that this phenomenon is not cell type specific (Figure 6C).

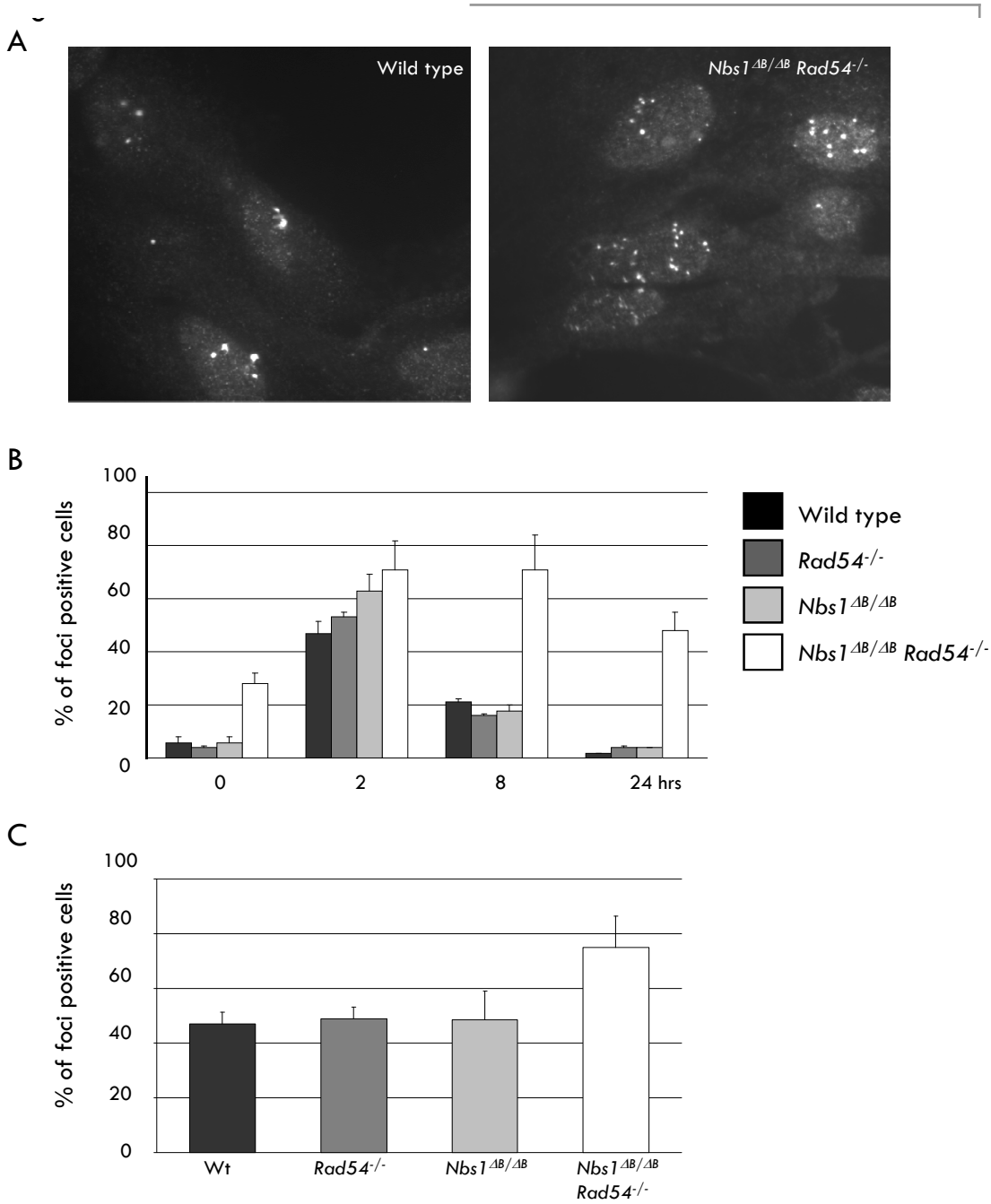

Figure 6: RAD51 foci persist in double mutant MEFs

(A) RAD51 foci formation in untreated MEFs of the indicated genotype. (B) Quantification of RAD51 focus formation at 0, 2, 8 and 24 hours after 2 Gy γ-ray irradiation in MEFs. (C) Spontaneous foci formation in ES cells. At least 75 cells were counted for each genotype in 3 independent experiments. Cells with 5 or more foci were scored as foci positive cells.

Persistent RAD51 foci are indicative of persistent DNA damage, which predicts that *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ cells should display a high incidence of chromosomal aberrations. Therefore, we made metaphase spreads of wild type and single mutant MEFs and ES cells (Table 2). Interestingly, double mutant cells exhibited a much higher level of spontaneous chromosomal instability than wild type or single mutant cells. The majority of these aberrations affected only one chromatid, which is consistent with the idea that the most deleterious type of damage for the double mutant cells is a SSB that is converted to a DSB in S phase, which frequently remains unjoined and finally can lead to cell death.

Cell type	Genotype	Metaphases analyzed	Aberrant metaphases (%)	Chromatid type aberrations (%)	Chromosome type aberrations (%)
ES cells	Wild type	58	3	1.5	1.5
	Rad54$^{-/-}$	60	3	1.5	1.5
	Nbs1$^{\Delta B/\Delta B}$	60	7	2	5
	Nbs1$^{\Delta B/\Delta B}$*Rad54*$^{-/-}$	60	13	8	5
pMEFs	Wild type	22	0	0	0
	Rad54$^{-/-}$	20	6	0	6
	Nbs1$^{\Delta B/\Delta B}$	20	0	0	0
	Nbs1$^{\Delta B/\Delta B}$*Rad54*$^{-/-}$	38	29.5	22	7.5
tMEFs	Wild type	56	2	0	2
	Rad54$^{-/-}$	52	0	0	0
	Nbs1$^{\Delta B/\Delta B}$	70	1	0	1
	Nbs1$^{\Delta B/\Delta B}$*Rad54*$^{-/-}$	55	60	51	20

Tabel 2: double mutant cells acquire spontaneous chromosomal aberrations
Chromatid type aberrations: aberrations restricted to a single chromatid, including gaps, breaks and radial structures. Chromosome type aberrations: aberrations involving both sister chromatids, including gaps and breaks. ES cells: embryonic stem cells, pMEFs: primary MEFs (MEF cultures up to passage 8), tMEFS: transformed MEFs (MEF cultures from passage 8 on).

Discussion

The MRN complex has multiple functions in the maintenance of chromosomal stability. We investigated how the *Nbs1*$^{\Delta B}$ mutation, which mimics the mutation found in NBS patients, affects genome stability and how this phenotype is influenced by a defect in HR (*Rad54*$^{-/-}$). We found that the double mutant cells are hypersensitive to many DNA damaging agents and show a high

level of chromosomal instability, even in an unchallenged situation.

We considered several possible reasons for the synergistic effects in *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ cells. Nbs1 mutations have been found to compromise the intra-S checkpoint, both in patient cells and in mouse cells. This has been interpreted as a defect in signaling to the replication machinery that should inhibit initiation of new replicons during S phase. We therefore reasoned that the hypersensitivity to various agents might be caused by such a signaling defect. However the hypersensitivity of double mutant cells to CPT could not easily be explained by such a defect: although CHK2 phosphorylation was decreased in *Nbs1*$^{\Delta B/\Delta B}$ cells this did not result in a measurable intra-S phase checkpoint defect. Therefore, we also considered other possible explanations for the observed hypersensitivities. From our analysis, it is clear that agents that induce S phase specific DSBs (CPT and PARP inhibitor) cause the highest level of hypersensitivity in the double mutant cells. This leaves three possible explanations: (1) NBS1 could signal from DSBs to the nearby replication fork, which might be necessary for proper restart of replication, especially when HR is compromised, or (2) NBS1 could prevent formation of DSBs from SSBs during S phase or (3) NBS1 might be necessary for the DSB repair reaction itself.

The first two possibilities would both result in increased formation of DSBs. This signaling could take place after DSB formation by a traversing replication fork, or one could imagine that a signal is generated from a SSB that would prevent DSB formation by halting the replication fork until the damage has been repaired. It is difficult to discriminate these two possibilities. In both scenarios one would expect an increase in persistent DSBs and chromatid type chromosomal aberrations in the double mutant cells. The classic signaling pathway that involves NBS1 is via ATM kinase activation, resulting in an impaired CHK2 phosphorylation. However, MRN dependent activation of the ATR kinase activity after replication fork stalling or UV irradiation has also been reported [18], suggesting that non-DSB type lesions may also require the MRN complex for efficient detection and/or signaling. Interestingly, depletion of the MRN complex from xenopus egg extracts caused many DSBs during replication [29]. A similar phenomenon was found after caffeine treatment, which inhibits both ATM and ATR kinases, or after depletion of these two protein kinases, suggesting that DSB formation or a lack of repair can result from an inability to activate the ATM and ATR kinases [29]. Both possibilities are not mutually exclusive and may contribute to the chromosomal instability observed in *Nbs1*$^{\Delta B/\Delta B}$ and double mutant cells.

