DNA Repair 8 (2009) 253–261

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Analysis of the relationships between ATM and the Rad54 paralogs involved in homologous recombination repair

Michal Kirshner[a], Moran Rathavs[a,b], Anat Nizan[a], Jeroen Essers[c], Roland Kanaar[c], Yosef Shiloh[b], Ari Barzilai[a,*]

[a] Department of Neurobiology, George S. Wise, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
[b] Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
[c] Department of Cell Biology & Genetics, Cancer Genomics Center and Department of Radiation Oncology, Erasmus MC, 3000 DR Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 7 September 2008
Received in revised form 4 November 2008
Accepted 4 November 2008

Keywords:
Ataxia-telangiectasia, A-T
ATM
Rad54
Rad54B
Homologous recombination
DNA damage response

ABSTRACT

Ataxia-telangiectasia is a pleiotropic genomic instability disorder caused by lack or inactivation of the ATM protein kinase and characterized by progressive ataxia, immunodeficiency, ionizing radiation sensitivity and cancer predisposition. ATM mobilizes the cellular response to DNA double strand breaks by phosphorylating key players in this response. Double strand breaks are repaired by either nonhomologous end-joining or homologous recombination (HR) in which the Rad54 and Rad54B paralogs function. Here, we investigated the functional relationships between Atm and the Rad54 proteins by constructing compound genotypes in mice. Mouse strains were generated that combined inactivation of the *Atm*, *Rad54* and *Rad54B* genes. All mutant genotypes were viable, but obtained at sub-Mendelian ratios. Double mutants for *Atm* and each *Rad54* paralog exhibited reduced body weight and shorter lifespan, but no distinct neurological phenotype. Concomitant inactivation of ATM and Rad54 did not increase IR sensitivity; however, the triple *Atm/Rad54/Rad54B* mutant exhibited a significant IR hypersensitivity compared to the other genotypes. Interestingly, *Atm*−/− animals also exhibited hypersensitivity to the crosslinking agent mitomycin C, which was increased by deficiency of either one of the Rad54 paralogs. Our results reveal a differential interaction of the ATM-mediated DNA damage response and Rad54 paralog-mediated HR depending on the DNA damaging agent that initiates the response.

1. Introduction

DNA in living cells is under constant attack from damaging agents. One of the most cytotoxic lesions is the double strand break (DSB), caused by oxygen radicals, ionizing radiation (IR) and radiomimetic chemicals [1]. Unrepaired DSBs may lead to cell death or neoplasia. DSBs evoke the DNA damage response (DDR) – a network of signaling pathways that modulates numerous aspects of cellular metabolism. The DDR is thought to be a hierarchical process that is executed through a series of steps: lesions are detected by *sensor* proteins that subsequently mediate the activation of *transducers* that convey the damage signal to a multitude of downstream effectors [2–5].

The transducers of the DSB response belong to a conserved family of proteins that contain a phosphatylinositol-3-kinase-like domain, most of which possess serine/theronine kinase activity [6]. A prominent member of this family is the ataxia-telangiectasia mutated (ATM) protein, which responds primarily to DSBs [2,4,5,7]. ATM was identified as the product of a gene mutated in the human genetic disorder ataxia-telangiectasia (A-T), an autosomal recessive disorder characterized by progressive cerebellar ataxia, immunodeficiency, premature aging, gonadal dysgenesis, extreme radiosensitivity, genomic instability and a high incidence of lymphoreticular malignancies [8,9].

Eukaryotic cells use two main mechanisms to repair DSBs: nonhomologous end-joining (NHEJ), an error-prone ligation mechanism that acts throughout the cell cycle [10], and a high fidelity process based on homologous recombination (HR) between two sister chromatids, which is functional in the late S and G2 phases of the cell cycle [3,11]. Both processes are carried out by multicomponent protein complexes.

Homologous recombination repair (HRR) is carried out by members of the *RAD52* epistasis group. In yeast and mammals some of the proteins members of this group interact with each other during DSB recombinational repair [12–15]. Major players in this process are the MRN (Mre11-RAD50-NBS1/XRS2) complex, RAD51, RAD52 (in yeast), RAD54 and the RAD51 paralogs [11,16,17].

* Corresponding author. Tel.: +972 3 6409782; fax: +972 3 6407643.
E-mail address: barzilai@post.tau.ac.il (A. Barzilai).

1568-7864/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2008.11.005

The Rad51 protein functions in homology recognition and performs strand exchange between recombining DNA molecules. A pivotal intermediate in these reactions is the Rad51 nucleoprotein filament formed when Rad51 polymerizes on single-stranded DNA that results from damage processing [18]. Rad54 possesses DNA-dependent ATPase which allows it to translocate on DNA, thereby affecting DNA topology. Biochemically, Rad54 has been implicated in multiple steps of HR. It stabilizes the Rad51 nucleoprotein filament in an early stage of recombination [19], and subsequently promotes chromatin remodeling [20–22] and stimulates Rad51-mediated formation of a joint molecule between the broken DNA and the repair template, referred to as a D loop [23]. In later stages of the reaction it can displace Rad51 from DNA [24,25].

The human genome has two *RAD54* paralogs, *RAD54* and *RAD54B*, which encode proteins of 747 [26] and 910 [27] amino acids, respectively, that possess similar activities [28]. The central part of these proteins contains seven helicase motifs found in members of the SNF2/SWI2 family; their amino-terminal regions are less similar to each other [27]. The expression patterns of the two proteins are similar, but not identical and coincide with those of other members of the *RAD52* epistasis group [28].

An important strategy to learn about the physiological roles of human proteins and functional links between them is to ablate them in the mouse using gene targeting techniques and combine several such mutations in the same animal. Atm-null mice that were generated by disrupting the *Atm* locus recapitulate most of the features of the human A-T phenotype and display growth retardation, male and female infertility, extreme predisposition to thymic lymphomas, and acute sensitivity to ionizing radiation. However, the neurological symptoms of the disease are very mild in the corresponding animal model, and can be observed only after challenging tests [29,30].

Similar to Rad54−/− and Rad54B−/− mice, Rad54−/−/Rad54B−/− mice display no overt phenotypes and appear normal. Mice lacking both Rad54 and Rad54B are not sensitive to IR however they display extreme sensitivity to the interstrand DNA crosslinker mitomycin C (MMC) [28].

In order to study the functional relationships between Atm and the two Rad54 proteins, we created mouse strains lacking Atm and one or both of the Rad54 paralogs. The results of DNA damage sensitivity studies reveal complex interactions. ATM and the Rad54 paralogs appear to function in separate DSB response pathways and at least at the cellular level the presence of the Rad54 paralogs is detrimental in the absence of ATM.

2. Experimental procedures

2.1. Generation of the Rad54/Atm and Rad54B/Atm double mutant mice

All mouse strains in this study have identical 129Sv background. Rad54- and Rad54B-deficient mice were described previously [32], [28]. These male and female mice are fertile, while *Atm*−/− animals are sterile. Initial crossing brought Rad54 and Rad54B deficiencies onto *Atm*+/− background, and subsequent crossing led to double knockout mice that were deficient for Atm and one of the Rad54 paralogs. *Rad54*/*Rad54B*/*Atm* triple null mice were generated by crossing the *Rad54*−/−//*Rad54B*−/−//*Atm*+/− genotypes. The single knockouts and wild type (WT) animals served as controls throughout the study.

