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DNA double-strand break repair *in vivo* assessed by γ-H2AX foci analysis in brain and lung tissue of repair-proficient and -deficient mouse strains

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INDEX

Abbreviation

A-T	ataxia-telangiectasia
ATLD	Ataxia telangiectasia-like disorder
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia mutated-Rad3-Related
BRCA1	Hereditary breast and ovarian cancer susceptibility gene
CT	Computed tomography
DNA-PK	DNA-dependent protein kinase
DSB	DNA double-strand break
HR	homologous recombination
LIG	Ligase
MRN	MRE11-RAD50-NBS1
NHEJ	non-homologous end-joining
PBS	phosphate-buffered saline
PFGE	pulsed-field gel electrophoresis
PIKK	phosphatidylinositol 3-kinase related kinase
PNKP	Polynucleotide kinase/phosphatase
SCID	severe combined immunodeficiency
TDP1	tyrosyl-DNA phosphodiesterase
WRN	Werner syndrome protein
XRCC	X-Ray Repair Cross Complementing

1. Summary

Purpose:

Double-strand breaks (DSBs) are the most deleterious form of DNA damage after ionizing radiation, and deficiencies in repairing DSBs lead to pronounced radiosensitivity. Organs and tissues show substantially varying levels of sensitivity during radiotherapy but only little is known about how DSBs are repaired differentially in various normal tissues. H2AX phosphorylation is an early step in the response to DNA damage and it has been demonstrated that enumerating γ-H2AX foci (the phosphorylated histone) can be used to measure the induction and repair of radiation-induced DSBs *in vitro*. In the present study the γ-H2AX foci approach was established in normal tissues of brain and lung of DSB repair-proficient and repair-deficient mice to evaluate if this approach is a precise index for the DSB repair *in vivo* and if there are differences in the DSB repair kinetics between the different tissues which may account for their considerably varying intrinsic radiosensitivities.

Methods:

For the DSB induction, brain and lung tissue of C57BL/6 mice was analyzed at 10 min after whole body irradiation with 0.1 Gy, 0.5 Gy and 1.0 Gy. For the DSB repair kinetics, brain and lung tissue of repair-proficient (C57BL/6 mice) and repair-deficient mouse strains (BALB/c, A-T and SCID mice) were analyzed at 0.5 h, 2.5 h, 5 h, 24 h and 48 h after whole body irradiation with 2Gy. Brain and lung tissue of sham-irradiated mice of each strain served as controls. γ-H2AX immunohistochemistry and γ-H2AX immunofluorescence analysis was used to measure DSBs formation and repair in the brain and lung tissue of the different mouse strains.

Results:

For the DSB induction, identical γ-H2AX foci levels with a clear linear dose correlation and very low backgrounds in the brain and lung tissue were observed. Scoring the loss of γ-H2AX foci allowed us to verify the different, genetically determined DSB repair deficiencies, including the minor impairment of BALB/c mice. Repair-proficient C57BL/6 mice exhibited the fastest decrease in foci number with time, and displayed only low levels of residual damage at 24 h and 48 h postirradiation.

In contrast, SCID mice showed highly increased γ-H2AX foci levels at all repair times (0.5 h to 48 h) while A-T mice exhibited a lesser defect which was most significant at later repair times (≥ 5 h). Radiosensitive BALB/c mice exhibited slightly elevated foci numbers compared with C57BL/6 mice at 5 h and 24 h but not at 48 h postirradiation. The DSB repair kinetics measured in brain and lung tissue were nearly identical, although these organs reveal clearly different clinical radiosensitivities.

Conclusion:

The results provide evidence that quantifying γ-H2AX foci in various normal tissues represents a sensitive tool for the detection of induction and repair of radiation-induced DSBs at clinically relevant doses *in vivo*. γ-H2AX foci kinetics measured in the brain and lung were similar to kinetics in peripheral blood lymphocytes (measured in previous experiments) demonstrating that data obtained in blood samples can be utilized to screen for DSB repair deficiencies as predictor for clinical radiosensitivity. Strikingly, the various analyzed tissues exhibited similar kinetics for γ-H2AX foci loss despite their clearly different clinical radiation responses. Hence, the distinct radiosensitivity of parenchymal cells does not rely on tissue-specific differences in DSB repair. Rather, it is suggested that differing down-stream events determine the characteristic radiation responses of different tissues.

2. Introduction

2.1. DNA double-strand breaks

DNA double-strand breaks (DSBs) are considered as the most biologically lethal of all DNA lesions, which if unrepaired severely threatens not only the integrity of the genome but also the survival of the organism. DSBs can be produced by exogenous agents such as ionizing radiation and certain chemotherapeutic drugs but also arise endogenously during replication, V(D)J recombination, and meiosis (HOEIJMAKERS et al., 2001; VAN et al., 2001). Appropriate cellular signaling responses to DNA damage and the ability to repair damaged DNA are fundamental processes that are required for organismic survival.

As shown in figure 1, cells respond to DSBs through the action systems that detect the DNA lesion and then trigger various downstream events. At least in some cases, these systems can be viewed as classical signal-transduction cascades in which a ‘signal’ (DNA damage) is detected by a ‘sensor’ (DNA-damage binding protein) that then triggers the activation of a ‘transducer’ system (protein kinase cascade), which amplifies and diversifies the signal by targeting a series of downstream ‘effectors’ of the DNA-damage response.

The DSB signaling systems need to be exquisitely sensitive and selective, as they must be triggered rapidly and efficiently by low numbers of, and maybe just one DSB, and must remain inactive under other conditions. If DSBs left unrepaired, they can result in permanent cell cycle arrest, apoptosis or mitotic cell death (OLIVE et al., 1998), and if repaired incorrectly, can lead to carcinogenesis through directly induced or delayed chromosomal rearrangements (MORGAN et al., 1998).

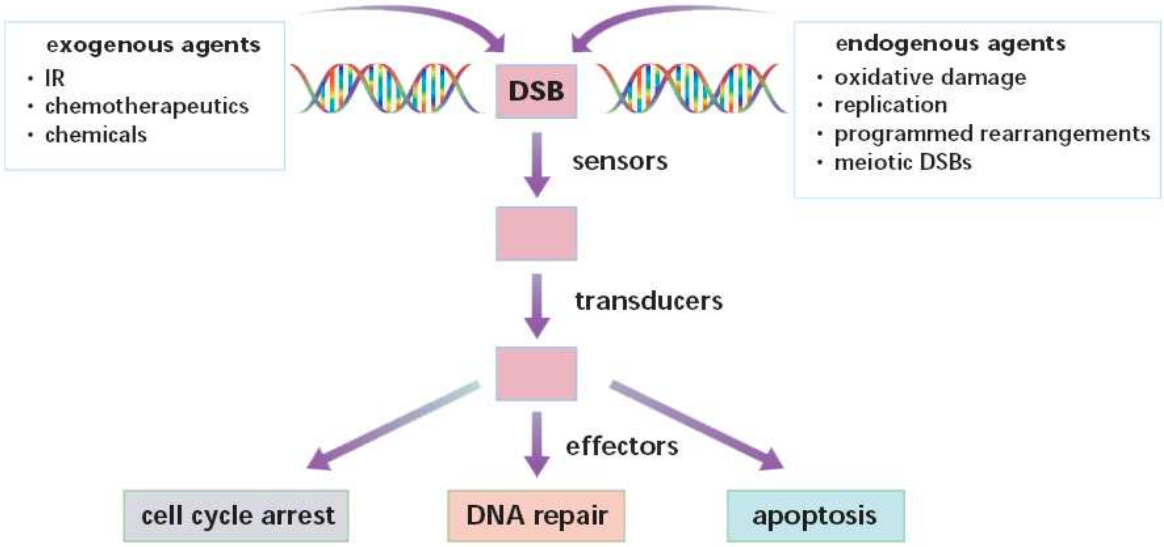

Fig.1 Signaling of DSBs. DSBs are recognized by different sensor proteins, which transmit the signal to a series of downstream effector molecules through a transduction cascade, to activate signaling mechanisms for cell-cycle arrest and induction of repair, or cell death if the damage is irreparable. Figure was taken from Khanna et al., 2001.

2.2. DNA double-strand break repair pathways

There are at least two mechanisms for DSBs repair: non-homologous end-joining (NHEJ) and homologous recombination (HR). HR and NHEJ are mechanistically distinct DSBs repair pathways function to maintain the integrity of DNA (LIEBER et al., 2003; WEST et al., 2003; O'DRISCOLL et al., 2006). Each pathway consists of a distinct array of different repair enzymes and associated factors (Fig. 2). Both pathways may be activated cooperatively repair DNA lesions (GOEDECKE et al., 1999; PIERCE et al., 2001; ROTHKAMM et al., 2003). Compared with HR, NHEJ is the predominant mechanism in mammalian cells and repairs broken ends with little or no requirement for sequence homology and involves the DNA-dependent protein kinase (DNA-PK) holoenzyme and the XRCC4-LIG4 complex.

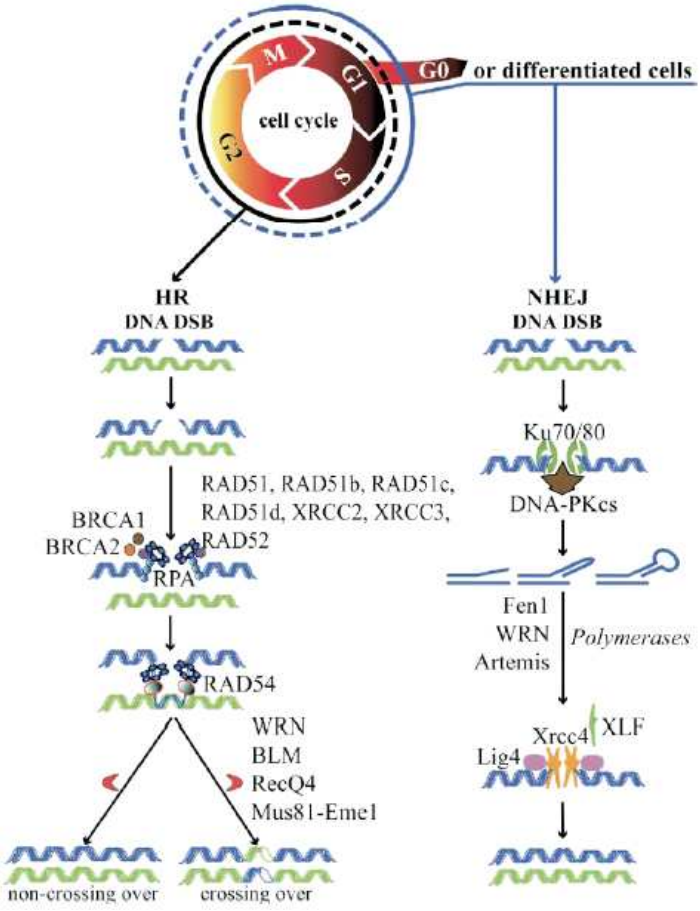

Fig. 2 Pathways of DSB repair. NHEJ rejoins the two broken ends directly and generally leads to small DNA sequence deletions. It requires the DNA-end-binding protein KU, DNA-PKcs, XRCC4, DNA ligase IV may also function in NHEJ, particularly if the DNA ends require processing before ligation. HR requires RAD52, RAD54, BRCA1, BRCA2 and RAD51, which forms filaments along the unwound DNA strand to facilitate strand invasion. Figure was taken from Lee et al., 2006.

The DNA-PK consists of the DNA end-binding heterodimer Ku70-Ku80 and the catalytic subunit DNA-PKcs (JEGGO et al., 1998; SMITH et al., 1999; KARRAN et al., 2000). HR is dependent upon homologous sequences flanking the breaksite, the nonhomologous ends are removed and aligned with regions of homology in the complimentary strand. DNA synthesis and ligation complete the process.

The choice of repair pathway that becomes activated is linked to the cell cycle, with HR being available in S and G2 phases. During HR, the damaged chromosome enters into synapsis with, and retrieves genetic information from an undamaged DNA molecule with which it shares extensive sequence homology. Simple eukaryotes such as the yeasts S.cerevisiae and S.pombe rely mainly on HR to repair radiation-induced DNA DSBs while NHEJ is more important in mammals. In mammals the NHEJ pathway predominates in many stages of the cell cycle-particularly in G0 and G1. Both pathways are highly conserved throughout eukaryotic evolution but their relative importance differs from one organism to another.