As a third possibility we considered that NBS1 may be required for DSB repair by HR and/or NHEJ. Previously, combined defect in HR and NHEJ was found to result in synergistic effects in mice and cells [9,10,30]. We excluded a role for NBS1 in homologous targeting, suggesting that the core HR functions are still intact. However, we cannot exclude that NBS1 is involved in a subpathway of HR that is important for replication-associated DSB repair. Although the increased IR sensitivity in double mutant MEFs might be explained by NBS1-related NHEJ defects, this does not explain the high level of hypersensitivity for agents that mainly cause DSBs

associated with S phase progression. Therefore, we suggest that the involvement of NBS1 in the direct mechanisms of HR or NHEJ is not a likely explanation for the phenotype of the double mutant cells. However, the MRN complex may have a function in keeping DNA ends together [31]. Moreover, it is notable that the MRN complex forms foci after various DSB inducing treatments, independently of other DSB repair proteins [32]. The MRN complexes interact via a Zinc-hook at the tip of their RAD50 subunit and can bridge two DNA ends [33,34]. This function might help to keep the DSB together until the HR (or NHEJ) repair machinery has properly aligned the DNA ends for the actual joining reaction. Since the NBS1 mutation does not support MRN foci formation, the microenvironment of DNA ends may have changed in such a way, that the ends may much more readily loose juxtaposition and therefore remain unrepaired. Obviously, this phenotype would be enhanced when HR is compromised. Although both DNA damage signaling and a changed microenvironment of the DSB may contribute to the various sensitivities of *Nbs1*$^{\Delta B/\Delta B}$ cells, we consider its function in holding the DNA ends in close proximity most important for sensitivity to replication associated DSBs.

Materials and Methods

Mouse breeding and cell culture

To investigate the effect of combined *Rad54* and *Nbs1* mutations in mice, we set up crosses to generate *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ double mutant mice. Because *Rad54*$^{-/-}$ mice are fully fertile and viable, [8] we used both *Rad54*$^{-/-}$ and *Rad54*$^{+/-}$ mice in the crosses. Since, *Nbs1*$^{\Delta B/\Delta B}$ females are subfertile we used *Nbs1*$^{\Delta B/+}$ females for the crosses [19].

Primary MEFs were obtained from embryos at E13.5 by cell dispersal following removal of organ block tissue. Cells were cultured in DMEM/Ham F10 1:1 medium supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml) in a humidified 37°C incubator maintained at 3% O_2 and 5% CO_2.

ES cells were isolated at E3.5 [10]. ES cells were cultured on gelatin coated dishes in a 1:1 mixture of DMEM and Buffalo Rat Liver (BRL) conditioned medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml)/streptomycin (100 μg/ml), 0.1 mM non-essential amino acids, 50 μM β-mercaptoethanol and 500 U/ml leukemia inhibitory factor.

Homologous targeting assay

The efficiency of homologous recombination was assessed by homologous gene targeting experiments to the *Rad54* locus. Targeting of the locus by RAD54-GFP was detected by FACS analysis of GFP expression in ES cells containing the targeted locus as described previously [28].

DNA damage sensitivity assays

Cellular clonogenic survival assays were performed in triplicate as described previously [8]. For UV and IR survival assays, cells were exposed to the specified dose of UV-C light or gamma rays from a 137Cs source at 0.8 Gy/min. For MMC survival assays, cells were incubated in medium containing the specified concentration of MMC for one hour. In the case of CPT, cells were exposed for 24 hours. The cells were then washed with phosphate-buffered saline and replenished with fresh medium. Hydrogen peroxide was added at the indicated concentration; this compound was not removed by changing the media. In the case of the KU58948 PARP inhibitor [35], ES cells were exposed continuously, and after 3 days the medium was replaced by fresh medium. The PARP inhibitor survival is plotted on a double logarithmic scale. For all survival assays, the cells were allowed to grow for 10 days, stained and colonies containing more than 50 cells were counted.

Wild type, *Nbs1*$^{\Delta B/\Delta B}$ ES cells, wild type and VH10-NE13 AT-fibroblasts were exposed to 10μM KU55933 ATM inhibitor [26,27] and after 30 minutes exposed to IR. Survival analysis was performed as described above.

Immunofluorescence and Western blotting

Immunostaining to detect RAD51 (α-hRAD51 nr. 2307, rabbit polyclonal antibody) [36] was performed as described previously [5]. Cell wells were counted positive by 5 of more foci. CHK2 phosphorylation was detected by SDS-PAGE separation of whole cell extracts and Western blotting using mouse monoclonal antibodies to CHK2 (BD Transduction).

Cytogenetic analysis

Frequencies of spontaneous and γ-ray-induced chromosomal aberrations were determined in exponentially growing cell cultures as described [37].

Radioresistant DNA synthesis assay

The RDS assay was performed essentially as described [38] but without the TCA-precipitation step. In short, duplicate 30 mm dishes (four for the unirradiated control) were prelabeled overnight with [14C]-thymidine (Amersham) in HEPES-buffered ES cell medium, then exposed to various doses of -rays using a 137Cs source (0.8 Gy/min), or treated with CPT for 1 hr, and subsequently labeled with [3H]-thymidine (Amersham) for 2 hrs. Free thymidine pools were chased by a further 30–45 min incubation in medium containing unlabeled thymidine. Scintillation-counted [3H] to [14C] radioactivity ratios of alkali-lysed cells were taken as a measure of DNA synthesis rates and plotted as percentages of unirradiated cells. The RDS assay with IR was performed 4 times and it was performed twice with CPT treatment.

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Chapter 6

Cooperative roles for NBS1 and RAD54 in meiosis

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Manuscript in preparation

Abstract

Nijmegen Breakage Syndrome (NBS) is an autosomal recessive disorder that results from a genetic defect in the *Nbs1* gene. The NBS1 protein functions as an early sensor of DNA double-strand breaks (DSBs). The NBS defect leads to a diverse clinical phenotype, including female infertility. We used a mouse model, which mimics the NBS mutation (*Nbs1*$^{\Delta B/\Delta B}$), to investigate the role of NBS1 in meiosis. The number of primordial and primary follicles is reduced in the ovaries of young *Nbs1*$^{\Delta B/\Delta B}$ females and this resulted in loss of all follicles in adult animals (18-weeks-old). *Nbs1*$^{\Delta B/\Delta B}$ males had normal fertility, indicating that the germ cell maturation defect in *Nbs1*$^{\Delta B/\Delta B}$ females was not caused by a general failure of meiosis. The *Nbs1*$^{\Delta B}$ mutation was crossed onto a *Rad54*-deficient background to determine the effect of an additional homologous recombination defect in meiosis. *Rad54* deficiency exacerbated the meiotic *Nbs1*$^{\Delta B/\Delta B}$ phenotype: the number of apoptotic cells in *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ ovaries increased and the males had smaller testes compared to wild type, but remained fertile. Still, large stretches, rather than transient foci, of RAD51 protein were observed in male meiotic prophase nuclei, indicating that meiotic DSB-repair may be affected. The early loss of primordial and primary follicles suggests that the oocytes either fail to arrest in prophase, or that female meiotic recombination is fundamentally different from male meiosis.

Introduction

MRE11-RAD50-NBS1 (MRN) is the mammalian homolog of the yeast Mre11-Rad50-Xrs2 complex. In yeast, the MRX complex plays at least two roles in meiotic recombination: it is required for the introduction of meiosis-specific DSBs by the SPO11 endonuclease and it is essential for the removal of SPO11 from the DNA followed by the resection of the broken DNA ends [1]. The MRN complex also plays a role in double-strand break (DSB) repair in somatic cells. Homologous recombination (HR) is an important form of DSB repair. Germ cells and embryonic stem cells rely more on HR for DSB repair than somatic cells [2], probably because more precise repair is required in these cells. NBS1, which is involved in HR has a sensing role for DSBs [3]. RAD51 is involved in the search for homology and strand pairing stages of the process [4]. RAD54 is probably involved in multiple steps in HR [4]. Although HR is indispensable for viability, mice lacking RAD54 are born, viable and fertile. Still, *Rad54*$^{-/-}$ mice show reduced recombination capacity and are hypersensitive to the crosslinking agent Mitomycin C (MMC), which can be explained by this HR defect [5].