2.2. Genotyping using PCR

At 3 weeks of age tail DNA was prepared using Genelute mammalian genomic DNA kit (Sigma). PCR primers and conditions are provided in the Supplementary information. The reaction products were separated on 2% agarose gel in TAE running buffer at 150 V for 30–50 min and visualized by ethidium bromide staining.

2.3. Growth measurements and pathology

Mice were weighed weekly from weaning through adulthood, i.e., from 3 to 100 weeks of age. For pathological analysis mice were perfused with 4% formaldehyde in PBS, and fixed tissues were examined, embedded in paraffin, sectioned into 6 μm sections, and stained with hematoxylin and eosin. Immunohistochemistry was performed on paraffin sections of formaldehyde-fixed tissues using an immunoperoxidase complex procedure with diaminobenzidine as chromogen.

2.4. Treatment of mice with ionizing radiation (IR) and mitomycin C (MMC)

Sensitivity to IR and MMC was assessed in 1–2-month-old littermates, with 10–20 mice for each genotype. The animals were irradiated with 4 Gy using CLINAC1800 irradiator (Varian Medical Systems, Palo Alto, CA) and monitored for 21 days, or intraperitoneally injected with 7.5 mg/kg body weight of MMC and monitored for 14 days. Control mice were injected with the same volume of PBS. The animals were housed together, weighed and observed periodically. Statistical tests employed were Chi squared analysis and the Mann–Whitney two-tailed test.

2.5. Primary fibroblast culture

Ear skin fibroblast cultures from 1 to 3-month-old mice were prepared as previously described [33]. The cells allowed to proliferate and were used for experiments between passages 5 and 7. At that stage we did not notice spontaneous transformation. Cell concentration was determined using hemocytometer, and about 5×10^6 cells were plated in 75 cm^2 flasks containing DMEM, 25% FCS, non-essential amino acids, gentamycin (50 μg/ml), and amphotericin B (2.5 μg/ml).

2.6. Clonogenic survival assays

Fibroblasts at the plateau phase of growth were replated at high dilutions in 60-mm dishes, and 6 h later exposed to increasing doses of IR or MMC. The number of cells per dish was chosen to ensure several scores of colonies in the final counting. The cultures were incubated for about 12 days and stained with crystal violet. Colonies containing >50 cells were counted.

2.7. Metabolic measurements

Seven 1-month-old animals of each genotype were weighed and placed for 5 days in individual metabolic cages (Tecniplast, Bugtggiate, Italy), which allow separation and collection of feces and urine. Food and water consumption and body secretions were recorded daily, metabolic activity scores (food and water consumption vs. amounts of body secretions) were calculated for the 3rd and 4th day at the metabolic cages, and normalized against body weight.

2.8. Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde following permeabilization with 0.5% triton X-100. Slides were incubated for an hour with blocking solution (1% BSA, 10% serum and 0.25%

triton X-100) and subsequently with a primary anti-Rad51 antibody overnight. Immunofluorescence of Rad51 foci was visualized using a Zeiss LSM-510 confocal microscope (Oberkochen, Germany). Rad51 foci were counted using the Image-Pro Plus program (Media Cybernetics, Bethesda, MD). The areas of the nuclei were specifically labeled (see Supplementary data Figs. S4 and S5), the foci were defined and only those foci which meet this definition were counted.

2.9. Rota-rod test for motor coordination and balance

Mice were tested during the light phase of their light–dark cycle. The Rota-rod test [29] was performed in 1- and 4-month-old mice. The animal was placed on a rotating drum and a record was made of the time it was able to maintain its balance on the rod. Following 1 min of training with a Rota-rod speed of 10 RPM, the mice underwent three rotating trials at 2 h intervals. Each trial consisted of three cycles of 1 min rotation and 1 min rest. Latency to fall-off during rotation was recorded. Statistical analyses were performed using two-tailed Student's *t*-test.

2.10. Balance beam test for motor coordination and balance

Mice were tested during the light phase of their light–dark cycle. The balance beam test was performed in 1- and 4-month-old male animals. The animal was placed on a 100-cm long horizontal beam (0.3, 0.45 and 0.6 cm in diameter) suspended 50 cm above the ground, and the time it took the animal to walk from one end to the other was recorded. Each trial consisted of three cycles of 1 min walking and 5 min rest. Statistical analyses were performed using two-tailed Student's *t*-test.

3. Results

3.1. HRR inhibition combined with Atm deficiency slightly exacerbated the Atm knockout phenotype

ATM−/− mice are obtained in the expected Mendelian inheritance ratio [29]. However, deleting either one or both Rad54 paralogs resulted in a significantly lower than expected recovery of the ATM−/− genotype (Table 1). Whereas the lifespan of Rad54 null mice is very similar to that of WT mice, the lifespan of the Rad54B−/− mice is slightly but not significantly longer. Deletion of the *Rad54B* gene in *Atm*−/− mice slightly but not significantly reduced lifespan compared to the *Atm*−/− genotype. On the other hand, the lifespan of *Rad54*/*Atm* double null mice was significantly shorter than that of *Atm*−/− mice ($p < 0.008$) (Fig. 1). Given that cancer was the major cause of death in all mutant genotypes deficient in Atm, the cumulative survival analysis indicates that deletion of Rad54 on the background of Atm deficiency increases cancer predisposition. Pathological analyses have indicated that Rad54 deficiency does not change the spectrum of tumor types arising in the Atm-deficient background. Interestingly, deletion of Rad54, Rad54B or both on *Atm*+/+ background did not affect the lifespan of the animals nor their cancer predisposition. Collectively, our results suggest that abrogation of the Rad54-dependent HRR pathway by itself does not increase cancer predisposition in mice but does enhance the oncogenic effect of Atm deficiency.

Atm−/− mice are 10–20% smaller than their wild type littermates [29]. Inactivation of Rad54, Rad54B or both genes did not reduce the body weight of the animals compared to the wild type genotype. Loss of Rad54 and Rad54B in Atm deficient mice affected the growth of both females and males compared to *Atm*−/− single mutants (Fig. 2). Importantly, there were no differences between the various genotypes in food consumption and excretions (data not shown).

Table 1
Genotype distribution of offspring in the various matings.

Genotype	Expected	Observed
Rad54/Atm		
−/−//+/+	65.75	74 (125%)
−/−//+/−	131.50	151 (115%)
−/−//−/−	65.75	38 (58%*)
Rad54B/Atm		
−/−//+/+	83.50	99 (118%)
−/−//+/−	167.00	180 (108%)
−/−//−/−	83.50	55 (66%*)
Rad54/Rad54B/Atm		
−/−//−/−//+/+	30.50	38 (125%)
−/−//−/−//+/−	61.00	72 (118%)
−/−//−/−//−/−	30.50	12 (39%*)

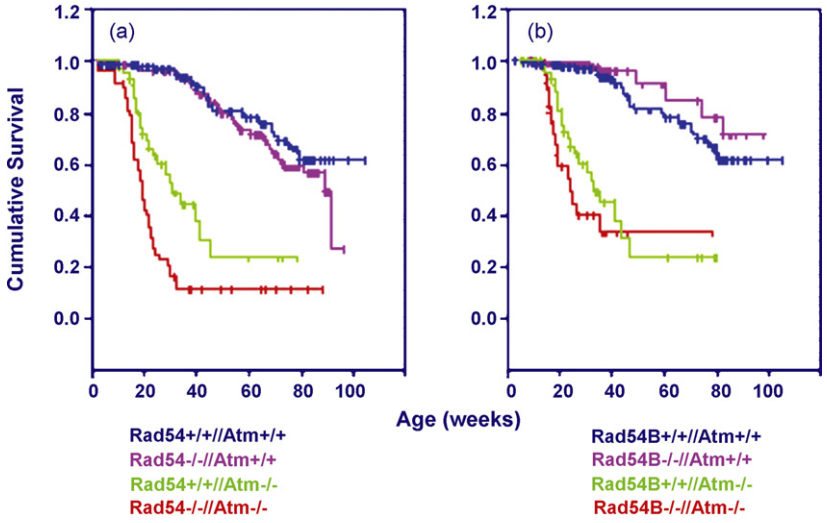

Fig. 1. Lifespan of mice with different Rad54/Atm and Rad54B/Atm genotypes. The cumulative survival of the Rad54/Atm (a) and Rad54B/Atm (b) genotypes plotted according to Kaplan–Meier analysis. Statistical significance was calculated using log-rank. The lifespan of the Rad54/Atm genotype is significantly shorter than that of Atm−/− mice ($p < 0.008$).