2.2.1. Homologous recombination (HR)

A major difference between HR and NHEJ repair mechanisms is the degree of fidelity of DNA repair. HR is an error-free mechanism depending on a homologous sequence as templates from a sister chromatids (WEST et al., 2003; THACKER et al., 2004). Hence, HR is limited to the late S to M phase of the cell cycle when sister chromatids are available. HR is the predominant DNA DSB repair pathway during early embryonic development when cells are rapidly dividing and error free/high fidelity repair is required (ORII et al., 2006). Figure 3 shows the process of HR. At damaged DNA termini, end-processing (unwinding and nucleolytic processing) generates a single strand with a 3' overhang upon which a recombinase complex is loaded. This pre-synapsis complex involves a group of proteins associated with Rad51 recombinase function, including a group of RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3), RAD52 and RAD54. BRCA2 is also essential for HR, and participates in RAD51 function (PELLEGRINI et al., 2002; WEST et al., 2003). Upon loading of RAD51 and its accessory proteins, the DNA strand invades the sister chromatid using the recombination-proficient DNA end to synapse with a homologous template. The final post-synapsis resolution of the four DNA strands of the two DNA duplexes (the Holiday junction) that ultimately result in repaired DNA involves RAD54 and RAD51C (LIU et al., 2004; WYMAN et al., 2004). However, the details of the resolution of the repair intermediates are unclear, and additional protein such as Mus81/Eme1 and the RecQ helicases are also involved (WEST et al., 2003; WYMAN et al., 2004).

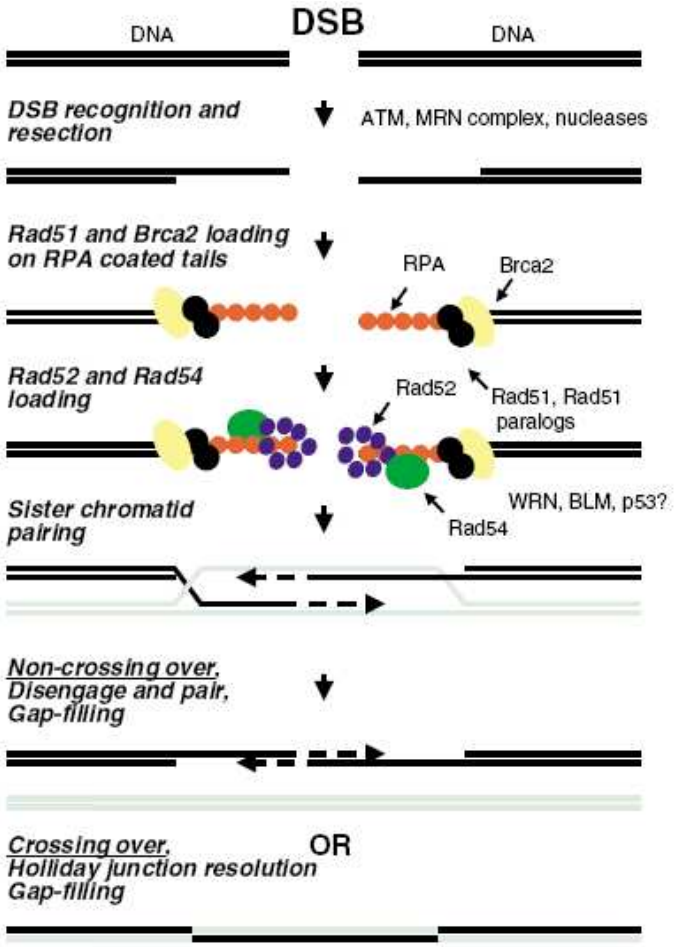

Fig. 3 Homologous recombination. In the earliest stage, ATM senses and perhaps binds to the DSB, and phosphorylates H2AX, which would then attract BRCA1 and NBS1. The MRN complex resects the DNA to provide ssDNA overhangs necessary for DNA pairing and strand exchange. BRCA2, attracted to the DSB by BRCA1, facilitates the loading of RAD51 onto RPA-coated DNA overhangs with the help of RAD51 paralogs that in turn attract RAD52 and RAD54. The tumor suppressor P53, known to interact with BRCA1, RAD51, BLM and WRN, is also likely found in this DNA–protein complex. Figure was taken from Valerie et al., 2003.

In addition to these factors known to be directly involved in HR, there are a number of molecules required for triggering DNA damage stress responses that act as ‘sensors’ for damage that are also important in cell cycle regulation and perhaps in the repair process itself. These sensors include ATM and ATR (ABRAHAM et al., 2001; DUROCHER et al., 2001).

Altogether, HR is critical for maintaining genomic integrity, because of its role in DSB repair. While the majority of DSBs are rejoined by NHEJ, the role of HR is enhanced in embryonic cells and late S/G2 phase cells. In some situations, such as during DNA replication, HR improves DSB repair fidelity and, as a result, increases cell survival following ionizing irradiation. Thus, HR may be required for efficient and accurate repair of a specific subset of DSBs that are refractory to NHEJ alone.

2.2.2. Non-homologous end-joining (NHEJ)

Despite the controversy over the relative importance of HR and NHEJ in mammalian cells, it is generally accepted that at least in the G1/G0 phase, the great majority of DSBs are rejoined by NHEJ (LEE et al., 1997; TAKATA et al., 1998). That the cell uses this somewhat error-prone process rather than HR is presumably due to the difficulty of matching the damaged sequence to its intact copy on the homologous chromosome (which is typically at a distant site in the nucleus) and bringing the two into close proximity. Figure 4 shows the process of NHEJ. NHEJ modifies the two DNA ends of a DSB so that they are compatible for direct ligation (LEE et al., 2003; LIEBER et al., 2003; O'DRISCOLL et al., 2006). NHEJ involves, among other proteins, KU, DNA-PKcs, DNA ligase IV, its cofactor XRCC4 (CHU et al., 1997; CALSOU et al., 1999; KARRAN et al., 2000; CHEN et al., 2001), and probably DNA polymerase μ (MAHAJAN et al., 2002) and/or polynucleotide kinase/phosphatase (PNKP) (CHAPPELL et al., 2002). Additional candidates for participation in NHEJ include Artemis (MA et al., 2002), the MRE11-RAD50-NBS1 (MRN) complex (HUANG et al., 2002), BRCA1 (ZHONG et al., 2002), tyrosyl-DNA phosphodiesterase (TDP1) (INAMDAR et al., 2002) and APE1 (DEMPLE et al., 1994; SUH et al., 1997).

One of the first proteins that localize to DNA ends at a DSB is the KU heterodimer. The Ku70/80 heterodimer recognizes and binds the free DNA ends, which forms an open ring-type that can be threaded onto a DNA end. One side of the ring forms a cradle that protects one surface of the DNA double helix, whereas the other side is much more open, presumably to allow other NHEJ factors to access the DSB. DNA-PKcs is recruited and activated by the Ku complex to form the DNA-PK holoenzyme (SMITH et al., 1999) and to act through phosphorylation events. They together protect broken DNA ends and facilitate recruitment of other end joining proteins.

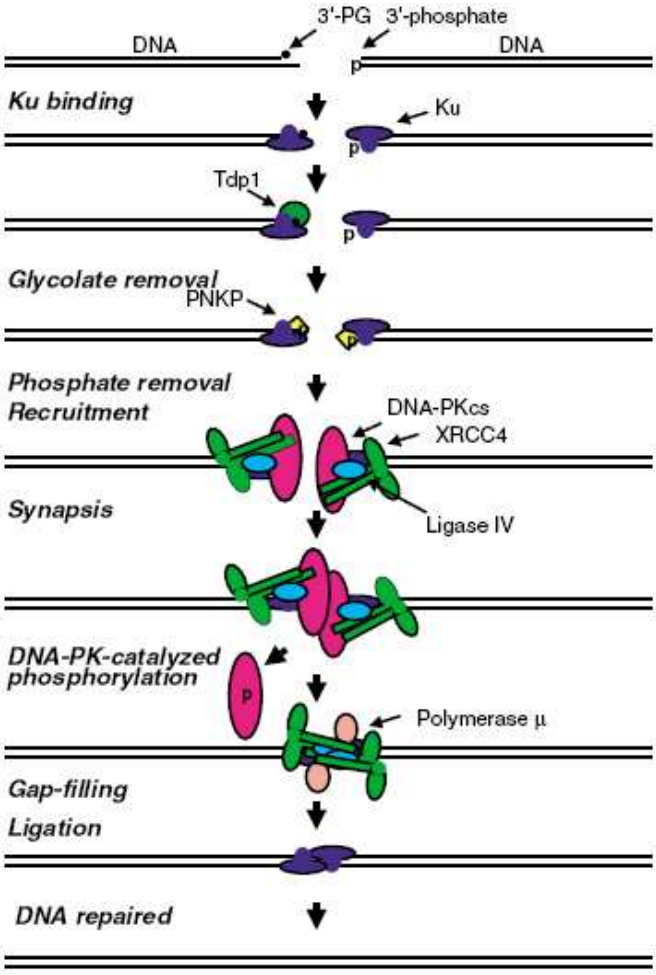

Figure. 4 Proposed model for accurate NHEJ of radiation-induced DSBs. Following initial binding of the DNA end by KU, KU recruits DNAPKcs, which blocks further processing and promotes uxtapositioning of the two DNA ends. DNA-PKcs phosphorylates itself and possibly other proteins in the complex, resulting in the release of DNA-PKcs from the extreme end of DNA, and possibly from the complex as a whole. A complex of KU, XRCC4, DNA ligase IV and a DNA polymerase then forms at the end-to-end junction and catalyzes alignment-based gap filling and ligation completing the repair process and restoring the original sequence. Figure was taken from Valerie et al., 2003.

DNA-PKcs is an 465 kDa polypeptide, the C-terminal region of which has homology to the catalytic domains of proteins of the phosphatidylinositol 3-kinase-likeprotein kinases (PIKKs), and its activity is stimulated by double-stranded DNA ends (ROUSE et al., 2002; SHILOH et al., 2003). More specifically, DNA-PKcs is most related to a sub-group of proteins in this family the PI 3-kinase related kinases (PIKK) which includes ATM, ATR and TRRAP. DNA-PKcs itself has affinity for DNA ends and its activation appears to be triggered by its interaction with a single-stranded DNA region derived from a DSB (HAMMARSTEN et al., 2000; MARTENSSON et al., 2002). As a consequence of this and the DNA binding properties of Ku, DNA-PK is activated by DNA DSBs *in vitro*, and presumably this is also the case *in vivo*.

Once bound to DNA DSBs, DNA-PK displays protein Ser/Thr kinase activity with preference for the consensus sequence Ser/Thr-Gln. KU stabilizes DNA-PKcs and this complex probably recruits and activates via its kinase activity proteins involved in DNA end processing and DNA ligation.

KU also recruits the LIG4/XRCC4 complex to broken DNA ends where this complex is required for ligation of the two DNA ends (NICK et al., 2000). These ends require modification to leave 5'-phosphate and 3'-OH termini prior to DNA ligation. There are several enzymes potentially involved in end-processing (LEES et al., 2003).

Werner syndrome protein (WRN), Artemis and MRE11 are all nucleases with putative roles in end-joining. The WRN is a RecQ like helicase, which can be stimulated by KU but inhibited by DNA-PKcs (YANNONE et al., 2001). WRN can displace DNA-PKcs from DNA-PK holoenzyme bound to a DNA end (LI et al, 2002).

Finally, there may be a requirement for the MRE11-RAD50-NBS1 complex in NHEJ. This complex, comprising the nuclease MRE11, the structural maintenance of chromosomes protein RAD50 and the protein NBS1, is rapidly recruited to DSB sites, where it tethers and processes the broken ends (STRACKER et al., 2004; MORENO-HERRERO et al., 2005). It has become apparent that, in addition to its DNA processing activities, the MRN complex controls the early steps in transduction of the DNA-damage signal. Additional factors as the sensor of the DSB response are recruited to the damaged sites, where they create rapidly expanding nuclear foci and take part in signaling damage to the transducers.