Mutations in the NBS1 gene cause Nijmegen breakage syndrome (NBS), which is characterized by microcephaly, increased sensitivity to ionizing radiation (IR) and predisposition to haematopoietic malignancy. A null mutation in Nbs1 leads to embryonic lethality in mice [6, 7] and only animals with hypomorphic mutations survive past early embryogenesis [8, 9]. The

Nbs1$^{\Delta B/\Delta B}$ mice mimic the patient mutation and have a mild phenotype; they are IR sensitive and cells derived from these mice are sensitive to various DNA-damaging agents, show increased levels of chromosomal aberrations after IR, and display DNA damage checkpoint defects [9]. DSBs can be visualized as γH2AX or RAD51 foci. MRN localizes to DSBs in response to IR [10] and subsequently activates ATM. This protein kinase is defective in Ataxia Telangiectasia (AT) patients and is required for the activation of the intra-S and G2/M checkpoints [11].

Female NBS patients and mice carrying *Nbs1* mutations show decreased fertility, indicating that NBS1 plays a role in meiosis. Meiosis is divided in two stages; meiosis I is a reductional division which consists of a prophase with the leptotene, diplotene, zygotene, pachytene and diakinesis stages, followed by metaphase I, anaphase I and telophase I. During prophase the duplicated chromosomes pair with their homologues, a process called synapsis. During this phase, a protein "zipper", the synaptonemal complex (SC), holds the homologous chromosomes tightly together along the chromosomal axes. SCs are assembled from two axial elements, one along each homolog, which are connected by numerous transverse filaments. When the SC disappears in late prophase, chiasmata can be observed. Meiosis II is the second part of the meiotic process, resulting in the germ cells.

Although the mechanism of meiotic recombination is largely similar in males and females, its timing is very different. In females, all oogonia enter meiotic prophase during fetal development and oocytes enter dictyate arrest just prior to metaphase I. This meiotic block continues until sexual maturation, when during each estrous cycle, the trigger for ovulation allows the oocytes in the dominant follicles to resume meiosis [12]. They complete the first meiotic division, extrude the first polar body and arrest again at metaphase II until fertilization. In contrast, male meiosis is an entirely postnatal event [12], which starts at puberty and continues throughout life. It is noteworthy that the dictyate arrest in prophase and the arrest in metaphase II are absent in the male germ line [12].

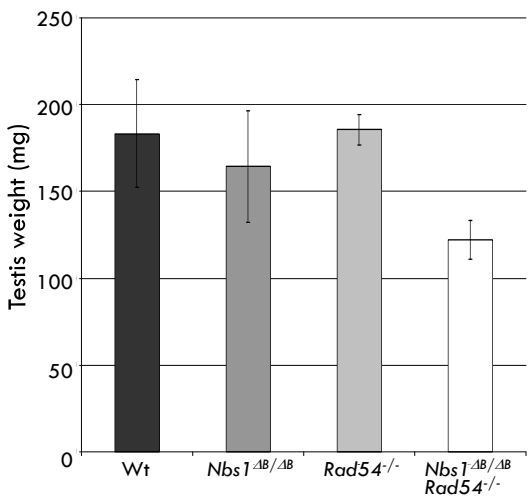


Figure 1: Analysis of testis weight
Testis weight of *Rad54*$^{-/-}$, *Nbs1*$^{\Delta B/\Delta B}$ and double mutant *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ in 3-month-old males (n=2). Error bars represent the standard error of mean.

In this chapter we describe the meiotic phenotype of *Rad54*$^{-/-}$, *Nbs1*$^{\Delta B/\Delta B}$ and double mutant *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ mice. *Nbs1*$^{\Delta B/\Delta B}$ females suffer from premature ovarian failure, caused by postnatally disturbed folliculogenesis. *Nbs1*$^{\Delta B/\Delta B}$ males have normal testes and are fertile. The HR defect of RAD54 on top of the *Nbs1*$^{\Delta B/\Delta B}$ mutation exacerbates the defect in females as well as in males.

Results

Analysis of the double mutant testis in mice

Previously, the MRN complex has been reported to have a function in meiosis [1, 13]. Therefore, we first analyzed male meiosis in the testis in detail. Male mice of all genotypes (*Nbs1*$^{\Delta B/\Delta B}$, *Rad54*$^{-/-}$ and double mutant) were fertile. However, the testis weight of *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ males was reduced compared to the wild type and the single mutants (Figure 1), suggesting that male germ cell spermatogenesis was affected in these animals.