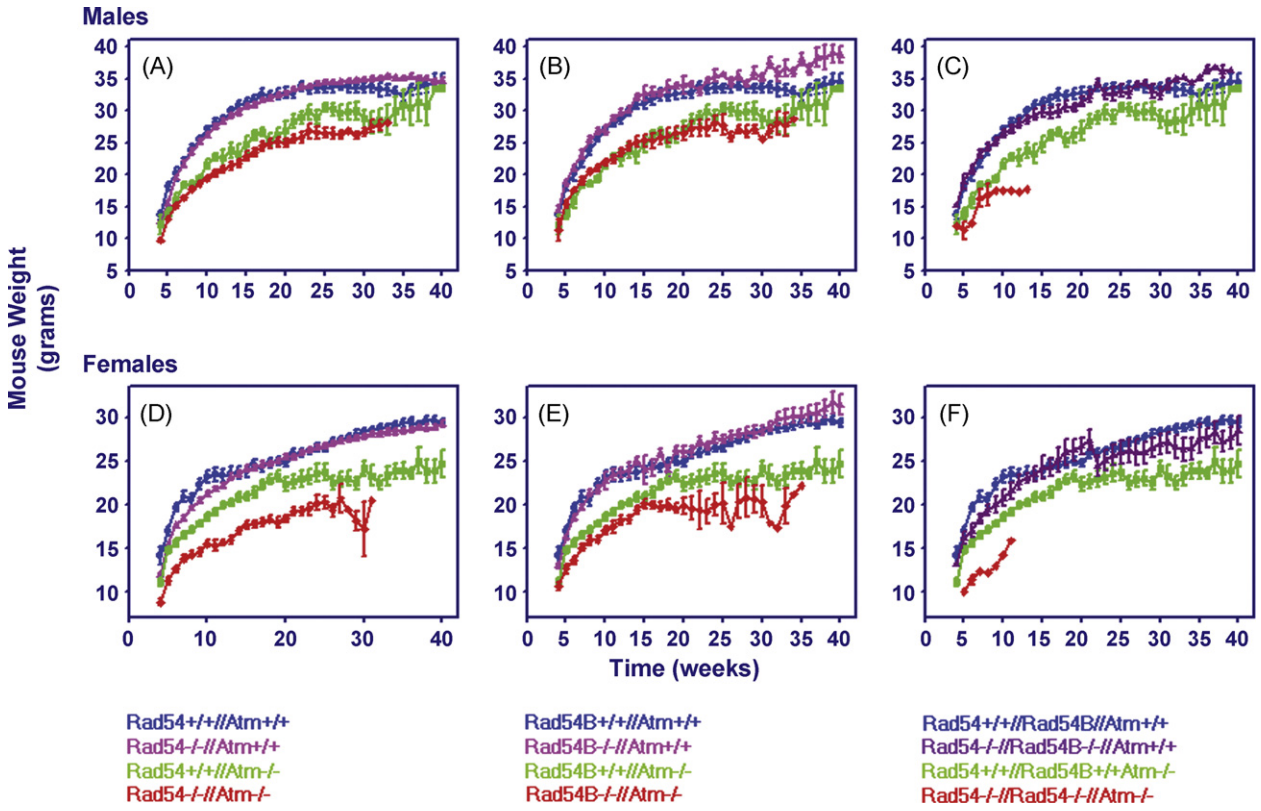

Fig. 2. Somatic growth of mice with various Rad54/Rad54B/Atm genotypes. Weight of the mice was measured from 3 to 40 weeks of age. Atm-deficient mice are 10–20% smaller than wild type animals. The removal of either Rad54 or Rad54B did not seem to contribute to growth retardation, but the combination of Rad54 or Rad54B and Atm deficiencies resulted in further growth retardation of about 10% compared to Atm-null mice. The effects of combined Rad54/Atm or Rad54B/Atm deletions were more profound in females. Error bars represent the SEM. Statistical analyses were performed with two-tailed Student's *t*-test (n = 10-20 mice).

Atm-deficient mice exhibit very minor, if any, neurological deficits [29,34]. We used the Rota-rod and balance beam tests to examine whether abrogation of HRR by Rad54 paralog deletion on *Atm*−/− background would affect the motor skills of the animals. All genotypes generated in this study demonstrated similar motor skills (not shown), and cerebellar histological analysis revealed similar numbers of Purkinje cells in all these genotypes (not shown). We conclude that the combined effect of these genotypes did not cross the threshold for exerting any overt effect on the structure and function of the mouse nervous system.

3.2. Effect of compound genotypes on organismal and cellular IR sensitivity

Hypersensitivity to IR is one of the hallmarks of A-T patients, Atm-deficient mice and cells lacking ATM [5,35]. To assess the effect on radiosensitivity of HRR abrogation when combined with Atm deficiency, animals with various Rad54/Rad54B/Atm genotypes were exposed to 4 Gy of IR and their survival was monitored. While Atm-deficient animals exhibited the expected profound radiosensitivity, IR sensitivity of Rad54- or Rad54B-deficient mice was similar to that of wild type animals, and combined defi-

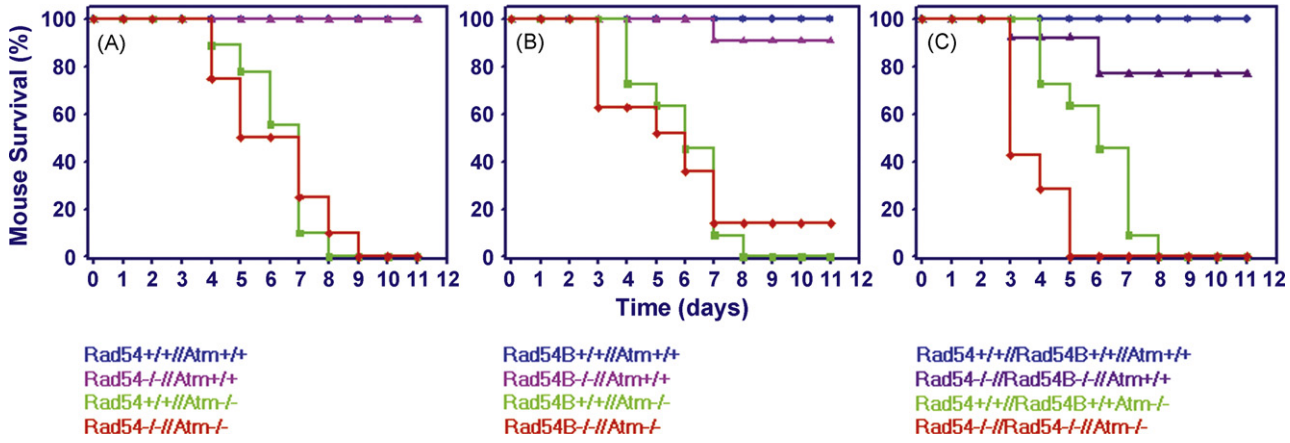

Fig. 3. Survival curve of various Rad54/Rad54B/Atm genotypes following IR treatment. Mice with the indicated Rad54/Rad54B/Atm genotypes were irradiated with 4 Gy of IR (10–20 mice of each genotype). Rad54/Rad54B/Atm triple mutant mice were significantly more sensitive to IR than Atm−/− animals. Using the Mann–Whitney test (two-tailed), assuming non-parametric data, the differences between the double or the triple null mice and the Atm−/− genotype were significant as noted in the text.