MRE11 is a nuclease with exonuclytic activity and may therefore process the complex DNA ends before ligation. It has also been shown to interact with Ku70 following DNA damage. Although NBS1 defective cells show a radiosensitive phenotype attributable to defective DSB repair (GIRARD et al., 2000), they are, however, proficient in their ability to undergo V(D)J recombination (YEO et al., 2000). Thus, the exact role that the MRE11-RAD50-NBS1 complex plays in NHEJ is still unclear, although it is undoubtedly an important factor in the repair of DNA DSBs, both through the initial sensing and through functional interactions with both NHEJ and HR factors (CARSON et al., 2003; UZIEL et al., 2003; HOREJSI et al., 2004; KOBAYASHI et al., 2004; LEE et al., 2004).

2.2.3. HR and NHEJ interactions

It should be noted that NHEJ and HR are not necessarily independent, since the coordinated action of both pathways is invoked by the cell in order to repair a DSB with minimal error (RICHARDSON et al., 2000; SAINTIGNY et al., 2001). Thus both HR and NHEJ repair pathways are required to maintain genomic integrity, even in the absence of a specific genotoxic insult. Several studies in hamster cells have suggested that when NHEJ is impaired, HR seems to increase and vice versa (RICHARDSON, 2000; ALLEN et al., 2002). Furthermore, cells obtained from DNA-PKcs-deficient SCID mice that are impaired in NHEJ and V(D)J recombination show more efficient levels of HR (PLUTH et al., 2001). There is a reported interaction between ATM and DNA breaks at the site of V(D)J recombination (PERKINS et al., 2002), suggesting that ATM also plays a role in NHEJ and V(D)Jrecombination. Altogether, different types of DSB repair are intricately linked together in a dynamic fashion with cell cycle regulation, but with sufficient flexibility to allow for redundancy and backups should one factor or type of repair fail.

2.3. DNA-damage response

The rapid DSB signal transduction response is well described, and involves a series of post-translational modifications such as phosphorylation that facilitate signal transduction via protein-protein interactions (Fig. 5).

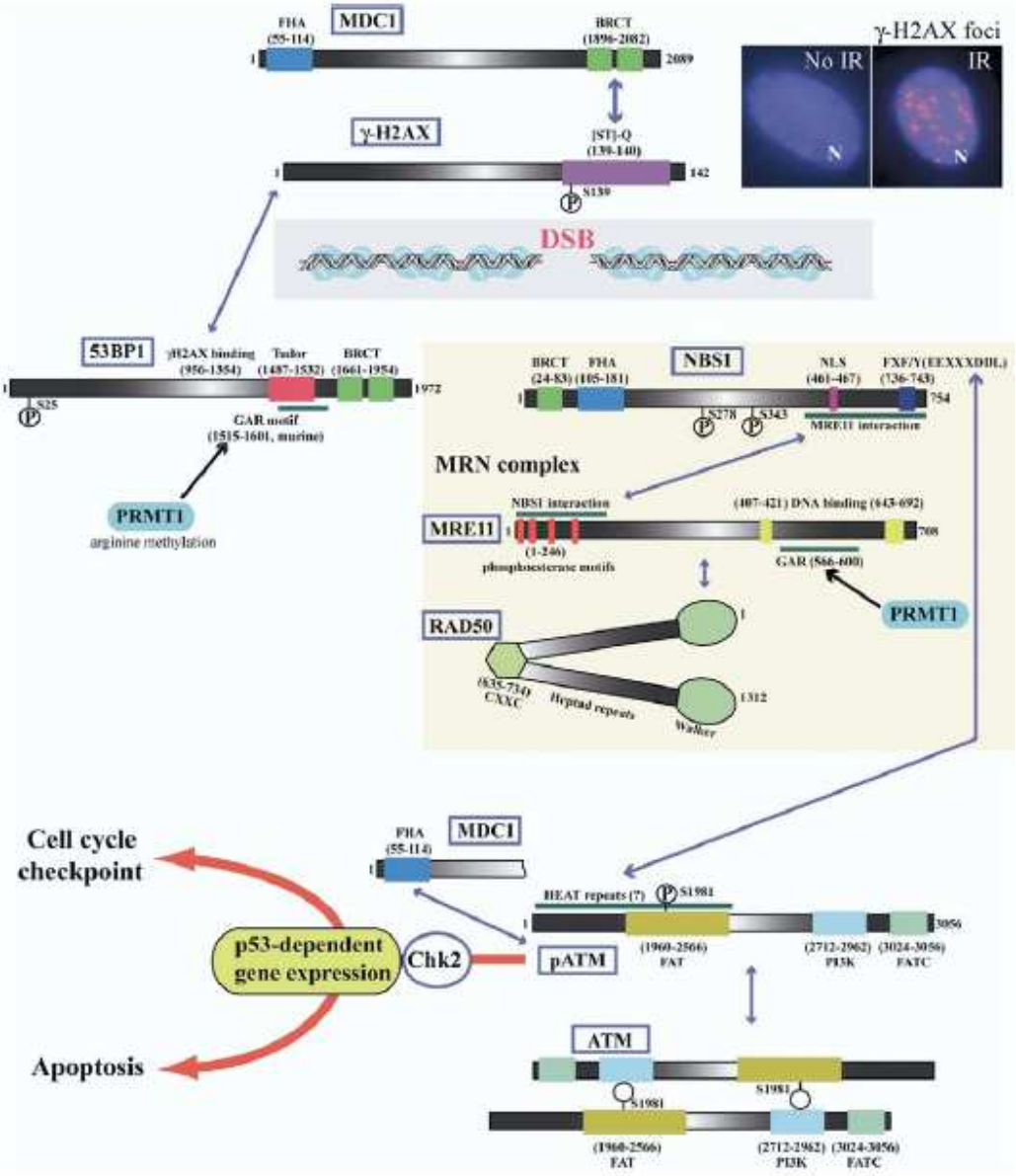

Fig. 5 Responding to DSBs. DNA damage induces multiple protein–protein interactions that collectively serve to modulate cellular signaling. Figure was taken from Lee et al., 2006.

2.3.1. ATM protein kinase

A crucial component of the DNA DSB signaling cascade in mammalian cells is the protein kinase, ATM, a protein kinase which DNA damage induced phosphorylation of various substrates involved in cell cycle regulation or maintenance of genomic stability (SHILOH et al., 2003; KASTAN et al., 2004; LAVIN et al., 2005). ATM is most renowned for its regulation of DSB-induced cell cycle checkpoint arrest that includes G1/S, intra S and G2/M checkpoint (SHILOH et al., 2001; KURZ et al., 2004). ATM also contributes to the regulation of apoptosis in response to DSBs. ATM deficiency leads to the human cancer predisposition and neurodegenerative syndrome ataxia-telangiectasia (A-T). At cellular level, ATM deficiency is manifested by increased sensitivity to ionizing radiation and other agents that yield DSBs but little or no hypersensitivity to other forms of DNA damage. Inactive ATM exists as a dimer that undergoes autophosphorylation on serine 1981 in response to DSBs to become an active monomer (BAKKENIST et al., 2003). Recent data suggest that ATM is recruited to and activated at sites of DSBs (ANDEGEKO et al., 2001). ATM activation is also intimately linked to other factors, principal among these is the MRN complex (D'AMOURS et al., 2002; PETRINI et al., 2003; VAN et al., 2003). Cells in which the MRN complex is compromised can affect ATM localization to DNA damage-induced foci or show decreased ATM activity and reduced phosphorylation of ATM substrates (CARSON et al., 2003; UZIEL et al., 2003; KITAGAWA et al., 2004; LEE et al., 2004; DIFILIPPANTONIO et al., 2005). While the exact details of ATM activation by the MRN complex have not to be fully elucidated, the structure of MRN that facilitates its role as a sensor of DNA breaks provides important insights. Critical for the function of this complex is a zinc-hook structure present in the flexible coiled-coil region of RAD50 (HOPFNER et al., 2002; WILTZIUS et al., 2005). The binding of MRN to DNA induces a conformational change leading to an inter-complex tether via RAD50 between distinct MRN complexes bound to opposing DNA strands (MORENO-HERRERO et al., 2005; WILLIAMS et al., 2005). The ability of the MRN complex to tether broken DNA ends provides a physical basis for recruitment of critical signaling kinases such as ATM. The association of ATM with the MRN complex involves interaction of ATM with FXF/Y motifs in the NBS1 carboxyl terminal region (FALCK et al., 2005; FERNANDES et al., 2005; YOU et al., 2005), and this interaction facilitates ATM localization to damaged DNA. Methylation of the MRN complex is also likely to influence its function. MRE11 can be methylated by protein arginine methyltransferase 1 after DNA damage, a modification that can influence the

checkpoint function of the MRN complex (BOISVERT et al., 2005).

Activated ATM phosphorylates numerous substrates. Downstream substrates for ATM include P53, MDM2, CHK1, CHK2, BRCA1 and NBS1, SMC1, which are involved in cell cycle progression, DNA repair or apoptosis (SHILOH, 2003; KASTAN et al., 2004). Collectively, these proteins function as key regulators of the DNA damage response, and a clear interdependency exists among them as inactivation of any renders cells hypersensitive to DSBs (PETRINI et al., 2003; SEDELNIKOVA et al., 2003; SHILOH et al., 2003; VAN et al., 2003; KITAGAWA et al., 2004).

It has been proved that ATM is absolutely required for repairing a subset of DSBs usually repaired slowly over 24 h (RIBALLO et al., 2004). Their results suggested that the majority of DSB rejoining in G1 cells is rapid, is LIG4 dependent, but has a less stringent requirement for DNA-PKcs. A distinct component of DSB rejoining (approximately 10%) occurs over a prolonged time and requires ATM, Artemis, and DNA-PKcs representing an "ATM-dependent" component of end-joining (RIBALLO et al., 2004).

2.3.2. γ-H2AX

The other one of the very early events following DSBs is the phosphorylation of histone H2AX (within minutes) to create γ-H2AX which functions to recruit DNA damage response factors to sites of DNA damage (SEDELNIKOVA et al., 2003; FERNANDEZ-CAPETILLO et al., 2004). Using a fluorescent antibody specific for the γ-H2AX, discrete nuclear foci can be visualized at sites of DSBs.

In eucaryotes, DNA is packaged into nucleosomes, which are in turn arranged in various higher order structures to form chromatin (PRUSS et al., 1995). The crystallographic structure of nucleosome has been elucidated (LUGER et al., 1997). Two copies of each histone protein, H2A, H2B, H3 and H4, are assembled into an octamer that has 145–147 base pairs (bp) of DNA wrapped around it to form a nucleosome core (of relative molecular mass 206K). In mammals, each histone family is encoded by multiple genes, which with few exceptions are expressed in concert with replication (HEINTZ et al., 1991). The various members of the H4, H3, and H2B families differ in few if any amino acid residues. In contrast, the H2A family includes three subfamilies whose members contain characteristic sequence elements that have been conserved independently throughout eucaryotic evolution (WEST et al., 1980;

THATCHER et al., 1994).The three H2A subfamilies are the H2A1-H2A2, the H2AZ, and the H2AX. Histone H2AX (REDON et al., 2002; SEDELNIKOVA et al., 2003), a minor histone H2A variant, encodes a conserved Ser-Gln-Glu (SQE) motif in the carboxyl-terminal tail. An early event in the DNA damage response after ionizing irradiation is the phosphorylation of serine 139 of H2AX (ROGAKOU et al., 1998; SEDELNIKOVA et al., 2002).

The phosphorylation is mediated by ATM (ROGAKOU et al., 1998; BURMA et al., 2001; SEDELNIKOVA et al., 2002), ATR (FURUTA et al., 2003), and/or DNA-dependent protein kinase (DNA-PK) (PARK et al., 2003), and occurs (in a domain of about one megabase) at the breaksites of DSB or single strand break (ROGAKOU et al., 1999). For many years, the analysis of DSBs repair relied on pulsed-field gel electrophoresis (PFGE), which reqired high irradiation doses (50Gy). Recently, the γ-H2AX foci have been used as an extraordinarily sensitive technique to detect DSB formation and repair even after very low dose irradiation (ROTHKAMM et al., 2003). DNA repair- and checkpoint associated proteins such as RAD50, RAD51 and BRCA1, as well as the 53BP1, colocalize with γ-H2AX (FURUTA et al., 2003; HUYEN et al., 2004).