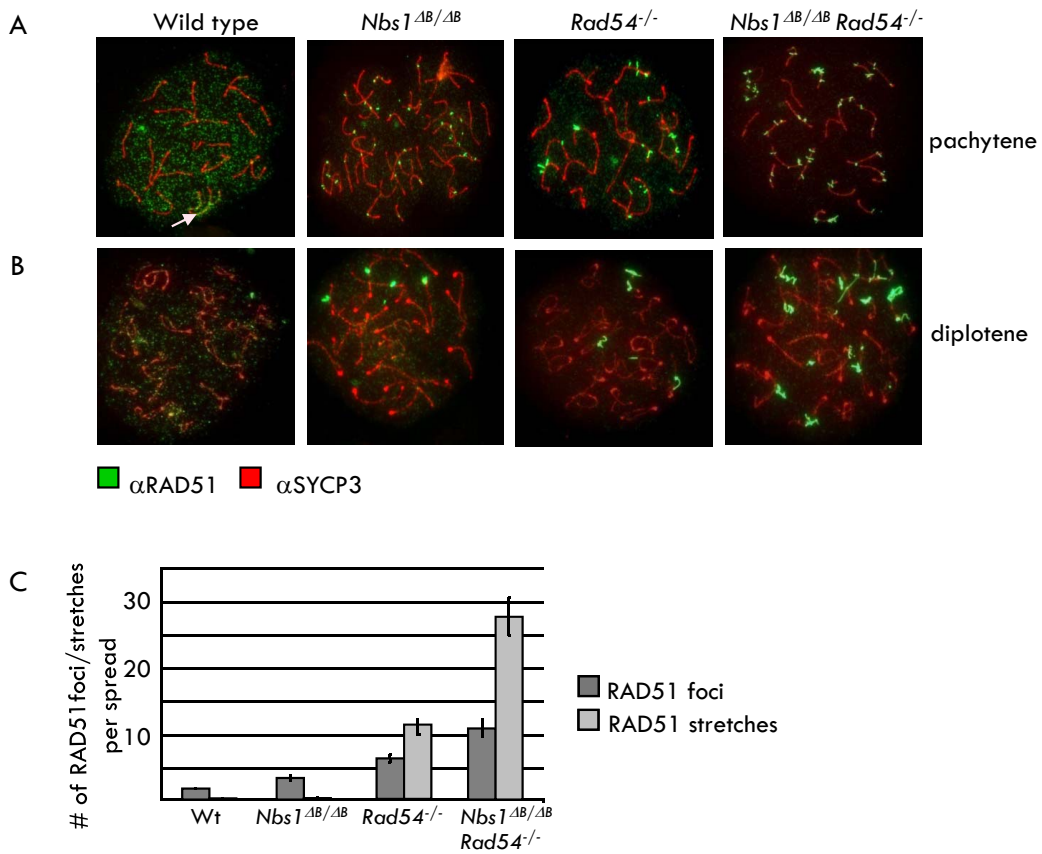


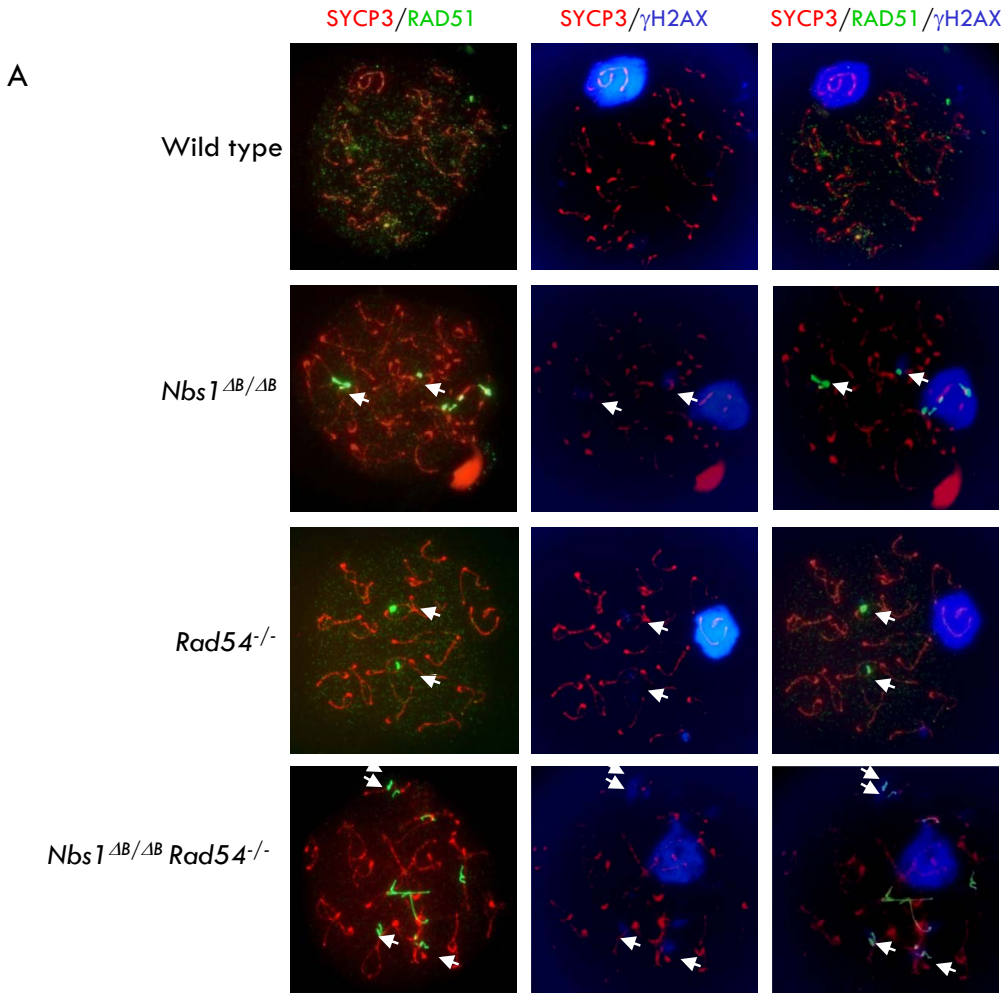
Figure 2: SYCP3 and RAD51 analysis in spermatocyte spreads

(A) late pachytene and (B) diplotene stage of meiosis. The spermatocyte spreads were stained for SYCP3 (red) and RAD51 (green). (C) Quantification of the RAD51 foci and stretches in late pachytene spermatocyte nuclei. Foci are small dots of RAD51. The XY body has not been counted, an error indicates the XY body. 30 nuclei were analyzed for each genotype. Error bars represent the standard error of mean.

First, we investigated the localization of RAD51 protein during meiotic progression. RAD51 is required for DNA strand exchange during meiotic recombination [14]. During early meiotic prophase, in leptotene nuclei, approximately 200-300 SPO11-dependent RAD51 foci can be observed [15]. These foci most likely represent the meiotic DSBs. As cells progress towards zygotene and pachytene this number gradually declines, and in pachytene only a very low number of foci remains, mostly on the unsynapsed part of the X chromosomal SC. We previously found aberrant RAD51 staining in pachytene and diplotene nuclei in *Rad54*$^{-/-}$ testis [16]. In these nuclei, apart from foci of normal size, larger stretches of foci of RAD51 were observed, that did not always co-localize with the SCs. We analyzed RAD51 and SYCP3 distribution on late pachytene and diplotene spread spermatocyte nuclei isolated from the different genotypes. At late pachytene, the SCs are fully assembled, and at diplotene, the homologous chromosomes start to separate while the SC gradually disassembles. The staining pattern of SYCP3 was used to distinguish between the different meiotic prophase stages. In wild type spermatocytes, we detected little RAD51 staining in pachytene and diplotene nuclei (Figure 2A, B). However, a higher number of RAD51 foci, and some RAD51 stretches were visible in the *Nbs1*$^{\Delta B/\Delta B}$ spermatocytes at late pachytene and diplotene. More RAD51 stretches were present in the *Rad54*$^{-/-}$ spermatocytes in late pachytene nuclei (Figure 2C). The RAD51 distribution was even more severely affected in both stages in the double mutant. This was visible not only by an increase in the number of RAD51 foci per nucleus but also by the presence of large stretches of RAD51 protein. We observed an additive effect on the pattern of RAD51 foci and stretches in pachytene and diplotene in spread nuclei of the double mutant spermatocytes, showing that NBS1 and RAD54 have non-redundant roles in meiotic recombination.

The presence of RAD51 patches in late pachytene and diplotene suggested that repair of meiotic double-strand breaks was delayed. Therefore, we used γH2AX accumulation as another marker for DSBs. We analyzed the distribution of γH2AX in late pachytene and diplotene and its co-localization with RAD51 (Figure 3A, B). During male meiotic prophase, the X and Y chromosomes remain largely unpaired, with the exception of the so-called pseudoautosomal regions. The XY body is formed when the X and Y chromosomes are transcriptionally inactivated [17]. As described previously, the XY body is marked by a very high level of γH2AX [18]. Furthermore, we found staining in a focal pattern on the other chromosome, suggesting that some meiotic DSBs were still present in these spermatocyte spreads. The highest number of γH2AX foci was detected in the double mutant spermatocyte spreads, whereas less γH2AX staining was detected in the single mutants; we found that approximately 35% of the RAD51 foci also contained γH2AX in the mutant cells, suggesting that these are DSBs (Figure 3B).

To determine whether the DSBs are repaired at all, we investigated crossover formation. For this purpose, we analyzed the mismatch repair protein MutL homologue 1 (MLH1). This protein



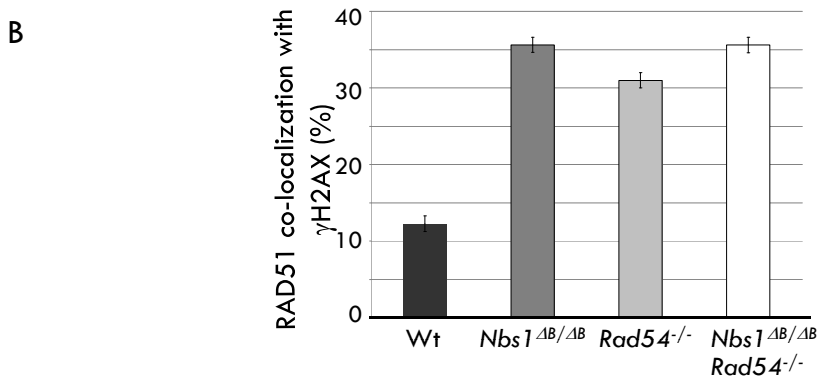


Figure 3: SYCP3, RAD51 and γH2AX analysis in spermatocyte spreads

(A) Staining of SYCP3 (red), RAD51 (green) and γH2AX (blue) in diplotene nuclei. The arrows indicate a co-localization of γH2AX and RAD51 staining (B) quantification of RAD51 foci that co-localize with γH2AX (%) in late pachytene nuclei.

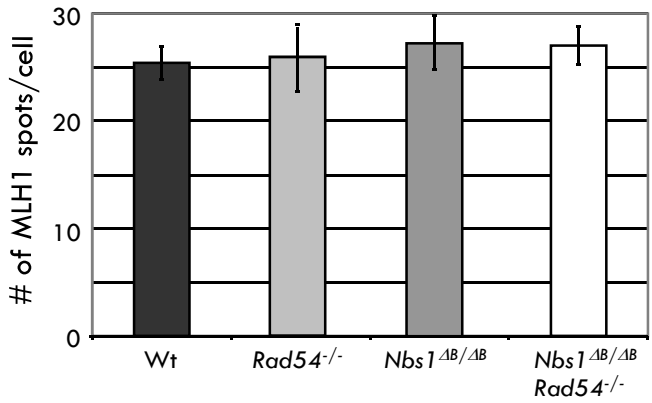


Figure 4: Analysis of the mismatch repair protein MLH1

The number of MLH1 spots per cell was plotted for all different genotypes. 5 cells were counted for each mouse, and two mice per genotype were analyzed. SYCP3 was co-stained to distinguish the pachytene nuclei in spermatocyte spreads. Error bars represent the standard error of mean.

previously found to form foci that correlated closely with the occurrence of genetically detected crossovers [19]. Unexpectedly, we did not observe any differences in MLH1 foci formation among the different genotypes (Figure 4). Cherry et al. 2007 observed a small increase in the number of crossovers in *Nbs1*$^{\Delta B/\Delta B}$ spermatocytes which is similar to our observations although the difference is not significant in our analysis [13].