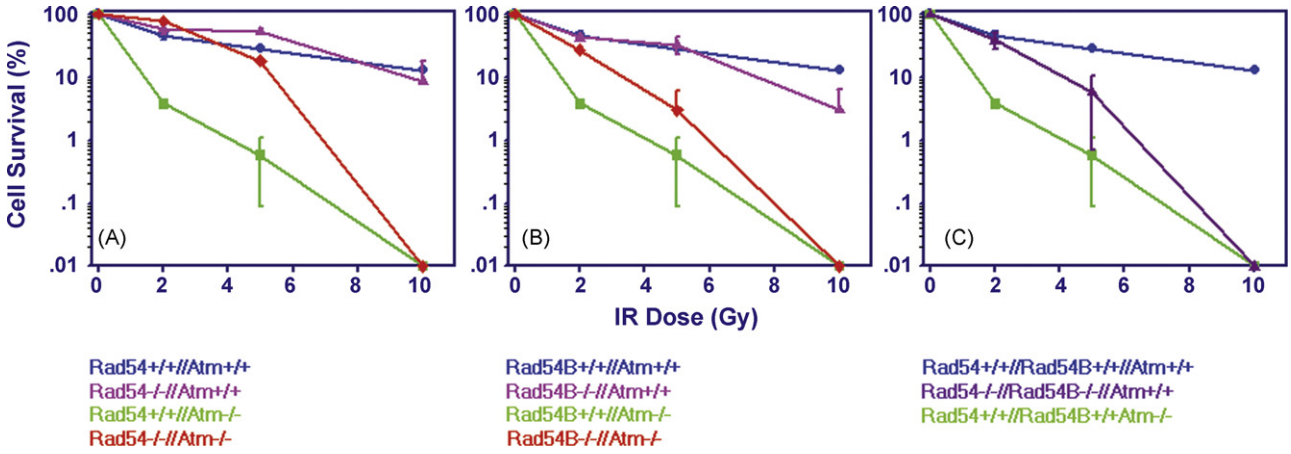

Fig. 4. Cellular radiosensitivity of various Rad54/Rad54B/Atm genotypes. Survival curves of fibroblast cells treated with IR while growing exponentially and asynchronously. Wild type cells, Rad54−/− and Rad54B−/− cells did not differ in radiosensitivity according to this assay. Rad54/Rad54B double null displayed higher radiosensivity than control cells. Atm−/− cells exhibited the expected radiosensitivity. Deletion of Rad54 or Rad54B on the background of Atm deficiency conferred protection against the toxic effects of IR.

ciencies of Rad54 and Rad54B slightly radiosentitized the mice (Fig. 3A–C). These results suggest that the contribution of Rad54 paralog-mediated HRR to the repair of IR-induced DSBs in mouse tissues is minimal [28,36]. Importantly, Rad54−/−//Atm−/− mice displayed radiosensitivity similar to that of Atm-null mice, while Rad54B−/−//Atm−/− mice were only very moderately, but significantly, hypersensitive to IR compared to Atm-null animals ($p < 0.04$) (Fig. 3A and B). In contrast, ablation of both Rad54 paralogs led to an increased radiosensitivity of Atm−/− mice. The mortality of the Rad54/Rad54B/Atm triple null mice following IR treatment was significantly higher than that of the Atm−/− mice ($p < 0.003$) (Fig. 3C). Thus, the impact of loss of both paralogs is enhanced by loss of Atm for IR sensitivity.

To compare the organismal sensitivity to IR of these genotypes to the sensitivity of the corresponding cell lines, we isolated fibroblast lines from the animals and tested their clonogenic survival following treatment with increasing IR doses. All single mutants recapitulated the results obtained at the organismal level. The Rad54 paralog mutant cells were not IR hypersensitive, while Atm deficient cells were (Fig. 4A and B). In addition, as observed with the mice, cells lacking both Rad54 paralog were slightly IR hypersensitive (Fig. 4C). Fibroblasts isolated from the Rad54/Rad54B/Atm triple null mice hardly proliferated and therefore their radiosensitive could not be assessed (Supplementary Fig. S1). Interestingly, however, ablation of any of the two Rad54 paralogs on *Atm*−/− background did not reduce cellular survival following IR treatment, but at intermediate doses of radiation reduced the radiosensitivity of Atm-deficient fibroblasts (Fig. 4).

3.3. *Effect of compound genotypes on organismal and cellular MMC sensitivity*

Mice deficient in Rad54 or Rad54B are hypersensitive to MMC [28,36]. Although Atm is thought to respond primarily to DSBs and not to interstand crolsslinks (ICLs) caused by MMC, a crosstalk between the pathways that respond to these lesions has been extensively documented [37,38]. We examined MMC sensitivity of mice with genotypes combining Atm and Rad54 or Rad54B deficiencies. Mice were injected intraperitoneally with 7.5 mg/kg body weight of MMC and monitored for 14 days. Surprisingly, Atm-deficient mice reacted more adversely to this treatment compared to wild type animals (Fig. 5). Inactivation of each Rad54 paralog rendered the animals MMC hypersensitive, while inactivation of both paralogs resulted in a more extreme MMC hypersensitivity, as previously established [28]. Interestingly, deficiency of each of the Rad54 paralogs acted additively with Atm loss to increase MMC