γ-H2AX extends to megabase regions of DNA around the lesions and can be visualized by using immunofluororescence microscopy as discrete nuclear foci at the site of DSBs, either induced by exogenous agents such as radiation (BURMA., 2001) or generated endogenously during programmed DNA rearrangements (CHEN et al., 2000; PETERSEN et al., 2001; MAHADEVAIAH et al., 2001).

Recent studies found that ATM and DNA-PK function redundantly to phosphorylate H2AX in response to DSBs. This overlapping function is observed in human, mouse, and vertebrate cells. ATM and DNA-PK both contribute to H2AX phosphorylation in response to DSBs (STIFF et al., 2004). The precise biological function of the foci is still unclear but H2AX phosphorylation is required for the retention of several damage response proteins at the break site (BASSING et al., 2002; CELESTE et al., 2002; CELESTE et al., 2003). Initial studies observed a close correlation between the number of foci and the number of DSBs produced by decay of ^{125}I incorporated into cellular DNA (SEDELNIKOVA et al., 2002), suggesting that each focus may represent an individual DSB and that each break may form a focus. A recent study found that the correlation is close to a 1:1 ratio, strongly suggesting that the number of foci formed after ionizing radiation at low doses is similar to the predicted number of DSBs induction (ROTHKAMM et al., 2003).

While the phosphorylation of H2AX occurs rapidly, additional recruitment and retention of γ-H2AX at the site of DNA DSB is dependent on another DNA damage-response protein: mediator of DNA damage checkpoint protein one (MDC1) (STUCKI et al., 2004). The C-terminal tandem BRCT domain of MDC1 binds the C terminus of γ-H2AX, and without MDC1 the kinetics of formation and removal of γ-H2AX is altered (STUCKI et al., 2005; LOU et al., 2006). Thus, γ-H2AX serves as a recruitment factor for the assembly of multiple factors that collectively act to enhance the efficiency of DNA repair.

2.3.3. DNA-dependent protein kinase

The DNA-dependent protein kinase (DNA-PK) is comprised of a regulatory subunit, containing the Ku70/80 subunits, and a 470 kDa catalytic subunit, DNA-PKcs. Mammalian cells that lack either component of the DNA-PK complex are defective in repairing DNA DSBs and hence are sensitive to the effects of exposure to ionizing radiation (SMITH et al., 1999).

DNA-PKcs, ATM, and ATR are members of the phosphatidylinositol kinase family. These proteins exhibit serine–threonine protein kinase activity, and are involved in the regulation of transcription, cell cycle progression, and genomic stability (DASIKA et al., 1999). One of the roles of DNA-PKcs is to protect and align broken ends of DNA alone or as part of a multi-protein complex (DEFAZIO et al., 2002). DNA-PKcs is autophosphorylated after cellular irradiation exposure (CHAN et al., 2002; DOUGLAS et al., 2002), which modifies its binding with Ku and has been implicated in the phosphorylation of a wide range of DNA damage/checkpoint proteins. This auto-phosphorylation event is critical for correct NHEJ activity within the cell (CHAN et al., 2002). After DSBs, in the initial step Ku binds the DNA ends, aligns them and thus prepares for ligation and protects from degradation. Ku also recruits DNA-PKcs to the DSBs, activating its kinase function. Although the exact target proteins of DNA-PKcs remain to be established, it has been proposed that DNA-PKcs can: (i) phosphorylate XRCC4 and remove or relocate the ligase IV/XRCC4 complex from Ku-bound DNA ends and thus allow necessary processing steps to occur, (ii) regulate accessibility of DNA ends to processing by its inactivation *via* autophosphorylation and/or by allowing translocation of Ku away from the DSB, and (iii) phosphorylate both Ku70 and Ku80 with as yet unknown consequences yet (CALSOU et al., 1999; NICK et al., 2000). DNA-PKcs

phosphorylates Artemis, which changes the function of Artemis, but there is no evidence for *in vivo* phosphorylation or a direct role in NHEJ (MA et al., 2002). There are also many reports of proteins that can regulate DNA-PK activity (caspase-3, Chk1, HSF1, MDC1, and so on).

Thus, signaling after DSBs involves a coordinated series of events that occur rapidly, and serve to activate key cellular effectors that effect cell-cycle arrest to allow repair via HR or NHEJ, or alternatively activate an apoptotic response.

2.4. DSB repair-proficient and -deficient mouse strains

In the present study γ-H2AX foci analysis was employed to investigate the repair of DSBs in SCID (severe combined immunodeficiency), BALB/c and A-T (Ataxia Telangiectasia) mice in comparison to control C57BL/6 mice. The highly radiosensitive SCID mice have a DSBs repair deficiency caused by a spontaneous mutation in the gene encoding the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). DNA-PKcs is a core protein of non-homologous DNA end joining (NHEJ). In contrast to SCID mice, imbred BALB/c mice possess two naturally occurring single-nucleotide polymorphisms in the DNA-PKcs gene, that reduce, but do not eliminate DNA-PKcs activity (OKAYASU et al., 2000). BALB/c mice have been consistently found to be unusually sensitive to the lethal effects of radiation and to the development of various types of spontaneous as well as radiation-induced tumours. A-T mice, defective for the Ataxia telangiectasia mutated (ATM) protein, are characterized by an increased radiosensitivity and a predisposition to cancer. ATM is the central component of the signal induction pathway responding to DSBs (LÖBRICH et al., 2005). Recent findings show that ATM-defective cell lines display not only cell cycle checkpoint defects responsible for their pronounced radiosensivity, but also a significant DSB repair defect (KÜHNE et al., 2004).

2.5. Diseases with DSB repair deficiencies

Human diseases can occur when components of the DNA DSB pathway are dysfunctional. Deficiencies in repairing DSBs lead particularly to pronounced clinical radiosensitivity. Most of these diseases show similar *in vitro* cellular phenotypes, such as sensitivity to ionizing radiation and other DSB-inducing chemicals, cell cycle checkpoint defects or a high frequency of chromosomal breaks and rearrangement.

2.5.1. Ataxia telangiectasia

Ataxia telangiectasia (A-T), a rare autosomal recessive syndrome resulting from mutations in the ATM kinase is a prime example of the consequences of an inappropriate response to DNA DSBs (CHUN et al., 2004; MCKINNON, 2004). A-T is a multisystem disorder that includes telangiectasia (dilated blood vessels, usually ocular), immunodeficiency and proneness to malignancies particularly lymphoma and leukemia. However, the most prominent clinical manifestation of A-T is the progressive ataxia characterized by the loss of Purkinje and granular cells in the cerebellum, confining A-T patients to the wheelchair generally by age of 10 years or younger. At cellular level, ATM deficiency is manifested by increased sensitivity to ionizing radiation and other agents that yield DNA DSBs but little or no hypersensitivity to other forms of DNA damage.

2.5.2. Ataxia telangiectasia-like disorder

Ataxia telangiectasia-like disorder (ATLD) shows similar features to those of A-T including neurodegeneration (TAYLOR et al., 2004). ATLD results from hypomorphic mutations of MRE11 that lead to attenuated levels of all three components of the MRN complex. Although an increased incidence of tumors is not reported in ATLD, the small number of individuals with this syndrome leaves this an open question. MRE11 is a member of the MRE11-RAD50-NBS1 (MRN) protein complex. MRN and its individual components are involved in responses to cellular damage induced by ionizing radiation and radiomimetic chemicals, including complexing with chromatin and with other damage response proteins, formation of radiation-induced foci, and the induction of different cell cycle checkpoints.

2.5.3. Nijmegen breakage syndrome

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder characterized by microcephaly, immunodeficiency, and an increased incidence of hematopoietic malignancies. NBS results from hypomorphic mutation of another MRN component, NBS1 (DIGWEED et al., 2004). NBS1 is located on human chromosome 8q21 and codes for a protein product termed nibrin, NBS1 or P95. Over 90% of patients are homozygous for a founder mutation: a deletion of five base pairs which leads to a frame shift and protein truncation. The protein NBS1 is suspected to be involved in the cellular response to DNA damage caused by ionizing irradiation, thus accounting for the radiosensitivity of NBS.

The phosphorylation of NBS1 by ATM would indicate that ATM acts upstream of the MRN complex. Consistent with this were the suggestions that ATM could be activated in the absence of fully functional NBS1 protein. In contrast, the regulation of some ATM target proteins, e.g. Smc1 requires the MRN complex as well as ATM. NBS1 may, therefore, be both a substrate for ATM and a mediator of ATM function. Recent studies that indicate a requirement of the MRN complex for proper ATM activation suggest that the relationship between ATM and the MRN complex in the DNA damage response is yet to be fully determined.

It has been proved that a small increased fraction of unrejoined double strand breaks and, more significantly, increased chromosome breaks in noncycling NBS cells at 24 h after irradiation (GIRARD et al., 2000). One of the NBS lines examined (347BR) was atypical in showing a nearly normal checkpoint response. In contrast to the mild checkpoint defect, 347BR displays marked γ-ray sensitivity similar to that shown by other NBS lines. Thus, the γ-ray sensitivity correlates with the repair defect rather than impaired checkpoint control. Taken together, the results provide direct evidence for a repair defect in NBS cells and are inconsistent with the suggestion that the radiosensitivity is attributable only to impaired checkpoint arrest. 347BR also displays elevated spontaneous damage that cannot be attributed to impaired G2-M arrest, suggesting a function of NBS1 in decreasing or limiting the impact of spontaneously arising double strand breaks.

2.5.4. LIG 4 Sydrome

Hypomorphic mutations of LIG4 that attenuate LIG4 activity lead to LIG4 syndrome whereby individuals display similar phenotypes to NBS such as unusual facial features, growth retardation and microcephaly (O'DRISCOLL et al., 2001). LIG4 is important in NHEJ and V(D)J recombination, and LIG4 syndrome patients display immunodeficiency; cells from these individual are radiosensitive and defective in NHEJ repair of DNA DSBs. Immunodeficiency also occurs in individuals with mutations in the XRCC4-binding protein, XLF/Cernunnos and a similar neuropathology to LIG4 syndrome is present (BUCK et al., 2006).

In a previous *in vitro* study, a DNA ligase IV (LIG4)-null human pre-B cell line and human cell lines with hypomorphic mutations in LIG4 have been shown to be significantly impaired in the frequency and fidelity of end joining (SMITH et al., 2003). Analysis of the null line demonstrates the existence of an error-prone DNA ligase IV-independent rejoining mechanism in mammalian cells. Analysis of lines with hypomorphic mutations demonstrates that residual DNA ligase IV activity, which is sufficient to promote efficient end joining, nevertheless can result in decreased fidelity of rejoining. Thus, DNA ligase IV is an important factor influencing the fidelity of end joining *in vivo*. The LIG4-defective cell lines also showed impaired end joining in an *in vitro* assay using cell-free extracts. Finally, they demonstrate that the ability of DNA ligase IV ± XRCC4 to protect DNA ends may contribute to the ability of DNA ligase IV to promote accurate rejoining *in vivo* (SMITH et al., 2003).

It has been shown that DNA ligase IV-deficient (180BR) cells have a pronounced repair defect after ionizing radiation doses between 10 and 80 Gy by PFGE. At higher doses, the mutation in 180BR cells leads to a more severe repair defect, indicating that the mutated DNA ligase IV protein can handle a small but not an excessive number of DSBs. The DSB repair of the LIG4 null pre-B cells N114P2 with targeted disruptions in both LIG4 alleles is significantly impaired in comparison with the time course of the DNA ligase IV-deficient primary fibroblasts. The difference is most pronounced for repair times of 8 and 24 h in N114P2 cells compared with 180BR cells, more than twice as many unrepaired DSBs (KÜHNE et al., 2004).