Analysis of the double mutant ovaries in mice

In contrast to the fertile males, *Nbs1*$^{\Delta B/\Delta B}$ females (n=8) did not produce any offspring at 8 weeks of age. Therefore, we analyzed the ovaries in more detail. Wild type adult ovaries contain three stages of follicle development: primordial, primary and secondary follicles. The primordial follicles are the earliest follicles observed, which consist of oocytes that are not surrounded by a granulosa cell layer. One layer of granulosa cells surrounds the primary follicles, while the secondary follicles contain multiple layers of granulosa cells.

We started to analyze ovaries from 8- and 18-week-old wild type and *Nbs1*$^{\Delta B/\Delta B}$ females. The wild type ovaries were filled with follicles, mainly primary and secondary follicles, whereas the *Nbs1*$^{\Delta B/\Delta B}$ ovaries (n=2) were smaller and contained very few follicles at 8 weeks and no follicles at 18 weeks (Figure 5).

To get a better understanding of the reason for this deficiency in follicles, we analyzed earlier developmental stages. All stages of follicle development were present in 3-week-old wild type ovaries. In contrast, we detected only secondary follicles in the *Nbs1*$^{\Delta B/\Delta B}$ ovaries (Figure 6).

Therefore, we analyzed even earlier stages of ovary development. Wild type ovaries showed a complete range of follicles at 10 days p.p., but the *Nbs1*$^{\Delta B/\Delta B}$ showed very few primordial follicles (Figure 7A, B). Primary follicles were present at this stage of development in the *Nbs1*$^{\Delta B/\Delta B}$ mutant ovaries but they were severely reduced in numbers compared to wild type and *Rad54*$^{-/-}$ (Figure 7C).

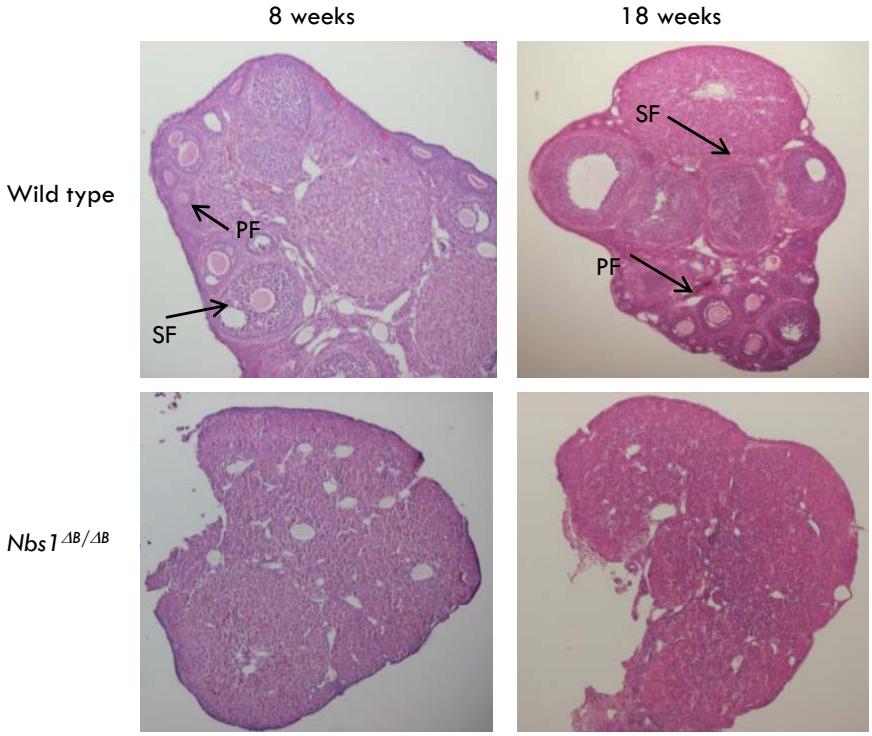


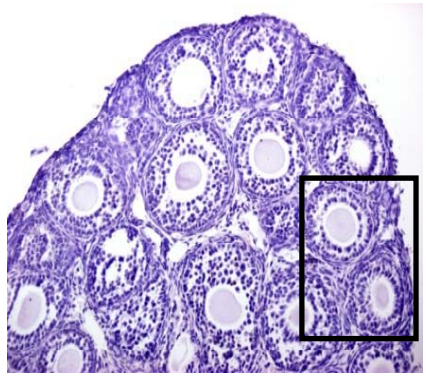
Figure 5: HE staining of wild type and *Nbs1*mutant ovaries at 8 and 18 weeks p.p.
Two mice were analyzed per genotype and four to six slides per genotype were analyzed. Abbreviations: PF-primary follicle, SF-secondary follicle.

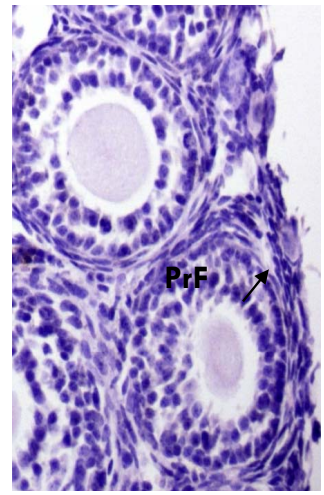
To investigate if there was already ovarian failure before birth, the oocytes were stained with the germ cell nuclear antigen 1 (GCNA1) [21]. Wild type, *Nbs1*$^{\Delta B/\Delta B}$ and *Rad54*$^{-/-}$ ovaries were indistinguishable just before birth (E18.5) (Figure 8). Moreover, a TUNEL staining to detect apoptotic cells was performed. The levels of apoptotic cells between the different genotypes was also the same before birth (data not shown), indicating that the disturbed folliculogenesis in *Nbs1*$^{\Delta B/\Delta B}$ ovaries developed after birth. The cells which were stained are the granulosa cells, which cannot explain the observed phenotype.

Synergistic effect of RAD54 and NBS1 in female meiosis

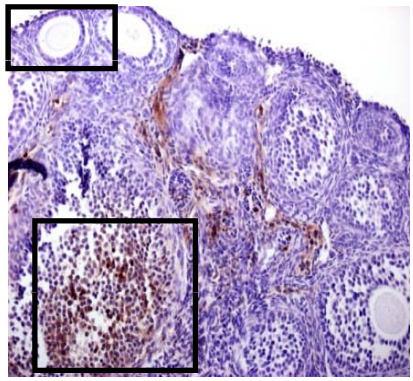
In order to get more insight into the role of NBS1 in ovary development, we combined the *Nbs1*$^{\Delta B}$ mutation with a *Rad54* deletion, which diminished HR [5]. *Rad54* deficiency did not cause any obvious abnormalities in ovary development. The *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ double mutant ovaries, on the other hand, resembled those of *Nbs1*$^{\Delta B/\Delta B}$ mice, but the defect was even more severe. No primordial follicles were found at day 10 p.p. and the number of primary follicles was also severely reduced compared to wild type and *Rad54*$^{-/-}$ (Figure 7). At 3 weeks p.p. no secondary follicles were present, which indicates that the reduced HR capacity of these oocytes causes follicle loss at an earlier stage in the double mutant ovaries (Figure 8). However, embryonic day E18.5 the double mutant has no problems either (Figure 8).

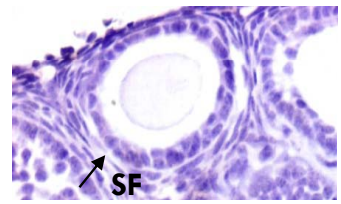
A Wild type

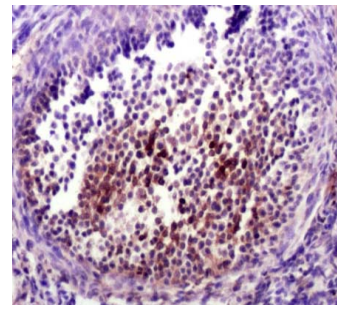




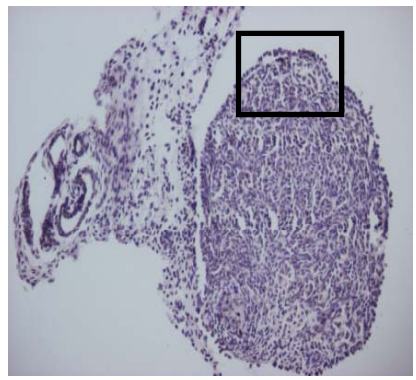
B *Nbs1*$^{\Delta B/\Delta B}$







C *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$



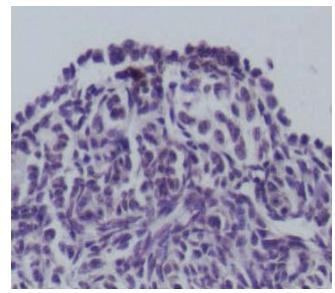


Figure 6: Histological sections of ovaries at postnatal day 21

Ovarian tissue is stained with HE. TUNEL-positive cells are stained brown, these cells are the granulosa cells. Abbreviations: PrF: primordial follicle, SF: secondary follicle. (A) wild type (B) *Nbs1*$^{\Delta B/\Delta B}$ (C) *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ Two mice were analyzed per genotype and four to six slides per genotype.