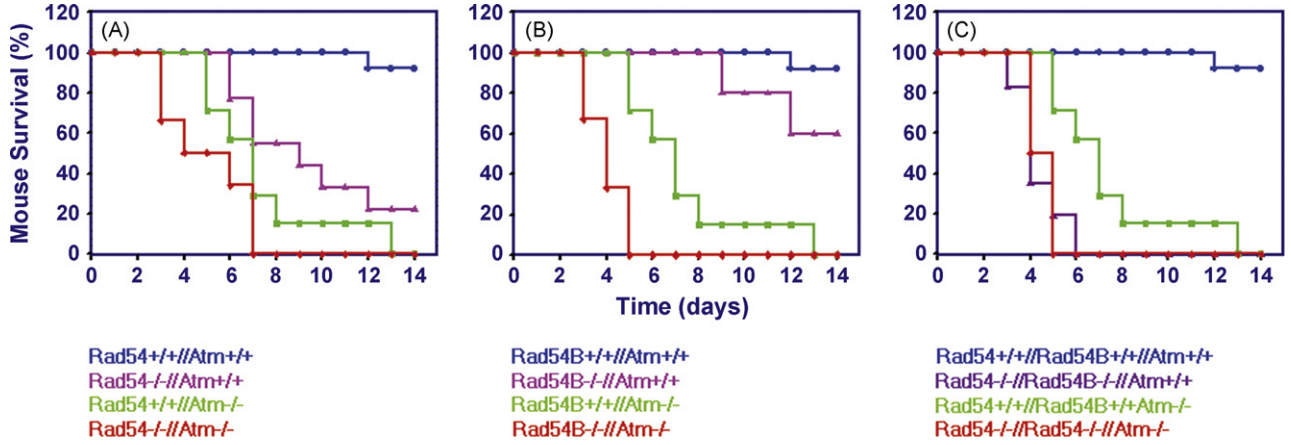

Fig. 5. Survival curve of various Rad54/Rad54B/Atm genotypes following MMC treatment. Mice with the indicated Rad54/Rad54B/Atm genotypes were injected peritoneally with MMC (7.5 mg/kg body weight, 4–11 mice of each genotype). Surprisingly, Atm−/− mice were more sensitive to MMC injection than Rad54−/− and Rad54B−/− mice. MMC-injected Rad54/Rad54B double mutant mice displayed markedly shorter survival rate than Atm−/− mice, and the rate was similar to that of Rad54/Rad54B/Atm triple mutant mice. The survival rate of MMC-injected Rad54/Atm and Rad54B/Atm was shorter than that of Atm−/− mice.

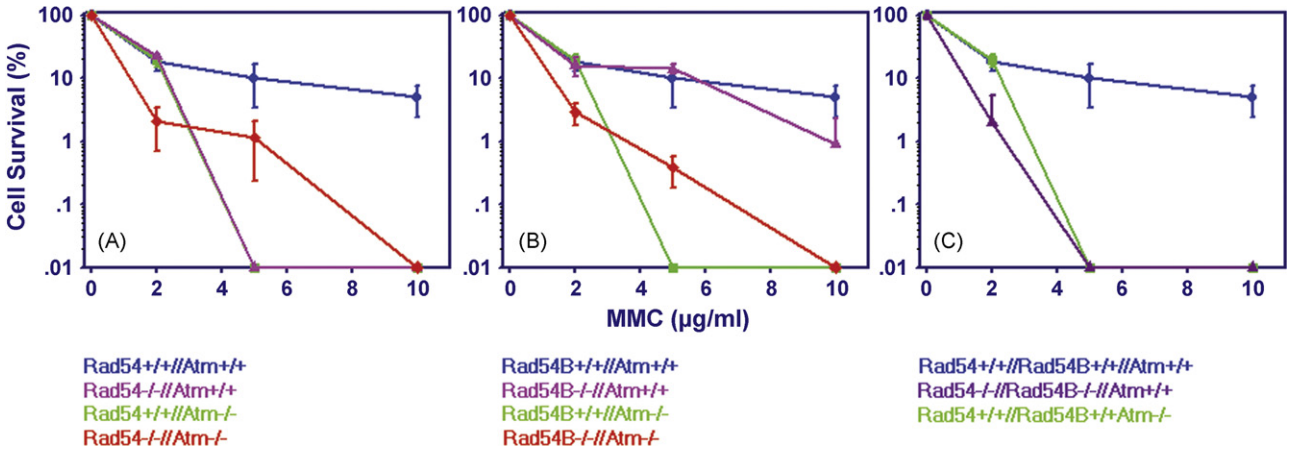

Fig. 6. Cellular sensitivity of various Rad54/Rad54B/Atm genotypes to MMC toxicity. Survival curves for fibroblast cells treated with MMC while growing exponentially and asynchronously. Wild type and Rad54B−/− cells were not hypersensitive to MMC. Rad54- and Atm-deficient cells were similarly hypersensitive to MMC. Most of these cells did not survive the dose of 5 μg/ml MMC. Rad54/Rad54B double null cells displayed a similar hypersensitivity to MMC as Atm−/− cells. Ablation of either Rad54 paralog conferred some resistance to MMC hypersensitivity of Atm deficient cells at high doses.

sensitivity. Finally, ablation of all three genes did not further sensitize the mice to MMC. The data indicate a significant role of Atm in responding to MMC-induced DNA damage and underscore the importance of HRR in this response. The highly increased MMC sensitivity conferred by Atm deficiency was also reflected in clonogenic survival of Atm−/− fibroblasts treated with this chemical (Fig. 6A–C). In these cells, Rad54 seemed to play a greater role in MMC response that Rad54B, as judged by cellular MMC sensitivity. As seen at the cellular level for the IR response (Fig. 4), combining Atm deficiency with that of any of the Rad54 paralogs reduced the MMC hypersensitivity, particularly Rad54B (Fig. 6A–C). These data indicate that Atm may play a greater role than previously thought in the cellular response to MMC-induced DNA damage.

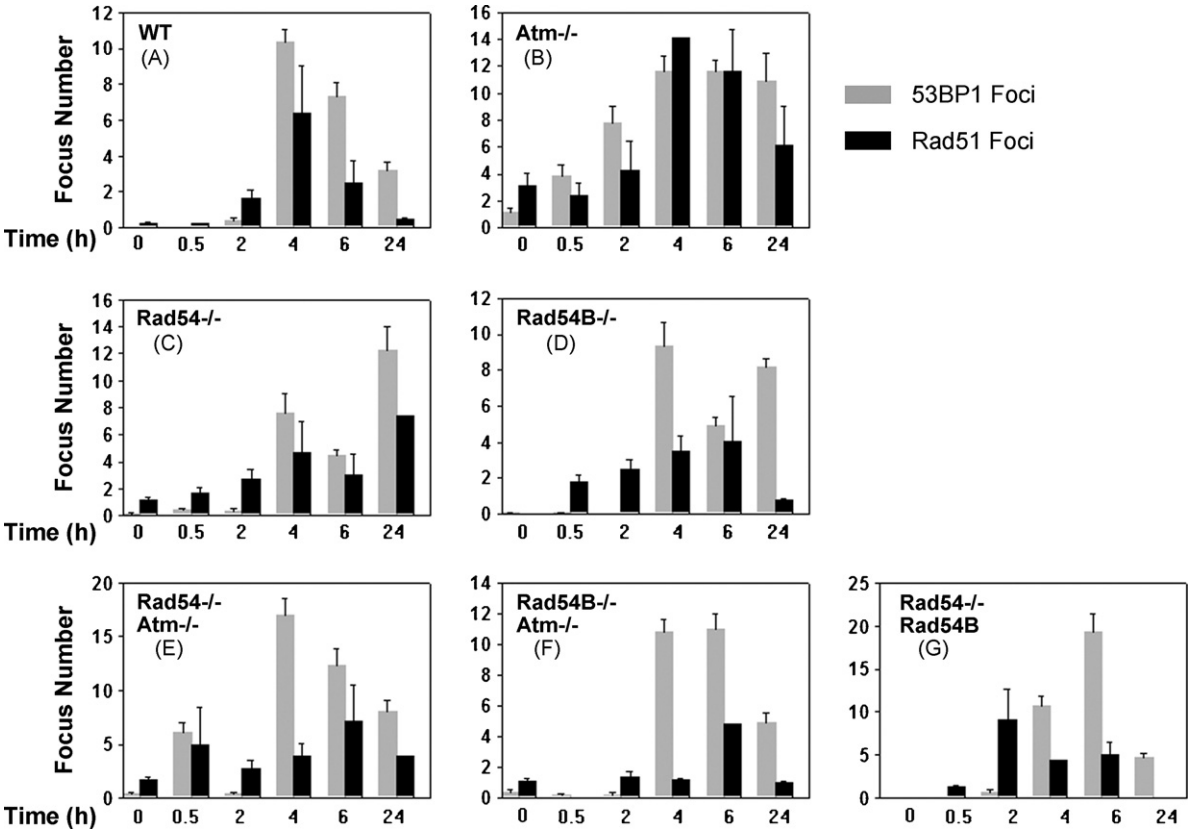

Fig. 7. 53BP1 and Rad51 focus formation and disappearance in cells with various Rad54/Rad54B/Atm genotypes in response to IR. Fibroblasts derived from the various Rad54/Rad54B/Atm were treated with 5 Gy and at the indicated time points the cells were fixed and immunoreacted with anti-53BP1 and anti-Rad51 antibodies. The number of 53BP1 foci per nucleus was measured using ImagePro software in 10–20 nuclei for each treatment/genotype. The experiments were performed in three different cultures prepared from different mice. Due to different sizes in the fibroblasts as detected in the confocal microscope, the foci were calculated to an average area of a cell which is about 500 μm^2.