2.5.5. Artemis

Mutations in the Artemis protein in humans result in hypersensitivity to DNA double-strand break-inducing agents and absence of B- and T-lymphocytes (radiosensitive severe combined immune deficiency [RS-SCID]) (MOSHOUS et al., 2001). The Artemis nuclease has been described as an additional NHEJ component (MOSHOUS et al., 2001). Artemis cleaves DNA hairpin intermediates during V(D)J recombination in an ATM-independent manner (MA et al., 2002); however, it mediates the repair of a fraction (10%) of DSBs incurred after ionizing radiation in an ATM-dependent manner (RIBALLO et al, 2004). Current models suggest that Artemis functions to process the ends of otherwise nonligatable DSBs prior to ligation by core NHEJ factors (LÖBRICH et al., 2005). The mechanism of Artemis activation *in vivo* is unclear, although Artemis is rapidly hyperphosphorylated in an ATM-dependent manner after exposure to DSB-inducing agents (POINSIGNON et al., 2004; RIBALLO et al., 2004; ZHANG et al., 2004; CHEN et al., 2005; MA et al., 2005; WANG et al., 2005). ATM and other phosphatidylinositol 3-kinase like kinases (PIKKs), including DNA-PKcs, preferentially phosphorylate serine or threonine followed by glutamine (S/TQ) motifs. Artemis contains 10 such sites, of which eight are located in the C-terminal 200 amino acids. Artemis cDNA mutated in seven of these sites was able to complement the radiosensitivity of Artemis-deficient cells (POINSIGNON et al., 2004). Despite this, other studies have suggested that phosphorylation of Artemis by DNA-PKcs leads to endonuclease activation (MA et al., 2002; 2004; 2005).

In a previous study, the major DNA-PK and ATM phosphorylation sites within Artemis (S503, S516 and S645) was identified under physiologically relevant ionic conditions, and showed that ATM-dependent Artemis phosphorylation at S645 occurs *in vivo* (GOODARZI et al., 2006). ATM cannot substitute for DNA-PK to support Artemis activity *in vitro*, supporting the *in vivo* dependency upon DNA-PKcs.

2.6. Radiation-induced acute and late toxicities of normal tissues

Radiotherapy is an important curative and palliative modality in the treatment of cancer, but associated acute and late toxicities of neighbouring normal tissues limit the deliverable dose intensity. Early responses (acute toxicities) usually arise in rapidly renewing tissues where stem cells divide regularly and provide a steady supply of daughter cells differentiating into

functioning cells. The proliferative damage of radiation-vulnerable stem cells causes a transient decrease in the number of specific functioning cells, but this acute radiation response heals by proliferation of surviving stem cells. Late normal tissue responses (late toxicities) usually arise in slowly renewing tissues composed of highly differentiated cell populations performing specialized functions; only certain functional cells maintain the capacity of proliferation. In contrast to transient, clinically manageable acute responses, late normal tissue injury is often progressive and does not respond to treatment. Even though an interplay of various phenomena accounts for the pathogenesis of late normal tissue damage, the clinically observed striking heterogeneity between different organs may depend on the intrinsic radiosensitivity of their non-dividing functional cell populations. There is evidence that the cellular radiosensitivity may to a considerable extent be determined by the repair capacity of radiation-induced DSBs (BANATH et al., 2004; OLIVE et al., 2004; TANEJA et al., 2004; KLOKOV et al., 2006). Furthermore, other molecular and cellular mechanisms like cell cycle progression, growth factor signal transduction, and apoptosis may contribute to the intrinsic radiosensitivity (ROSEN et al., 1999; LÖBRICH et al., 2006).

In the present study, the DSB repair capacity of brain and lung tissue representative for late-responding organs characterized by clearly different clinical radiation responses and tolerance doses was analyzed (SCHERER et al., 1991). The highly complex organs lung and brain are composed of many different cell types, and our limited knowledge of their cellular population kinetics as well as their intrinsic cellular radiosensitivities is based predominantly on histopathological alterations observed in irradiated tissues (SCHERER et al., 1991). In clinical cancer treatment, the radiosensitive lung represents a critical tissue, with radiation pneumonitis and lung fibrosis representing two major, often lethal complications after radiotherapy in the thoracic region. The brain, in contrast, tolerates larger doses of radiation, but the functional impairment with neurological deficits developing in a slowly progressive manner over months to years represents a severe radiation sequelae.

3. Material and methods

3.1. Animals

12 weeks old C57BL/6 (wild-type, C57BL/6NCrl), BALB/c (BALB/cAnNCrl) and SCID (severe combined immunodeficiency, CB17/Icr-Prkdc scid/Crl) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Homozygous A-T (129S6/SvEvTac-A-T -tm1Awb-/J, homozygous recessive $Atm^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, Main, USA). The mice were housed 4-5 per cage in laminar flow hoods in pathogen-free rooms to minimize the risk of infections and maintained under identical housing conditions (temperature 22 ± 2^{o}C, 55 ± 10% humidity, and light-dark cycle 12:12) for the whole course of the experiments and supplied with standard laboratory diet and water. The mice were allowed to acclimatize from shipping for 1 week before use.

mice strain	number	dose(Gy)	time point (hour)
C57BL/6	15	2	0.5/2.5/5/24/48
	3	0	control
Balb/c	15	2	0.5/2.5/5/24/48
	3	0	control
A-T	10	2	0.5/2.5/5/24/48
	2	0	control
SCID	15	2	0.5/2.5/5/24/48
	3	0	control
C57BL/6	6	0.1/0.5/1.0	0.1
	2	0	0.1

Tab.1. Number of mice used for the different experiments.

For the DSBs induction, the brain and lung tissue of C57BL/6 mice was analyzed 10 min after irradiation with 0.1 Gy, 0.5 Gy and 1.0 Gy compared to unirradiated controls. For the DSB repair kinetics two (A-T) or three (C57BL/6, BALB/c, SCID) different mice per strain were analyzed at 0.5 h, 2.5 h, 5 h, 24 h and 48 h after 2 Gy irradiation. Sham-irradiated mice served as controls.

3.2. Radiation schedule

Before irradiation, the isodose distributions of the whole body irradiation were evaluated by ADAC Pinnacle three-dimensional treatment planning system (Fig 6). The dose distribution revealed that the 95% isodose enclosed the whole body of each individual mouse (Fig 7). A special plastic cylinder was used to restrain the mice, the cylinder's diameter was 20 cm and height was 5 cm. By the use of tissue-equivalent plastic material (thickness 1.5 cm), an acceptable dose uniformity throughout the whole body of the mice was achieved. The irradiation characteristics were as follows: beam energy 6 MV photons; dose-rate: 2.0 Gy/min; source skin distance (SSD): 99 cm; size of irradiation size: 30×30 cm^2. Groups of sham-irradiated served as experimental controls for each time period without anaesthesia. All animal studies were performed according to Institutional Animal Care and Use Guidelines, and the experimental protocol was approved by the Medical Science Animal Care and Use Committee of the University of Saarland.

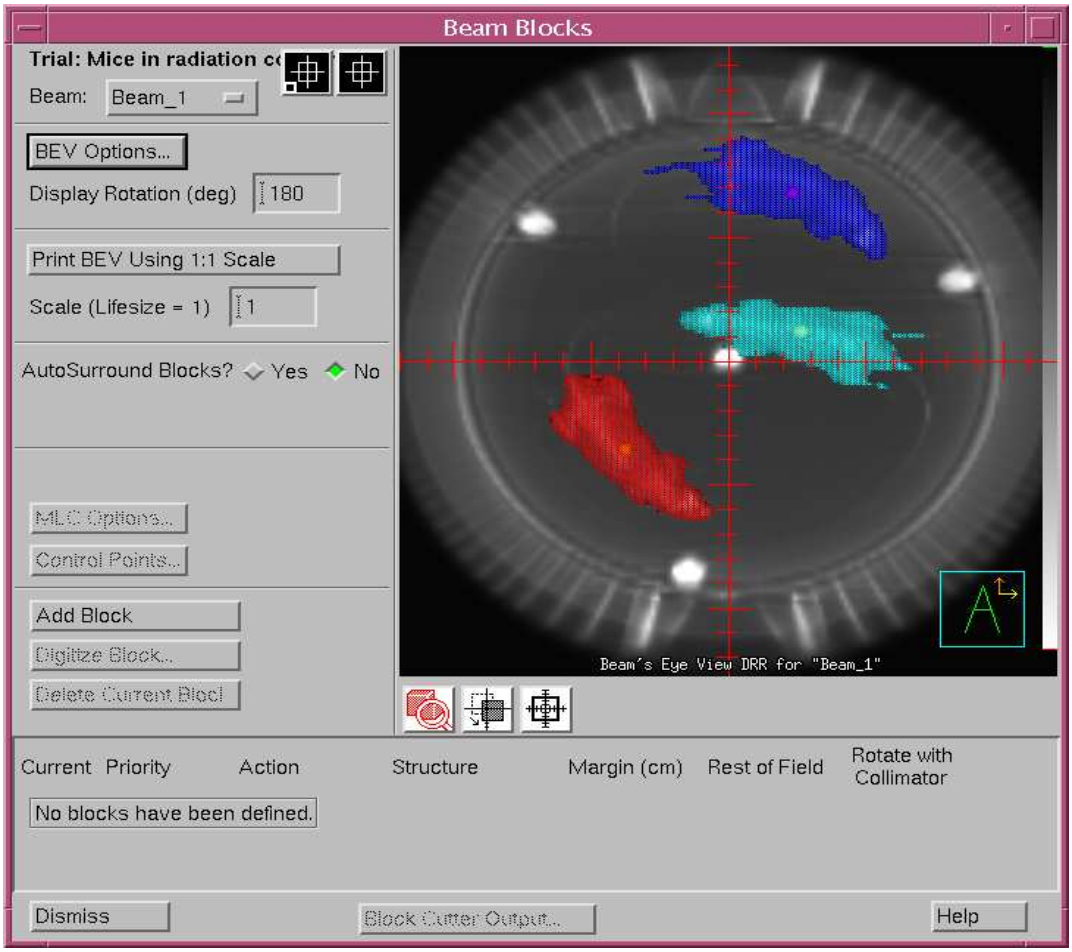

Fig. 6 CT plan of the whole body irradiation.

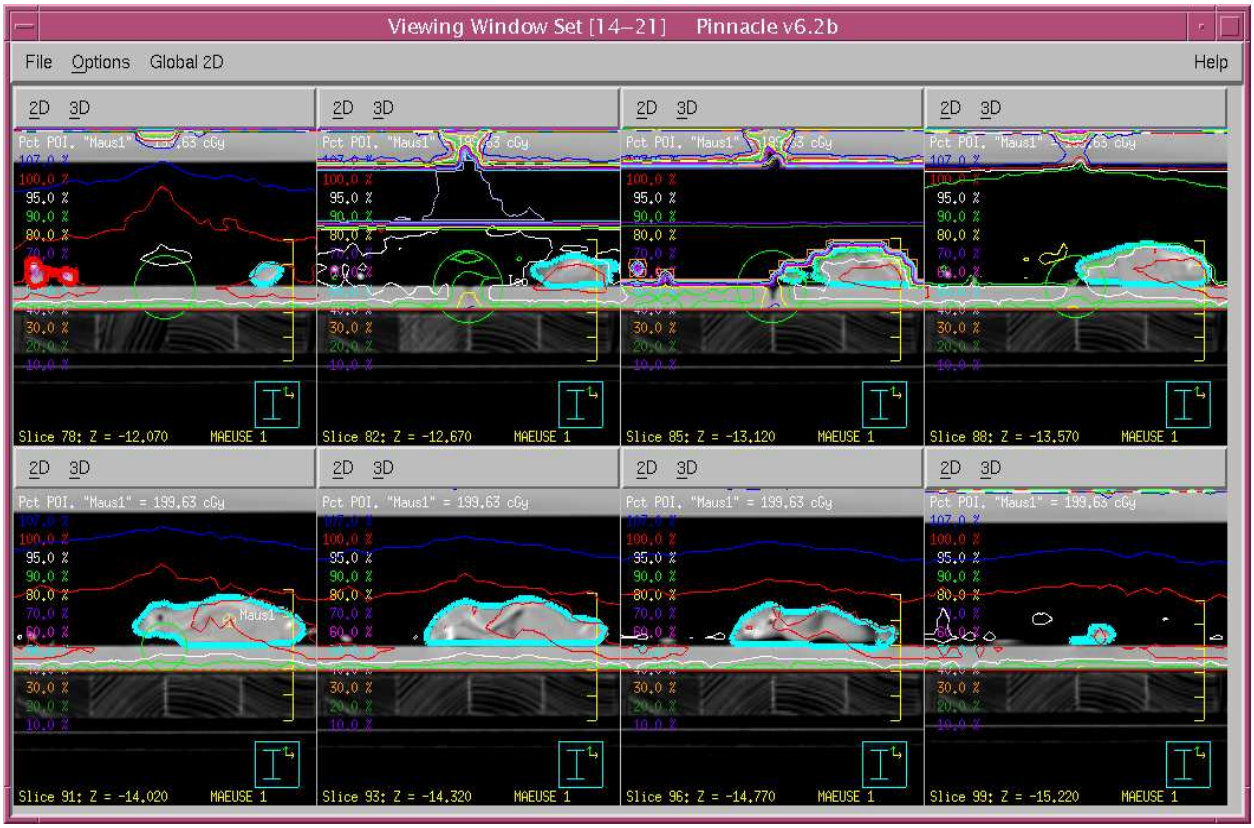

Fig. 7 The dose distribution revealed that the 95% isodose enclosed the whole body of each individual mouse.