Discussion

Nbs1$^{\Delta B/\Delta B}$ mice are IR sensitive and cells derived from these mice are sensitive to various DNA damaging agents, show increased levels of chromosomal aberrations after IR treatment and display cell cycle checkpoint defects [9]. *Rad54*$^{-/-}$ mice are viable and therefore provide a suitable model system to study the biological significance of a defect in the mammalian HR pathway [5].

Herein, we describe the meiotic phenotype of the *Nbs1*$^{\Delta B/\Delta B}$, *Rad54*$^{-/-}$, and double mutant mice. *Nbs1*$^{\Delta B/\Delta B}$ females showed a severe loss of follicles within the first few weeks of life, whereas spermatogenesis in *Nbs1*$^{\Delta B/\Delta B}$ males appeared normal. In the double mutant females, ovarian dysgenesis was more severe and male spermatogenesis was mildly affected as well.

Persistent RAD51 patches in double mutant spermatocytes

Since the testis weight of *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ mice was slightly, but statistically significantly reduced, we analyzed the RAD51 distribution in spermatocyte spreads. It was already known [16] that RAD51 distributes abnormally in *Rad54*$^{-/-}$ meiotic spreads in late pachytene and diplotene. *Nbs1*$^{\Delta B/\Delta B}$ males did not exhibit gross changes, only a small difference in diplotene was observed in RAD51 localization. Cherry et al., 2007 found a slight increase in the proportion of pachytene cells with RAD51 foci in *Nbs1*$^{\Delta B/\Delta B}$ spermatocyte spreads during early prophase [13].

Interestingly, we observed a much more severe excess of foci and patches of RAD51 in the double mutant males, than in the *Rad54*$^{-/-}$ single mutant. A relatively large fraction of the RAD51 patches in double mutant diplotene nuclei contained γH2AX, suggesting that DSBs persist longer in these animals. We conclude, that the meiotic defect in *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ males is mainly caused by delayed and/or inefficient HR. The HR defect can result in more apoptotic spermatocytes which may in turn explain the smaller testis size in the double mutant mice.

Specific function of NBS1 in spermatogenesis and oogenesis

As RAD54 and the MRN complex have both been found to be required for meiosis in yeast (where Xrs2 is the third partner in the Mre11/Rad50 complex), we first considered the possibility that the mouse phenotypes could be explained by delayed or defective meiotic recombination. In the double mutant we observed an exacerbation of the single mutant phenotypes. This could be explained by the function of RAD54 in homologous recombination. The *Nbs1*$^{\Delta B/\Delta B}$ females have a more severe phenotype compared to *Nbs1*$^{\Delta B/\Delta B}$ males, which is unique for a gene involved in DNA repair and meiosis. *Nbs1*$^{\Delta B/\Delta B}$ females loose most of their oocytes very soon after birth, a phenotype which is comparable to what has been observed in *Spo11*$^{-/-}$ ovaries [22]. However, *Spo11* knockout males display a more severe meiotic phenotype, with a complete block of spermatocyte development during early meiotic prophase.

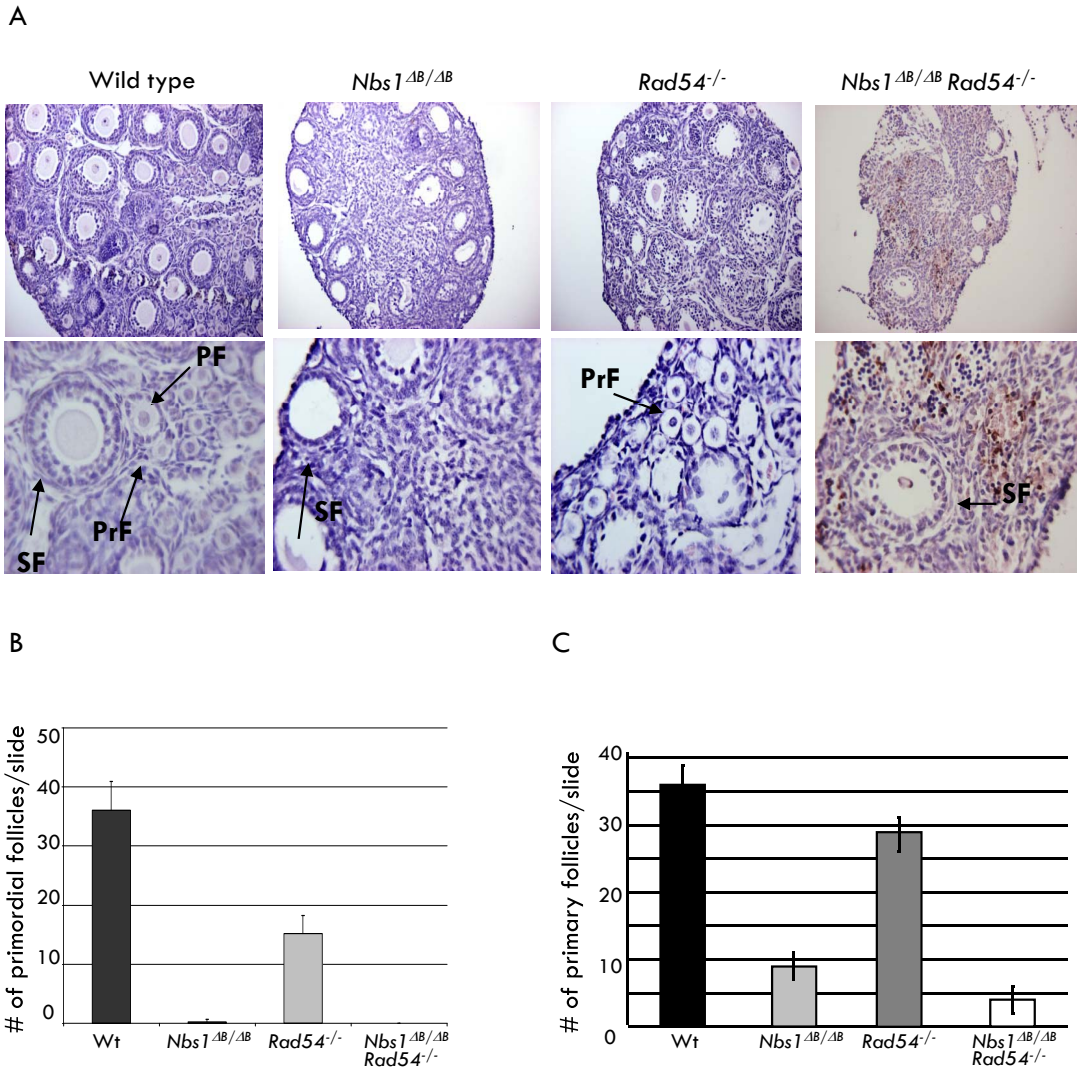


Figure 7: Analysis of 10-day-old ovaries

(A) Ovarian tissue is stained with HE. TUNEL-positive cells are stained brown, these cells are the granulose cells. Abbreviations: PrF-primordial follicle PF-primary follicle, SF-secondary follicle. (B) Quantification of the amount of primordial follicles per slide in all genotypes. Two mice were analyzed per genotype, in the case of the double mutant only one ovary was analyzed. (C) Quantification of the number of primary follicles per slide in all genotypes. Two mice were analyzed per genotype and four to six slides per genotype. In the case of the double mutant only one ovary was analyzed. Error bars represent the standard error of mean.