3.4. 53BP1 and Rad51 focus formation in response to IR in cells with various Atm/Rad54 genotypes

The generation 53BP1focus formation is a reliable quantitative measurement of DSB formation and repairs regardless the mechanisms of repair [39,40]. To analyze the role of Rad54, Rad54B and Atm on the general DSB formation and repair, we measured the generation and disintegration of 53BP1 foci. Temporal analysis of the formation and disappearance of 53BP1 foci showed a number of differences among cells with the various Rad54/Rad54B/Atm genotypes. Whereas no signs of 53BP1 nuclear foci could be detected in untreated WT fibroblasts, a low level 53BP1 foci was already detected in untreated Atm−/− cultures. (Fig. 7A and B, grey bars). The kinetics of 53BP1 focus formation in WT and Rad54/Atm double mutant fibroblasts which were exposed to 5 Gy IR was similar except that the amount of 53BPI foci was markedly higher in Rad54/Atm double null cells. The cells reaching their peak number of 53BP1 foci at 4 h and the number of foci slowly declined thereafter. Exposure of Atm−/− cells to IR resulted in steady accumulation of 53BP1 foci peaking at 4 h, but unlike WT cells the number of foci remained high for at least 24 h (Fig. 7B, grey bars). Rad54 and Rad54B null cells accumulated 53BP1 foci over 24 h (Fig. 7C and D, grey bars). The accumulation of 53BP1 foci in Rad54B/Atm double null cells was almost identical to WT cells, however, the rate of decline was somewhat reduced (Fig. 7E, grey bars). Retarded accumulation of 53BP1 foci was observed in Rad54/Rad54B double null mice followed by quick rise (6 h) in 53BP1 focus number which sharply disappeared (Fig. 7G, grey bars).

DSB repair via HRR is based on coordinated action of the RAD52 group of proteins, including the DNA strand exchange protein Rad51, whose accumulation at DSB sites is visible as prominent nuclear foci [16]. In WT mouse fibroblasts IR-induced Rad51 foci peaked 4 h after irradiation and subsequently declined to base level (Fig. 7A, black bars). The pattern and time course of Rad51 focus formation in *Atm*−/− fibroblasts was somewhat different to those observed WT cells, with a higher number of foci 4 h post-irradiation. Also, in *Atm*−/− cells the amount of foci 24 h post-irradiation remained markedly higher than the initial level observed in untreated cells. The number of Rad51 foci in Atm−/− cells was the highest among the various genotypes (Fig. 7B, black bars). Exposure of Rad54−/− fibroblasts to IR resulted in slow accumulation of Rad51 foci, peaking 4 h after treatment, moderately declining and then slowly re-accumulating over time (Fig. 7C, black bars). IR-treated *Rad54*/*Atm*-null cells steadily accumulated Rad51 foci peaking at 6 h post irradiation and slowly declined (Fig. 7E, black bars). Slow accumulation of Rad51 foci was detected in irradiated *Rad54B*−/− cells, peaking at 6 h and then a decline to control levels was observed (Fig. 7D, black bars). Similar pattern of Rad51 focus accumulation and disappearance was measured in *Rad54B*/*Atm* (Fig. 7F, black bars). *Rad54*/*Rad54B*-null cells exhibited rapid focus accumulation, peaking at 2 h and declines to below control levels. (Fig. 7G, black bars).

4. Discussion

All the genotypes that we generated – compound mutant mouse strains deficient in Atm and the Rad54 paralogs involved in HRR – were viable, suggesting that combined inactivation of Atm and some component(s) of the HRR system is not critically important for normal development and survival of the compound mutant mice. In contrast, combined inactivation of Atm and NHEJ results in embryonic lethality. Atm/Ku70, Atm/Ku80 and Atm/DNA-PKcs double null mice die during their embryonic development [41]. Our experiments show that the Rad54 paralogs provide a valuable model system to study affects of attenuated HRR because, inactivation of central components of the HRR system, such as Rad51, result in embryonic lethality or cell death [18], demonstrating the importance of the HRR system for embryonic development. These findings are compatible with the notion that not all proteins involved in HRR are essential, implying redundancy in function, or the existence of sub-pathways of recombination that by themselves are not essential for cell viability.

In chicken DT40 cells Atm has a role in controlling HRR [31]. Whereas Ku70(NHEJ$^-$)//Atm−/− DT40 cells display radiosensitivity and high radiation-induced aberration frequencies, Rad54 (HRR$^-$)//Atm−/− cells show only slightly elevated aberration levels after irradiation, suggesting that Atm is epistatic with the HRR pathway [31]. Our results further expand on this point and demonstrate that Atm has different contributions to the various HRR pathways.

Inactivation of Rad54 paralog-mediated HRR in the presence of intact Atm does not reduce the birth rate of either Rad54−/−, Rad54B−/− or Rad54−/−//Rad54B−/− mice. On the other hand, deletion of the Atm gene on the background of various Rad54/Rad54B genotypes results in reduced birth rate of the double and triple Rad54/Rad54B/Atm mutants, suggesting that Atm and some components of the HRR pathways are not fully epistatic. If Atm is essential for HRR, Atm deficiency should result in embryonic lethality, since HRR is crucial at early embryogenesis. Following this line of thinking, it is possible that Atm is not involved in the activation of the HRR system during early embryogenesis. This notion is supported by a recent report showing that HRR is dissociated from Atm functions but dependent on Nbs1 activity [42]. Interestingly, combined deletions of Atm and Rad54, Rad54B or both result in significant growth retardation that is more pronounced in female mice, due perhaps to differences in metabolic rates or hormonal regulations between males and females.

Around 70% of the Atm−/− succumbs to thymic lympomas between the ages of 20 and 60 weeks [43] (Fig. 1). In contrast, none of the Rad54-, or the Rad54B-deficient mice develop malignant tumors, suggesting that deletion of these genes does not lead to cancer predisposition. Furthermore, we did detect an increase in cancer predisposition in Rad54−/−//Atm−/− mice but not in Rad54B−/−//Atm−/−.