3.3. Tissue sampling

After anaesthesia by an intraperitoneal injection of Rompun and Ketamine (Rompun 1ml and Ketamine 0.75 ml, diluted in 8.25 ml 0.9% natrium chloride solution, 0.1 ml/10 g), the blood was harvested by left ventricle puncture, and the different organs (lung, spleen, kidney, intestine, heart and brain) were quickly harvested. Each tissue was fixed in 4% neutral buffered formaldehyde overnight at room temperature and then cut into pieces. In order to analyze the different regions of brain, before embedding the brains were cut in four parts from rostral to caudal, the different sections contain the cerebral hemispheres, the hippocampus region, the ventricular system and the cerebellum. After dehydrated with a graded series of ethanol and xylene, the tissues were embedded in paraffin and cut into an average thickness of 4 μm sections.

3.4. γ-H2AX immunofluorescence staining

After deparaffinization and rehydration using the following series of washes: two xylene washes (10 min each), followed by 100% ethanol, 96% ethanol, 90% ethanol, 80% ethanol, 70% ethanol rinses (2 min each), the sections were washed by H_2O for 1 min. Antigen retrieval was performed by heating the sections for 60 min with citrate buffer (DAKO Retrieval puffer, #S-2031, Glostrup, Denmark pH 6.0) at 96°C for unmasking the antigenic sites. After washing the sections with PBS for 5 min on a shaker, samples were blocked with normal goat serum (cat. #642921 ICN, Irvine, CA, USA) at room temperature for 60 min for saturation of non-specific binding sites. Afterwards the tissue sections were incubated with primary mouse monoclonal antibody against γ-H2AX (antiphospho-H2AX [Ser 139]; Upstate Biotechnology, Lake Placid) at 1:1600 in PBS in the humidified chamber overnight at 4°C, then the sections were incubated with biotinylated Alexa Fluor 488-conjugated goat anti-mouse secondary antibody IgG (Invitrogen, cat. #A11001) diluted 1:200 in PBS in a humidified chamber for 60 min at room temperature in dark. Finally the sections were counterstained with DAPI and mounted in Entellan (Merck, Darmstadt, Germany) at 4°C overnight protected in the dark. Between each step the sections were washed by PBS (3x10 min).

3.5. γ-H2AX immunohistochemical staining

The formalin-fixed and paraffin-embedded tissue sections were dewaxed in xylene for 2 × 10 min and rehydrated in graded ethanols (100%, 96%, 90%, 80%, 70% for 2 min each). Then antigen retrieval was performed by heating the sections for 60 min with citrate buffer (DAKO Retrieval puffer #S-2031, Glostrup, Denmark, pH 6.0) at 96°C. After washing slides with PBS for 5 min on a shaker, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. The sections were blocked with the normal rabbit serum (cat. #642921 ICN, Irvine, CA, USA) at room temperature for 60 min for saturation of non-specific binding sites after rinse water washing 2 min. The primary anti-γ-H2AX antibody (antiphospho-H2AX [Ser 139]; Upstate Biotechnology, Lake Placid) diluted 1:800 in PBS was applied to each section and incubated overnight in the humidified chamber overnight at 4°C. After washing with PBS 3 × 10min, the sections were incubated with biotinylated goat-anti-rabbit antibody (Invitrogen, cat. #A11001) diluted 1:200 in PBS in a humidified chamber for 60 min at room

temperature, then incubated with avidin-biotin-peroxidase complex (ABC complex, Dako, Glostrup, Denmark) for 30 mins at room temperature. After washing with PBS 2 × 10 min, the sections were added with diaminobenzidine (DAB, Sigma) and then put into PBS to stop the reaction as soon as brown colour appeared. Finally, the sections were counterstained with hematoxylin for 45 seconds. After rinsing for 2 min under floating water, the sections were dehydrated with ascending alcohols and mounted in Entellan (Merck, Darmstadt, Germany).

3.6. Quantification of γ-H2AX foci

Fluorescence images were captured by using Nikon E600 epifluorescent microscope equipped with charge-coupled device camera and acquisition software (Nikon, Düsseldorf, Germany). For quantitative analysis, γ-H2AX foci were counted by eye using objective magnification of x60 and x100. For each data point, the brain and lung tissue of two (A-T) or three (C57BL/6, SCID, BALB/c) different mice were analyzed. Cell/foci counting was performed until at least 40 cells and 40 foci (blood lymphocytes) or 80 cells and 40 foci (tissue) were registered, the error bars represent the SEM from the number of cells analyzed, for data points obtained after background substraction.

4. Results

4.1. γ-H2AX immunohistochemistry and γ-H2AX immunofluorescence

To examine DSB repair kinetics in complex organs, γ-H2AX immunohistochemistry and γ-H2AX immunofluorescence detection was established in brain and lung tissue. Combining these two staining techniques permits both the accurate identification of cells in the complex tissue morphology and the precise quantification of γ-H2AX foci numbers per cell.

Figure 8 shows the immunohistochemical staining of γ-H2AX in the brain and lung tissue of animals analysed at 0.5 h and 5 h post 2 Gy irradiation compared to unirradiated controls. While unirradiated tissues were almost completely negative for γ-H2AX, the degree of γ-H2AX staining clearly increased with irradiation dose and decreased with post-irradiation repair time. After testing different antigen retrievals and optimising staining procedures that nearly 100% of the cells in the brain, and lung stained positively was achieved for γ-H2AX at 0.5 h after irradiation with 2 Gy (Fig. 8). Figure 9 shows representative examples of γ-H2AX immunofluorescence staining in the different tissues at 0.5 h and 5 h after irradiation with 2 Gy compared to unirradiated controls. While unirradiated normal tissues were predominantly negative for γ-H2AX, a homogeneous pattern of discrete nuclear γ-H2AX foci was observed at 0.5 h post irradiation. Additionally, a reduction in foci number was apparent between 0.5 h and 5 h in all analyzed tissues (Fig. 9) suggesting that γ-H2AX immunofluorescence analysis can be used to quantify DSBs. γ-H2AX immunohistochemistry, in contrast, does not provide a quantitative assessment of DSBs but can assist in identifying the different cell types of the tissues.

In the brain**,** counting γ-H2AX foci in various regions composed of different cell types revealed identical repair kinetics (data not shown), consistent with results of Nowak *et al* (NOWAK et al., 2006). As a consequence, the enumeration of γ-H2AX foci was confined to the cerebral cortex of the midbrain, consisting of functional neuronal cells and supporting glial cells (Fig. 9). In the respiratory parenchyma of the lung, the alveoli are lined by very thin, membrane-like type I pneumocytes and endothelial cells. As their small, often overlapping cells impede the visual discrimination of different nuclei, the enumeration of γ-H2AX foci was confined to the bronchiolar epithelium where larger, more clearly separated nuclei allowed a reliable quantitative analysis (Fig. 9).

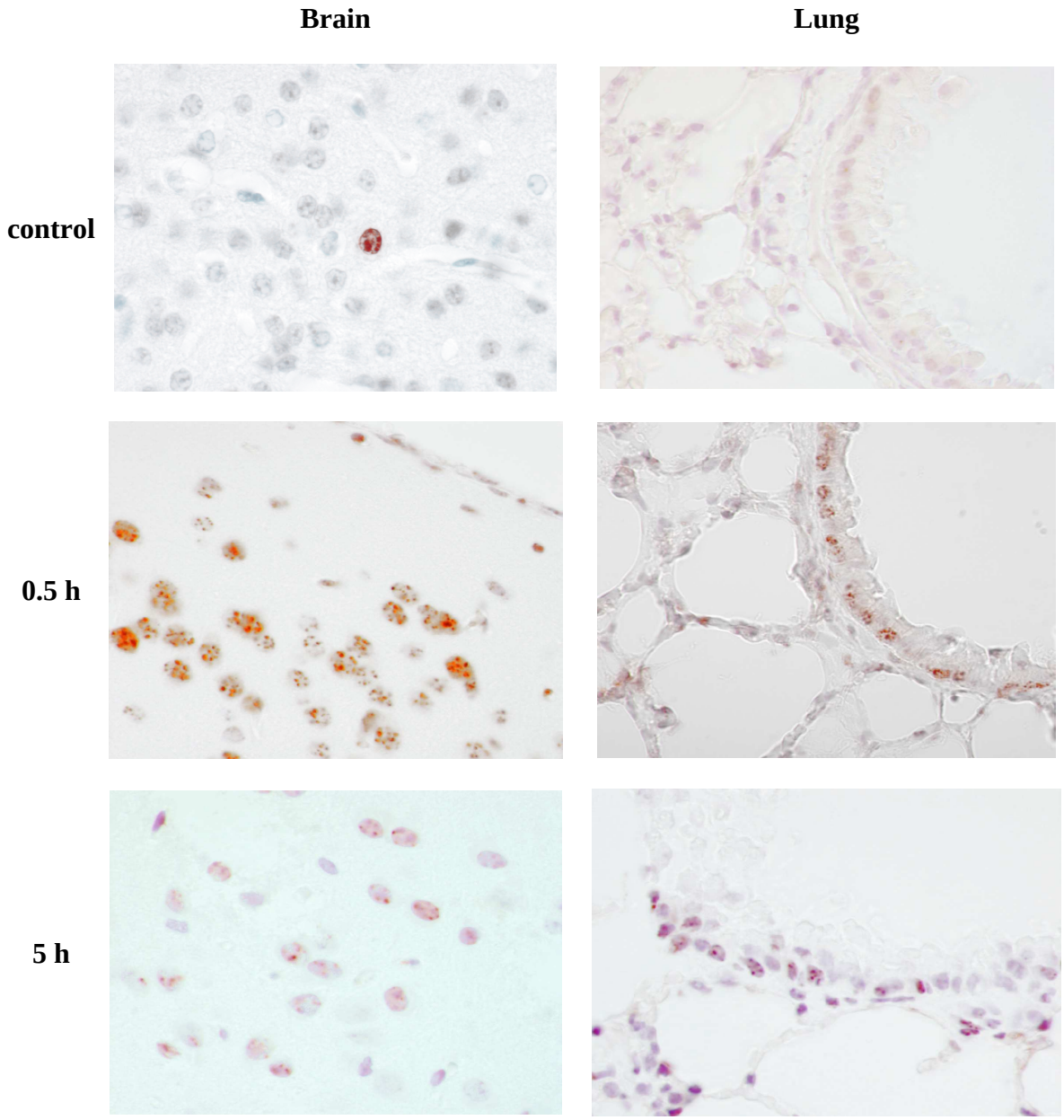

Fig. 8 Immunohistochemical staining of γ-H2AX in brain and lung tissue at 0.5 h and 5 h after irradiation with 2 Gy compared to unirradiated controls. While unirradiated normal tissues were almost completely negative for γ-H2AX, an intense granular staining of nearly all nuclei was apparent after irradiation. Original magnification x600.