NBS1, CDC25B and the dictyate arrest

It is well established that NBS1 can activate ATM, and the activation of this kinase leads to checkpoint activation. Therefore, we conclude that the NBS1 mutant protein may no longer be able to activate the ATM-dependent checkpoint. If this would be the case, the *Nbs1* mutant phenotype would be expected to resemble that of the Atm knockout. In *Atm*$^{-/-}$ females,

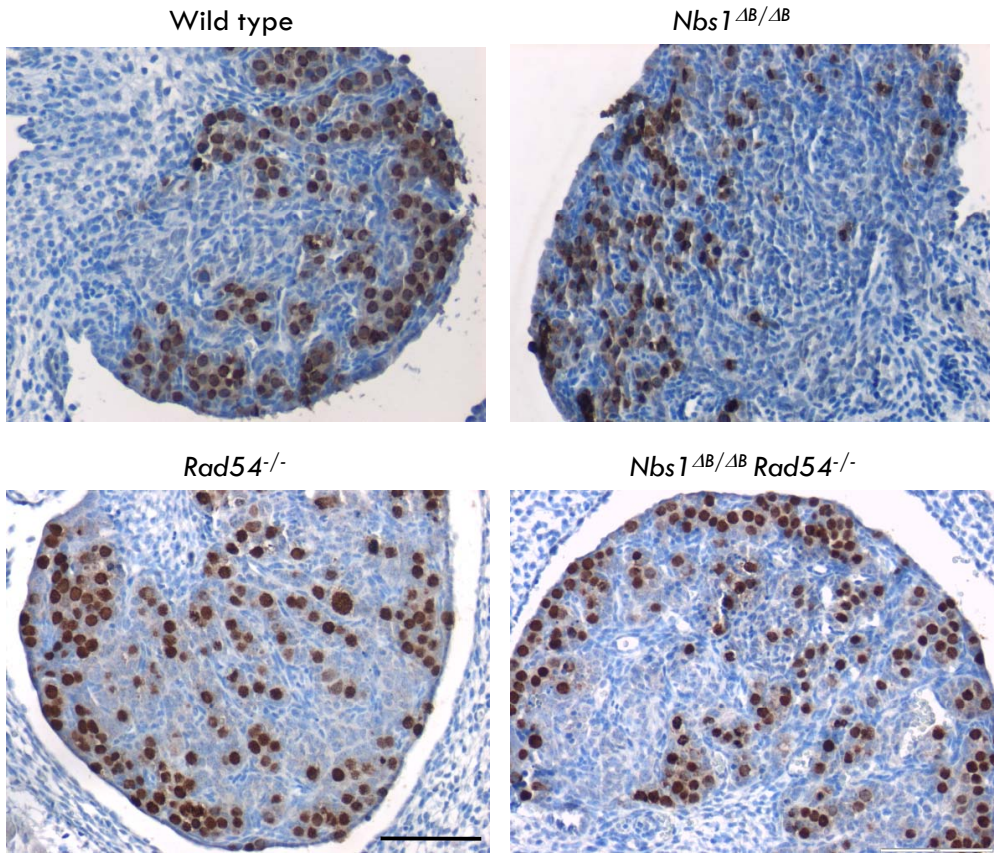


Figure 8: GCNA staining on histological section of ovaries at embryonic day 18.5
Ovarian tissue is stained with GCNA1, which stains oocytes nuclei in brown. The bar represents 100μM.

degenerate in prophase I prior to dictyate arrest, resulting in infertility [23]. In *Atm*$^{-/-}$ males, spermatocyte degeneration begins between postnatal days 8 and 16.5, soon after entry of the cells into prophase I of meiosis. Based on these data it appears unlikely that the *Nbs1*$^{\Delta B}$ mutation will interfere with this early ATM function.

As *Atm*$^{-/-}$ mice never reach the end of prophase I, we searched for other genes downstream of ATM that might shed light on the cause of the *Nbs1*$^{\Delta B/\Delta B}$ phenotype. The CDC25 phosphatases are ATM targets in somatic cells. Interestingly, the *Cdc25b*$^{-/-}$ mouse also shows female infertility. The *Cdc25b*$^{-/-}$ oocytes show normal dictyate arrest at prophase I, but they are not able to progress further through meiosis in response to the normal stimulus that occurs just prior to ovulation [24].

Interestingly, NBS1 is indirectly involved in the regulation of CDC25A, B and C in somatic cells [25]. NBS1 activates ATM, and subsequently [26] ATM phosphorylates CDC25A, which decreases CDC25A activity. This results in a decreased cyclin-dependent kinase 1 (CDK1)

dephosphorylation and subsequent arrest of the cell cycle. Thus, when NBS1 is not functioning properly, the cell cycle may continue, even in the presence of DNA damage. Normally, oocyte maturation arrests at late prophase of meiosis I (diplotene) [27]. Resumption of meiosis requires activation of CDK1. The phosphatases CDC25A, CDC25B and CDC25C activate CDKs in meiosis and mitosis [28]. If NBS1 also negatively regulates CDC25B in oocytes, impaired NBS1 function may lead to an overstimulation of CDC25B, which could lead to an impaired ability of the oocytes to enter or maintain dictyate arrest. The *Nbs1*$^{\Delta B/\Delta B}$ females are fertile early in life [9], but all follicles disappear within the first 8 weeks of postnatal ovary development. This rapid loss of primordial and primary follicles might be explained by an inability to maintain dictyate arrest. Follicles containing oocytes that fail to maintain the meiotic arrest will undergo a degenerative process called atresia, and the complete pool of oocytes will be depleted rapidly. The absence of an equivalent of the dictyate arrest in males may explain their near normal fertility.

Materials and Methods

Mice

The *Rad54*$^{-/-}$, *Nbs1*$^{\Delta B/\Delta B}$ and double mutant mice have been described previously [9, 29].

Immunocytochemistry of meiotic chromosomes

Testes were isolated from 12-weeks old wild type, *Rad54*$^{-/-}$, *Nbs1*$^{\Delta B/\Delta B}$ and *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ double mutant mice, as described previously [30]. Immunofluorescence was performed on spermatocyte spreads as described [31]. The slides were blocked with PBS containing 0.5% BSA and 0.5% non-fat dry milk, and were stained with rat polyclonal anti-SYCP3 [32], MLH1 (BD Pharmingen, San Diego, USA), mouse monoclonal γH2AX (Upstate, Temecula, USA) and/or rabbit anti-human RAD51 [33]. For rabbit polyclonal primary antibodies, the secondary antibodies were fluorescein isothiocyanate (FITC) labeled goat anti-rabbit IgG antibodies (Sigma, St Louis, USA); the secondary antibodies used for the rat polyclonal anti-SYCP3 (IgG) and mouse monoclonal anti-MLH1 (IgG) were Alexa 594-labeled goat anti-rat IgG and FITC-labeled goat anti-mouse IgG respectively. Primary antibodies were diluted in 10% BSA in PBS and incubated overnight in a humid chamber. Subsequently, slides were washed in PBS, blocked in 10% normal goat serum (Sigma) in blocking buffer (5% non-fat dry milk in PBS), and incubated with secondary antibodies in 10% normal goat serum in blocking buffer at room temperature for 2 h. Next, slides were washed in PBS and embedded in vectashield with or without DAPI (vector laboratories, Burlingame, USA).

Ovary processing and immunofluorescence assays

For immunohistochemistry, E18.5, P10 and P21 mouse ovaries were dissected and fixed overnight

in 4% paraformaldehyde in PBS at 4oC, dehydrated, paraffin embedded, sectioned (5 µm), and mounted on SuperFrost Plus microscope slides. 4-6 different slides were analyzed, all from two ovaries per genotype. Immunohistochemistry was performed as described previously [34]. TUNEL staining was performed according to manufacturer's protocol (using ApopTag Plus Peroxidase in Situ Apoptosis detection kit, S7101. Chemicon international, Inc. USA and Canada). GCNA1 staining was performed as described previously [21].