Inactivation of Atm results in a significant increase in mouse radiosensitivity. Inhibition of the HRR system by inactivation of Rad54 and Rad54B slightly increased the radiosensitivity of the double mutant mice (Fig. 3) and fibroblasts (Fig. 4). These results suggest that the irradiated mice and cells can activate one of the several sub-pathways of the HRR systems or NHEJ to increase their survival. It has been shown that activation of the Rad54-dependent HRR pathway in response to IR is important in embryonic stem cells but not in mature cells [32]. Interestingly, inactivation of Rad54 in Atm−/− mice does not exacerbate their radiosensitivity, whereas inactivation of Rad54B in Atm-deficient mice significantly shortened their survival following IR. These results suggest that Atm and Rad54 are epistatic in the specific pathway that is activated in response to IR treatment, whereas Rad54B and Atm are not fully epistatic. Although Rad54 and Rad54B have similar biochemical activities [23,44–46], this does not rule out the possibility that Rad54 and Rad54B operate in distinct subpathways of HRR. This assumption is supported by the finding that Rad54/Rad54B/Atm triple null mice display higher radiosentivity than the rest of the genotypes (Fig. 3). The assumption of multiple HRR sub-pathways is further supported by our finding of different sensitivities of Rad54−/− and Rad54B−/− mice and fibroblasts to MMC (Figs. 5 and 6). Inactivation of one of the pathways can be tolerated by the IR-treated mice or fibroblast, whereas Rad51 inactivation resulted in embryonic lethality. Because Rad51 is such an essential component of the HRR system,

it is possible that all the sub-pathways converge upstream to Rad51 activation.

Combined deletions of Atm and Rad54, and to lesser extent Atm and Rad54B, rendered the mutant fibroblasts more resistant to the cytotoxic effect of IR. It has been suggested that in certain circumstances Atm is a proapoptotic protein, for instance during CNS development [47]; or is an activator of apoptotic pathways as reflected by the phosphorylation of Bid [48,49]. Here we speculate that in the absence of Rad54 or Rad54B, Atm assumes a proapoptotic role and in Atm absence, the Atm-dependent apoptotic pathways are markedly attenuated. Our results show that the irradiated Rad54−/−//Atm−/− fibroblasts are capable of forming colonies in the presence of DSBs, evidenced by the slow accumulation of Rad51 foci (Figs. 4 and 7). The current opinion in the field of DNA repair portrays HRR and NHEJ as distinct pathways. The fact that Rad51 foci are formed and accumulate in response to IR suggests that the HRR system has been activated. It is also thought that a single unrepaired DSB is sufficient to halt cell proliferation and to lead to its demise. Thus, the cells that are capable of forming colonies have repaired their DNA. On the assumption that Rad54 deletion significantly inhibits the HRR system [32], we speculate that the initial DNA repair is performed by the HRR system, and in the absence of key components of that system the processed DSBs are repaired by NHEJ, enabling the cells to survive and proliferate. In addition to the cytotoxic effects of IR, Atm−/− mice and cells are also sensitive to MMC, suggesting that Atm is also involved in the DDR pathways initiated by MMC. While HR is an important DNA repair pathway for two-ended breaks in rapidly dividing ES cells, such as those induced by IR, NHEJ is much better suited to repair these lesions in the many non-dividing and mature cells of the adult mice. In contrast, MMC-induced DNA interstrand cross-links are processed into single-ended DSBs by DNA replication [50]. Single-ended DSBs cannot be acted upon efficiently by NHEJ and require the HRR system for repair [51]. Collectively, the results from the IR and the MMC experiments suggest that Atm is involved in the activation of both the HRR and NHEJ systems.

It is known that MMC is capable of generating DSBs [38,50]. In the absence of Atm, Rad54 or Rad54B, the cells are still capable of forming Rad51 foci. Our results are compatible with findings that the effects of MMC – S-phase arrest, γH2AX focus formation and Rad51 focus formation – are Atm-independent. Interestingly, the MMC effects were found to be dependent on ATR [52], highlighting the importance of the redundancy among the DDR transducers and explaining why Atm inactivation is not embryonic lethal. Contradictory observations were reported by Chen et al. [53] and Yuan et al. [54] showing that IR-induced Rad51 foci were defective in Atm-deficient cells but not in NBS cells. Different cell lines and protocols might account for these discrepancies. However, our data are consistent with the notion that Rad51 focus formation is largely Atm independent, despite the fact that Atm−/− cells are highly sensitive to IR and MMC. These results suggest that Atm is not necessary for the activation of events upstream of Rad51 focus formation, but rather for those events that occur downstream to the formation of Rad51 foci. Rad54 and Rad54B were reported to physically interact with Rad51 [28,55–57], but our results suggest that these proteins are not necessary for Rad51 focus formation in response to IR or MMC. Interestingly, there are marked differences in the pattern of Rad51 focus formation between Rad54−/−, Rad54B−/−, and Rad54/Atm and Rad54B/Atm double null mice. In the absence of Rad54B, the quantity of Rad51 foci was markedly lower than in Rad54−/− cells. These results are consistent with the notion that Rad54 and Rad54B are not epistatic and operate in different HRR sub-pathways.

Our data demonstrate the complexity of the interrelations between Atm, Rad54 and Rad54B. The interrelations seem to be dependent on the type of insults to which the mice or cells are exposed. It is known that proteins form complexes in order to exert their biological function. IR and MMC exert their toxic effect through the generation of DNA DSBs. DSB repair is carried out by very intricate protein complexes. Based on our findings we speculate that the identity of the proteins that form protein complexes is dependent on the type of damage that leads to DNA DSBs.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

This work was supported by research grants from the A-T Children's Project, the Israel Science Foundation and the US-Israel Binational Science Foundation (to A.B.), and The A-T Medical Research Foundation, The A-T Children's Project, the A-T Medical Research Trust and the A-T Ease Foundation (to Y.S.). Work in JEs and RKs laboratory is supported by grants from the Dutch Cancer Society (KWF), the Netherlands Organization for Scientific Research (NWO), the Netherlands Genomic Initiative/NWO, and the European Commission (Integrated Project 512113).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2008.11.005.

References

[1] C.H. Bassing, F.W. Alt, The cellular response to general and programmed DNA double strand breaks, DNA Repair (Amst.) 3 (2004) 781–796.
[2] Y. Shiloh, The ATM-mediated DNA-damage response: taking shape, Trends Biochem. Sci. 31 (2006) 402–410.
[3] J.C. Harrison, J.E. Haber, Surviving the breakup: the DNA damage checkpoint, Annu. Rev. Genet. 40 (2006) 209–235.
[4] C.J. Bakkenist, M.B. Kastan, Initiating cellular stress responses, Cell 118 (2004) 9–17.
[5] Y. Shiloh, ATM and related protein kinases: safeguarding genome integrity, Nat. Rev. Cancer 3 (2003) 155–168.
[6] R.T. Abraham, PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways, DNA Repair (Amst.) 3 (2004) 883–887.
[7] E.U. Kurz, S.P. Lees-Miller, DNA damage-induced activation of ATM and ATM-dependent signaling pathways, DNA Repair (Amst.) 3 (2004) 889–900.
[8] T.O. Crawford, Ataxia telangiectasia, Semin. Pediatr. Neurol. 5 (1998) 287–294.
[9] H.H. Chun, R.A. Gatti, Ataxia-telangiectasia, an evolving phenotype, DNA Repair (Amst.) 3 (2004) 1187–1196.
[10] M.R. Lieber, Y. Ma, U. Pannicke, K. Schwarz, The mechanism of vertebrate non-homologous DNA end joining and its role in V(D)J recombination, DNA Repair (Amst.) 3 (2004) 817–826.
[11] C. Wyman, D. Ristic, R. Kanaar, Homologous recombination-mediated double-strand break repair, DNA Repair (Amst.) 3 (2004) 827–833.
[12] Z. Shen, K.G. Cloud, D.J. Chen, M.S. Park, Specific interactions between the human RAD51 and RAD52 proteins, J. Biol. Chem. 271 (1996) 148–152.
[13] M.K. Dosanjh, D.W. Collins, W. Fan, G.G. Lennon, J.S. Albala, Z. Shen, D. Schild, Isolation and characterization of RAD51C, a new human member of the RAD51 family of related genes, Nucleic Acids Res. 26 (1998) 1179–1184.
[14] N. Liu, J.E. Lamerdin, R.S. Tebbs, D. Schild, J.D. Tucker, M.R. Shen, K.W. Brookman, M.J. Siciliano, C.A. Walter, W. Fan, L.S. Narayana, Z.Q. Zhou, A.W. Adamson, K.J. Sorensen, D.J. Chen, N.J. Jones, L.H. Thompson, XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages, Mol. Cell 1 (1998) 783–793.
[15] S.L. Hays, A.A. Firmenich, P. Berg, Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 6925–6929.
[16] C. Wyman, R. Kanaar, DNA double-strand break repair: all's well that ends well, Annu. Rev. Genet. 40 (2006) 363–383.
[17] C. Wyman, R. Kanaar, Homologous recombination: down to the wire, Curr. Biol. 14 (2004) R629–631.
[18] L.S. Symington, Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair, Microbiol. Mol. Biol. Rev. 66 (2002) 630–670, Table of contents.