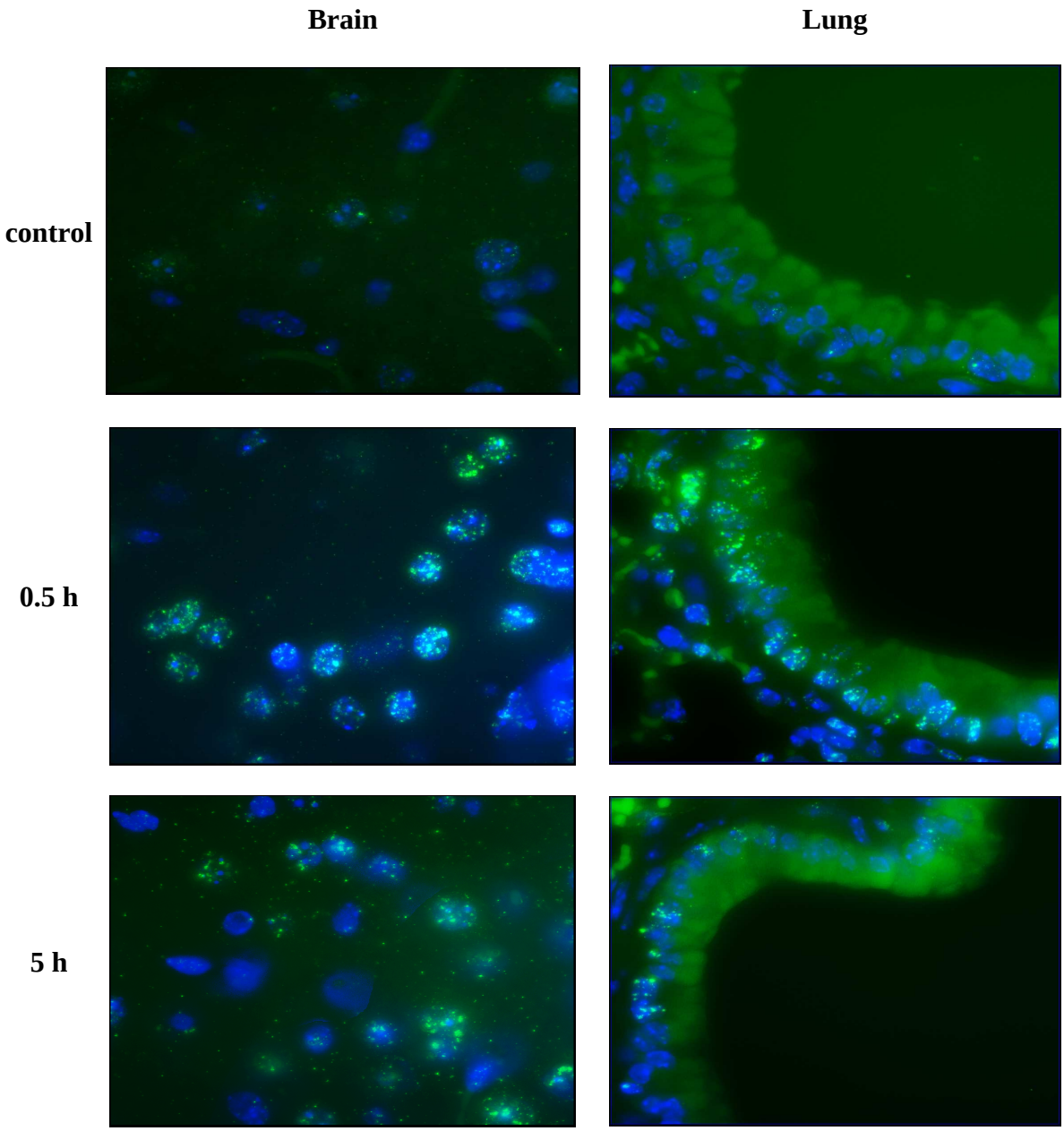

Fig. 9 Immunofluorescence staining of γ-H2AX in brain and lung tissue at 0.5 h and 5h after whole body irradiation with 2Gy compared to unirradiated control. While unirradiated normal tissues were predominantly negative for γ-H2AX, a homogenous pattern of discrete nuclear γ-H2AX foci was observed at 0.5 h postirradiation. The reduction in foci number was apparent between 0.5 h and 5 h in all analysed tissues suggesting that γ-H2AX immunofluorescence analysis can be used to quantify DSBs.

4.2. DSB induction in brain and lung tissue

For quantifying the DSB induction γ-H2AX foci were enumerated in the brain and lung tissues at 10 min following whole body irradiation of C57BL/6 mice. 10 min was the minimum time necessary to harvest the mice and retrieve the organs. Figure 10 shows the quantitative relationship between the number of γ-H2AX foci per cell of the brain and lung and the irradiation dose. For brain and lung tissue the same linear dose correlation from 0.1 Gy (≈ 0.8 foci/cell) to 1 Gy (≈ 8 foci/cell) and very low background levels with approximately 0.04 foci per cell were observed. The mean foci numbers in the brain were 0.93 ± 0.10, 4.20 ± 0.13 and 8.13 ± 0.09 respectively, and the corresponding values in lung were 0.90 ± 0.06, 4.23 ± 0.30 and 8.10 ± 0.15 after 0.1 Gy, 0.5 Gy, 1.0 Gy irradiation.

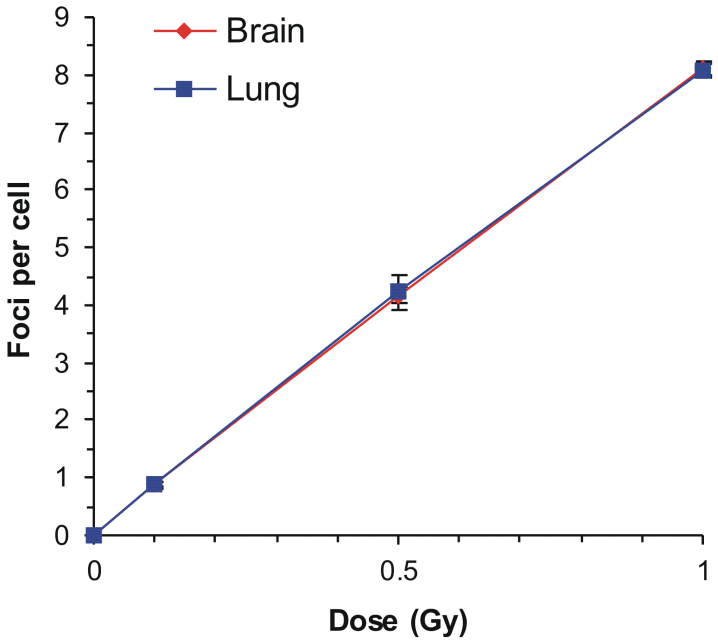

Fig. 10 DSB induction quantified by enumerating γ-H2AX foci in brain and lung tissue at 10 min following whole body irradiation of C57BL/6 mice. Brain and lung tissue reveal the same linear dose correlation from 0.1 Gy to 1 Gy. Error bars represent the SEMs of three experiments.

4.3. DSB repair in different mouse strains

The DSB repair kinetics of the different mouse strains are shown in figure 11, evaluated by counting γ-H2AX foci in the brain and lung tissue at defined time points (0.5 h - 48 h) postirradiation. Significantly, brain and lung tissue revealed nearly identical kinetics for γ-H2AX foci loss which were similar to the kinetics obtained in peripheral blood lymphocytes (measured in previous experiments). Wild-type C57BL/6 mice exhibited the fastest decrease in foci number with time, and displayed only low levels of residual damage at 24 h and 48 h postirradiation. In contrast, SCID mice showed highly increased γ-H2AX foci levels at all repair times (0.5 h to 48 h) while A-T mice exhibited a lesser defect which was most significant at later repair times (≥ 5 h). Interestingly, the magnitude of the A-T repair defect *in vivo* is similar to the ~10-15% repair defect previously obtained with *in vitro* studies (estimated from ~2-3 additional foci at 24 h and 48 h, and ~20 initial foci at 5-10 min post 2 Gy) (KÜHNE et al., 2004; RIBALLO et al., 2004). Similar to our lymphocyte data (Fig. 12), radiosensitive BALB/c mice exhibited slightly elevated foci numbers compared with C57BL/6 mice at 5 h and 24 h but not at 48 h postirradiation.

In summary, the DSB repair kinetics of the different mouse strains measured in the brain and lung tissue were qualitatively and quantitatively similar to the kinetics obtained from peripheral blood lymphocytes. Therefore, data obtained in lymphocytes can be utilized to predict the DSB repair capacity of complex solid tissues of different organs. Unexpectedly, the analyzed tissue specimens of the brain and lung revealed nearly identical DSB repair kinetics, even though these organs are characterized by clearly different clinical radiation responses (Fig 13).

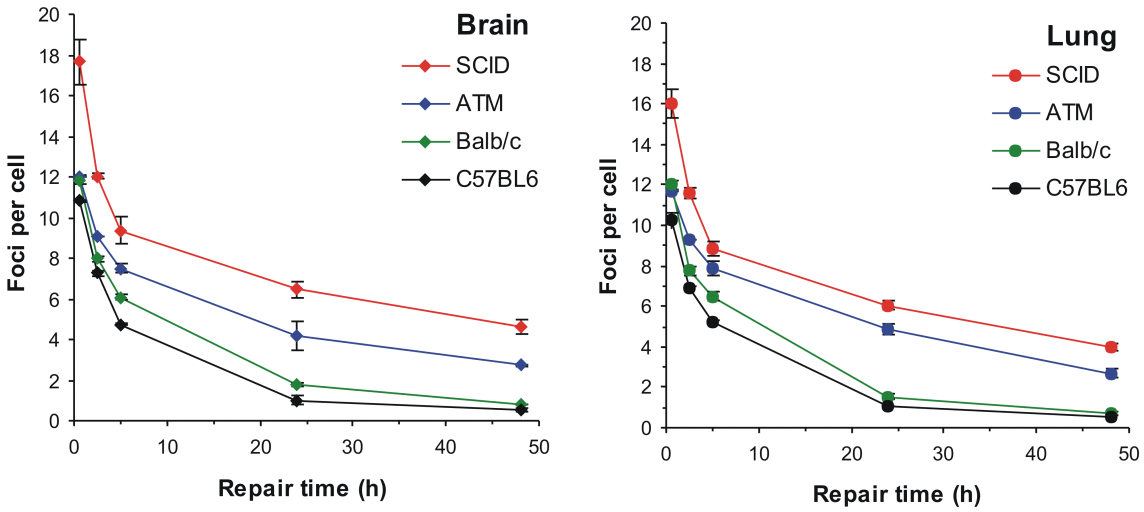

Fig. 11 DSB repair kinetics of the different mouse strains evaluated by counting γ-H2AX foci in the brain and lung at defined time-points after whole body irradiation with 2Gy. Similar to the kinetics obtained in blood lymphocytes (measured in previous experiments), the different repair deficiencies of the analyzed mouse strains can be demonstrated in the various tissues in almost the same manner. Strikingly, the analysed tissues exhibited similar kinetics of γ-H2AX foci loss, despite their clearly different clinical radiation responses. Error bars represent the SEM from two (A-T) or three (C57BL/6, SCID, BALB/c) experiments.

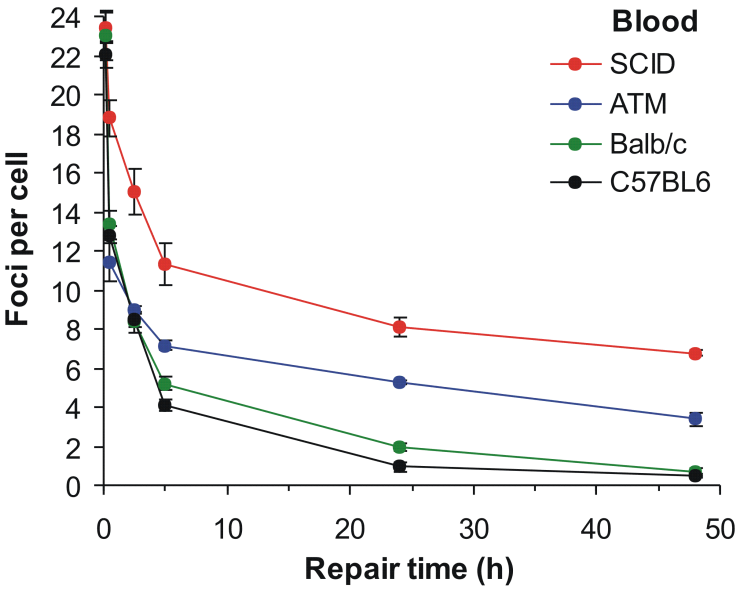

Fig. 12 DSB repair kinetics of the different mouse strains evaluated by counting γ-H2AX foci in the peripheral blood lymphocytes at defined time-points after whole body irradiation with 2Gy. The identical DSB formation was observed 5 min postirradiation in repair-proficient and -deficient mouse strains.