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Chapter 7

Summary

In this thesis, we describe novel findings with regard to the DNA damage response. We focused on proteins involved in DNA double strand break (DSB) repair. The main part is about the NBS1 protein, which is a component of the MRE11-RAD50-NBS1 (MRN) complex. The MRN complex is a central player in various cellular responses to DNA damage, including DSB repair, apoptosis and DNA damage checkpoint activation. DSBs are lesions which can be induced by many DNA damaging agents, including ionizing radiation (IR) and Mitomycin C (MMC). DSBs are also generated during DNA replication, V(D)J recombination and meiosis. There are at least two DSB repair pathways, homologous recombination (HR) and nonhomologous end-joining (NHEJ). Repair via HR uses homologous DNA sequences as a template for repair, whereas NHEJ ligates the broken ends together without using a repair template. To efficiently counteract DSBs, cells can activate several pathways, including DNA repair and cell cycle checkpoints, and these functions can interact with each other. Chapter 1 describes the DNA damage response, in particular DNA repair. In chapter 2 we focused on the biological relevance of DSB repair, especially after IR treatment, in mammalian cells derived from DSB repair deficient mice and the potential overlap between NHEJ and HR. In chapter 3 we investigated NHEJ in more detail. Therefore, we set up an assay in which we were able to analyze repair of a blunt ended DSB after Tn5 transposon excision. We showed that NHEJ is a precise DSB repair process and that the microhomology-mediated joining pathway could not substitute for the regular NHEJ pathway. The end-joining factors KU80 and XRCC4, and to a lesser extent DNA-PKcs, were required for efficient rejoining. In chapter 4 we investigated the DNA-PK complex in more detail. This complex consists of DNA-PKcs and the KU70/KU80 heterodimer. We observed a link between KU70 and the pro-apoptotic protein BAX which suggests that KU70 may link DSBs to the apoptotic pathway. Moreover, we found an interaction between KU70 and the MRN complex. In chapter 5 we aimed to elucidate the function of the MRN complex, by concentrating on the function of NBS1. To investigate the NBS phenotype we combined the mouse mimic of the most common patient mutation (*Nbs1*$^{\Delta B/\Delta B}$) with a *Rad54* null mutation, which diminishes HR. We exposed the double mutant cells to DNA damaging agents and observed that these cells were extremely sensitive to agents creating single-strand breaks (SSBs). SSBs can be converted to DSBs during replication. The persistent presence of the HR protein RAD51 and the increased levels of chromosomal aberrations in the double mutant cells indicated that these replication-associated DSBs are not repaired properly in *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ cells. Our results indicate that NBS1 and RAD54 function cooperatively, but in separate pathways to counteract chromosomal instability as a result of replication problems. Because the NBS defect leads to female infertility in patients, the meiotic phenotype of *Nbs1*$^{\Delta B/\Delta B}$ mice was analyzed in chapter 6. In *Nbs1*$^{\Delta B/\Delta B}$ females the folliculogenesis was disturbed, whereas the *Nbs1*$^{\Delta B/\Delta B}$ males showed normal fertility. *Rad54* deficiency exacerbated the meiotic defect in *Nbs1*$^{\Delta B/\Delta B}$ females, and *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ males had smaller testes. The disturbed folliculogenesis resulted in an early loss of follicles. This suggests that the oocytes either fail to accomplish the normal prophase I arrest, or suffer from

a female-specific defect in meiotic recombination.

While both HR and NHEJ function in mammalian somatic cells to repair DSBs, and the pathways are not mutually exclusive, their relative importance differs in the different cell cycle phases: NHEJ plays a dominant role in repairing DSBs induced in G1 and early S phases of the cell cycle, whereas HR is most efficient in late S and G2 phases.

Chapter 8

Samenvatting

In dit proefschrift, beschrijven we nieuwe bevindingen met betrekking tot het DNA herstel mechanisme. We hebben ons gericht op eiwitten die betrokken zijn bij het DNA dubbelstrengs breuk (DSB) herstel proces. Het grootste gedeelte gaat over het NBS1 eiwit, dat een component is van het MRE11-RAD50-NBS1 (MRN) complex. Het MRN complex is een centrale speler in verschillende cellulaire reacties op DNA schade, waaronder het dubbelstrengs breuk herstel proces, de geprogrammeerde celdood (apoptose) en celcyclus checkpoint activatie na DNA schade. Dubbelstrengs breuken zijn beschadigingen die geïnduceerd kunnen worden op verschillende manieren, waaronder ioniserende straling en Mitomycine C. Dubbelstrengs breuken kunnen ook gegenereerd worden gedurende DNA replicatie, V(D)J recombinatie en meiose. Er zijn tenminste twee herstelmechanismen om deze dubbelstrengse breuken te repareren: homologe recombinatie (HR) en 'nonhomologous end-joining' (NHEJ). Herstel via HR gebruikt een homologe DNA sequentie als een template voor het herstelproces. NHEJ daarentegen zorgt ervoor dat de gebroken uiteinden direct aan elkaar geplakt worden zonder een template. Om efficiënt om te gaan met deze dubbelstrengs breuken kunnen cellen enkele processen activeren, zoals DNA herstel en cel cyclus checkpoints, die een interactie met elkaar kunnen aangaan. Hoofdstuk 1 beschrijft het gehele proces dat optreed na DNA schade, met de nadruk op DNA dubbelstrengs breuk herstel. In hoofdstuk 2 hebben we aandacht besteed aan de biologische relevantie van dubbelstrengs breuk herstel. In het bijzonder hebben we gekeken naar het herstel na ioniserende straling in zoogdiercellen afkomstig van dubbelstrengs breuk herstel deficiënte muizen en de potentiële overlap tussen NHEJ en HR. In hoofdstuk 3 hebben we NHEJ wat nauwkeuriger bestudeerd. Daarvoor hebben we een assay opgezet waarin het mogelijk was het herstel na een blunte dubbel strengs breuk na Tn5 transpositie te analyseren. We hebben laten zien dat NHEJ een precies DNA dubbelstrengs breuk herstel mechanisme is en dat het mechanisme dat gebruik maakt van microhomologie het reguliere NHEJ mechanisme niet kan vervangen. De NHEJ-factoren KU80, XRCC4 en in een iets minder mate DNA-PKcs zijn vereist voor efficiënt herstel van de blunte dubbelstrengs breuk. In hoofdstuk 4 hebben we het DNA-PK complex in meer detail bestudeerd. Dit complex bestaat uit DNA-PKcs en de KU70/KU80 heterodimeer. We hebben een link gevonden tussen KU70 en het pro-apoptotische eiwit BAX dat suggereert dat KU70 het dubbelstrengs breuk herstel aan apoptose zou kunnen koppelen. Bovendien hebben we een interactie gevonden tussen KU70 en het MRN complex. In hoofdstuk 5 hadden we tot doel om de rol van dit MRN complex op te helderen door te concentreren op de functie van NBS1. Om het fenotype van NBS te onderzoeken, hebben we een muis gecreëerd die dezelfde mutatie bevat als de mutatie die gevonden is in de meeste patiënten (*Nbs1*$^{\Delta B/\Delta B}$) en deze gecombineerd met een *Rad54* nulmutatie die HR verstoort. Dubbelmutante cellen zijn geïsoleerd en deze hebben we vervolgens blootgesteld aan DNA beschadigende stoffen. We hebben gevonden dat deze cellen extreem gevoelig zijn voor stoffen die enkelstrengs breuken maken. Deze enkelstrengs breuken kunnen gedurende de replicatie omgezet worden in dubbelstrengs breuken. Persisterende foci van het HR eiwit RAD51

en het verhoogde niveau van chromosomale afwijkingen in dubbelmutante cellen impliceert dat deze replicatie-geassocieerde dubbelstrengs breuken niet goed hersteld worden. Onze resultaten laten zien dat NBS1 en RAD54 samenwerken om de chromosomale instabiliteit als resultaat van deze replicatie-geassocieerde problemen op te lossen.

Het NBS defect leidt tot onvruchtbaarheid in vrouwelijke patiënten. Daarom werd het meiotische fenotype van *Nbs1*$^{\Delta B/\Delta B}$ muizen geanalyseerd in hoofdstuk 6. In *Nbs1*$^{\Delta B/\Delta B}$ vrouwtjes is de folliculogenese verstoord, terwijl de *Nbs1*$^{\Delta B/\Delta B}$ mannen normaal vruchtbaar zijn. Rad54 deficiëntie versterkt het meiotische defect in *Nbs1*$^{\Delta B/\Delta B}$ vrouwen. Verder blijken *Nbs1*$^{\Delta B/\Delta B}$ *Rad54*$^{-/-}$ mannen kleinere testes te hebben. De verstoorde folliculogenese resulteerde in een vroegtijdig verlies van follikels, wat suggereert dat de oocyten niet in staat zijn om het profase I arrest te handhaven of dat de *Nbs1*$^{\Delta B/\Delta B}$ vrouwen leiden aan een vrouw-specifiek defect gedurende de meiotische recombinatie.

Terwijl zowel HR als NHEJ functioneren in somatische zoogdiercellen voor het herstel van dubbelstrengs breuken, varieert hun relatieve bijdrage in de verschillende fasen in de celcyclus; NHEJ is dominant in alle fasen van de celcyclus, alleen dubbelstrengs breuken die tijdens de replicatie gemaakt worden hebben HR echt nodig.

Curriculum Vitae

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