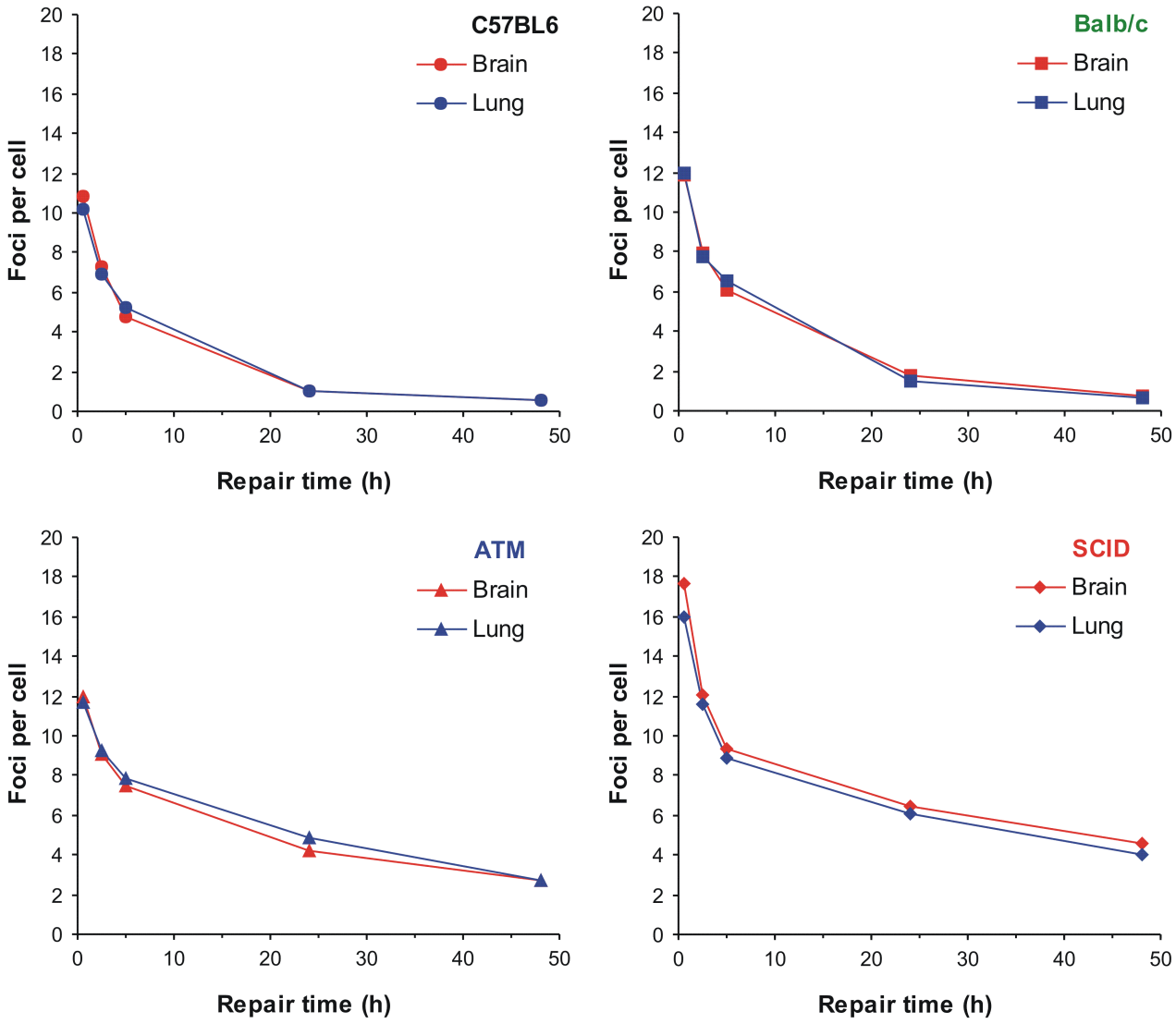

Fig. 13 The differences of DSB repair in brain and lung in different moouse strains. After 2 Gy irradiation at 0.5h, 2.5 h, 5 h, 24 h and 48 h, the DSB reapir of brain and lung in C57BL/6 mice, BALB/c mice, A-T mice and SCID mice. The data was derived from figure 11. There are no error bars displayed here in order to show the difference of DSB repair between the brain and lung.

5. Discussion

Efforts to measure the induction and rejoining of DSBs *in vivo* by PFGE or similar techniques have been discouraged so far by the high doses required, generally in excess of 50 Gy. The ability to detect DSBs by measuring γ-H2AX foci provides, for the first time, an opportunity to assess the induction and repair of DNA damage *in vivo* after relevant radiation doses.

5.1. γ-H2AX analysis as a predictive assay for radiosensitivity

Here, different DSB repair-proficient and -deficient mouse strains were used to test if γ-H2AX foci analysis can serve as a sensitive approach to assess DSBs *in vivo*, in normal tissues of various organs. Importantly, it is demonstrated that the different repair capacities can be detected by γ-H2AX foci analysis after whole body irradiation with 2 Gy. This includes the repair defect of SCID and A-T mice as well as the slight DSB repair impairment of BALB/c mice which is caused by a natural genetic variation in the NHEJ pathway (OKAYASU et al., 2000; MORI et al., 2001). Peripheral blood lymphocytes (as measured in previous experiments (KÜHNE et al., 2007)) and various normal tissues show nearly identical kinetics for γ-H2AX foci loss. This suggests that γ-H2AX foci analysis of peripheral blood lymphocytes is suitable to screen for various DSB repair deficiencies in other tissues.

5.2. Validation of γ-H2AX foci analysis in vivo

It has been shown that radiation-induced DSBs can be monitored in various normal tissues *in vivo*, directly by visualising γ-H2AX foci in formalin-fixed, paraffin-embedded tissue specimens. In contrast to previous studies quantifying relative amounts of γ-H2AX (QVARNSTRÖM et al., 2004; OLIVE et al., 2004), the absolute number of γ-H2AX foci formed per cell was counted. Thus, variations in staining intensity as well as background staining influence the quantification only to a minor degree. As expected, defined radiation doses induced identical numbers of DSBs per cell in the different tissues regardless of their intrinsic radiosensitivity. A clear linear dose correlation between 0 and 1 Gy in all analyzed

tissues was observed which underlines the precision of this approach. However, the γ-H2AX foci numbers counted in murine lymphocytes (measured in previous experiments: e.g. ~11.4 foci/cell/Gy after 5 min) and normal tissues *in vivo* were consistently lower compared to those observed in human lymphocytes (17.9 foci/cell/Gy after 5 min (LÖBRICH et al., 2005). For the most part, this difference can be attributed to the higher DNA content of human cells compared to murine cells (max. 3.2 Gb compared with 2.5 Gb [International Human Genome Sequencing Consortium 2001; Mouse Genome Sequencing Consortium 2002]). The remaining discrepancy can be explained through a slight background staining in the murine lymphocytes and the fact that partially truncated cells in the tissue sections were analyzed, which will lead to a slight underestimation of the real foci numbers.

In contrast to testis specimens where γ-H2AX is involved in meiotic recombination and accumulates in sex bodies resulting in high staining intensities (data not shown) (HAMER et al., 2003; MAHADEVAIAH et al., 2001), the background level of γ-H2AX foci in the normal tissues was very low, regardless of the proliferative capacity or the differentiation status. The low background foci level is remarkable and permits the accurate measurement of even very low radiation doses. It contrasts, however, to studies utilizing genomically unstable tumour cells which typically exhibit higher spontaneous γ-H2AX foci numbers (WARTERS et al., 2005; MAHRHOFER et al., 2006; YU et al., 2006).

Previous studies by us and others have established that γ-H2AX foci analysis represents a highly useful tool to monitor *in vitro* DSB induction and repair after low radiation doses (ROTHKAMM et al., 2003). Although regions of single-stranded DNA cause H2AX phosphorylation by ATR activation, these lesions are not generated by ionizing radiation to any significant extent. Moreover, ATR-dependent H2AX phosphorylation is typically observed after UV irradiation or replication stress and does not form discrete foci. Finally, the analysis of different mouse strains with defined mutations in DSB repair factors allowed us to correlate the rate at which γ-H2AX foci are lost with the DSB repair capacities of the different strains. Importantly, the kinetics for γ-H2AX foci loss reflect the different DSB repair capacities providing indirect evidence that γ-H2AX foci represent DSBs. Although it is possible that γ-H2AX foci analysis could overestimate DSBs remaining if repair is completed prior to the loss of visible foci, the time difference is unlikely to be greater than a few hours and would not affect our conclusions.

5.3. Similar DSB repair in tissues of differing radiosensitivity

This study was aimed to investigate tissue-specific differences in DSB repair and to ask if they correlate with the clinical radiation response. The clinical radiation response of normal tissues varies widely and likely depends both on the turn over times of the cell populations and the intrinsic radiosensitivity of the parenchymal cells. In cooperation with the working group of Prof. Löbrich, Institute of Biophysics, the DSB repair capacity of various organs representative for both early- and late-responding tissues were analyzed, characterized by clearly different clinical radiation responses (KÜHNE et al., 2007). The rapidly proliferating tissue of the small intestine is a radiosensitive, early-responding tissue. The cells in the crypts divide rapidly and provide a continuous supply of daughter cells that move up the villi, differentiate and mature into functioning cells. The radiation insult stops the mitotic activity in the stem compartement of the crypts, and therefore affects the cell renewal of the mucosal parenchyma. The brain, lung and heart by contrast, are late-responding tissues. The myocardium of the heart, for example, is composed entirely of mature, highly differentiated cells, which have lost the ability to divide. This fixed postmitotic cell population of myocytes is rather resistant to radiation. On the other hand, the highly complex organs lung and brain are composed of many different cell types, and our limited knowledge of their cellular population kinetics as well as their intrinsic cellular radiosensitivities is based predominantly on histopathological alterations observed in irradiated tissues (SCHERER et al., 1991). In clinical cancer treatment, the radiosensitive lung represents a critical tissue, with radiation pneumonitis and lung fibrosis representing two major, often lethal complications after radiotherapy in the thoracic region. The brain, in contrast, tolerates larger doses of radiation, but the functional impairment with neurological deficits developing in a slowly progressive manner over months to years represents a severe radiation sequelae.

Strikingly, it was observed nearly identical DSB repair kinetics regardless of the proliferative capacity, the differentiation status or the clinical radiation responses of the analysed tissues. In addition to differences in proliferation, parenchymal cells are characterized by varying intrinsic radiosensitivities which may be related to different DSB repair capacities. However, this has never been shown *in vivo*, in individual cells remaining in their complex tissue environment. Although we have been able to detect minor, genetically determined, DSB repair impairments (as shown for the BALB/c mice), we did not observe a significant difference in DSB repair kinetics between the various analyzed tissues characterized by clearly different

clinical radiation responses. These findings are of significant radiobiological interest because they challenge the widely held view that tissue-specific differences in radiosensitivity are related to differences in DSB repair. Thus, we conclude that the distinct radiosensitivity of parenchymal cells does not rely on tissue-specific differences in DSB repair and suggest that down-stream events determine their characteristic cellular response. Nevertheless, our analysis of different tissues exhibiting identical repair kinetics emphasizes the fundamental role of DSB repair to maintain genomic integrity, thereby contributing to cellular viability and tissue homeostasis.

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