[19] A.V. Mazin, A.A. Alexeev, S.C. Kowalczykowski, A novel function of Rad54 protein. Stabilization of the Rad51 nucleoprotein filament, J. Biol. Chem. 278 (2003) 14029–14036.
[20] A. Alexeev, A. Mazin, S.C. Kowalczykowski, Rad54 protein possesses chromatin-remodeling activity stimulated by the Rad51-ssDNA nucleoprotein filament, Nat. Struct. Biol. 10 (2003) 182–186.
[21] V. Alexiadis, A. Lusser, J.T. Kadonaga, A conserved N-terminal motif in Rad54 is important for chromatin remodeling and homologous strand pairing, J. Biol. Chem. 279 (2004) 27824–27829.
[22] M. Jaskelioff, S. Van Komen, J.E. Krebs, P. Sung, C.L. Peterson, Rad54p is a chromatin remodeling enzyme required for heteroduplex DNA joint formation with chromatin, J. Biol. Chem. 278 (2003) 9212–9218.
[23] G. Petukhova, S. Stratton, P. Sung, Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins, Nature 393 (1998) 91–94.
[24] J.A. Solinger, K. Kiianitsa, W.D. Heyer, Rad54, a Swi2/Snf2-like recombinational repair protein, disassembles Rad51:dsDNA filaments, Mol. Cell 10 (2002) 1175–1188.
[25] D.V. Bugreev, F. Hanaoka, A.V. Mazin, Rad54 dissociates homologous recombination intermediates by branch migration, Nat. Struct. Mol. Biol. 14 (2007) 746–753.
[26] R. Kanaar, C. Troelstra, S.M. Swagemakers, J. Essers, B. Smit, J.H. Franssen, A. Pastink, O.Y. Bezzubova, J.M. Buerstedde, B. Clever, W.D. Heyer, J.H. Hoeijmakers, Human and mouse homologs of the Saccharomyces cerevisiae RAD54 DNA repair gene: evidence for functional conservation, Curr. Biol. 6 (1996) 828–838.
[27] T. Hiramoto, T. Nakanishi, T. Sumiyoshi, T. Fukuda, S. Matsuura, H. Tauchi, K. Komatsu, Y. Shibasaki, H. Inui, M. Watatani, M. Yasutomi, K. Sumii, G. Kajiyama, N. Kamada, K. Miyagawa, K. Kamiya, Mutations of a novel human RAD54 homologue, RAD54B, in primary cancer, Oncogene 18 (1999) 3422–3426.
[28] J. Wesoly, S. Agarwal, S. Sigurdsson, W. Bussen, S. Van Komen, J. Qin, H. van Steeg, J. van Benthem, E. Wassenaar, W.M. Baarends, M. Ghazvini, A.A. Tafel, H. Heath, N. Galjart, J. Essers, J.A. Grootegoed, N. Arnheim, O. Bezzubova, J.M. Buerstedde, P. Sung, R. Kanaar, Differential contributions of mammalian Rad54 paralogs to recombination, DNA damage repair, and meiosis, Mol. Cell. Biol. 26 (2006) 976–989.
[29] C. Barlow, S. Hirotsune, R. Paylor, M. Liyanage, M. Eckhaus, F. Collins, Y. Shiloh, J.N. Crawley, T. Ried, D. Tagle, A. Wynshaw-Boris, Atm-deficient mice: a paradigm of ataxia telangiectasia, Cell 86 (1996) 159–171.
[30] P.R. Borghesani, F.W. Alt, A. Bottaro, L. Davidson, S. Aksoy, G.A. Rathbun, T.M. Roberts, W. Swat, R.A. Segal, Y. Gu, Abnormal development of Purkinje cells and lymphocytes in Atm mutant mice, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 3336–3341.
[31] C. Morrison, E. Sonoda, N. Takao, A. Shinohara, K. Yamamoto, S. Takeda, The controlling role of ATM in homologous recombinational repair of DNA damage, EMBO J. 19 (2000) 463–471.
[32] J. Essers, R.W. Hendriks, S.M. Swagemakers, C. Troelstra, J. de Wit, D. Bootsma, J.H. Hoeijmakers, R. Kanaar, Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination, Cell 89 (1997) 195–204.
[33] P.J. Stambrook, C. Shao, M. Stockelman, G. Boivin, S.J. Engle, J.A. Tischfield, APRT: a versatile in vivo resident reporter of local mutation and loss of heterozygosity, Environ. Mol. Mutagen. 28 (1996) 471–482.
[34] S.E. Browne, L.J. Roberts 2nd, P.A. Dennery, S.R. Doctrow, M.F. Beal, C. Barlow, R.L. Levine, Treatment with a catalytic antioxidant corrects the neurobehavioral defect in ataxia-telangiectasia mice, Free Radic. Biol. Med. 36 (2004) 938–942.
[35] Y. Shiloh, M.B. Kastan, ATM: genome stability, neuronal development, and cancer cross paths, Adv. Cancer Res. 83 (2001) 209–254.
[36] J. Essers, H. van Steeg, J. de Wit, S.M. Swagemakers, M. Vermeij, J.H. Hoeijmakers, R. Kanaar, Homologous and non-homologous recombination differentially affect DNA damage repair in mice, EMBO J. 19 (2000) 1703–1710.
[37] A.M. Gurtan, A.D. D'Andrea, Dedicated to the core: understanding the Fanconi anemia complex, DNA Repair (Amst.) 5 (2006) 1119–1125.
[38] K. Hanada, M. Budzowska, M. Modesti, A. Maas, C. Wyman, J. Essers, R. Kanaar, The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks, EMBO J. 25 (2006) 4921–4932.
[39] J. Kobayashi, K. Iwabuchi, K. Miyagawa, E. Sonoda, K. Suzuki, M. Takata, H. Tauchi, Current topics in DNA double-strand break repair, J. Radiat. Res. (Tokyo) 49 (2008) 93–103.
[40] O. Zgheib, Y. Huyen, R.A. DiTullio Jr., A. Snyder, M. Venere, E.S. Stavridi, T.D. Halazonetis, ATM signaling and 53BP1, Radiother. Oncol. 76 (2005) 119–122.
[41] J. Sekiguchi, D.O. Ferguson, H.T. Chen, E.M. Yang, J. Earle, K. Frank, S. Whitlow, Y. Gu, Y. Xu, A. Nussenzweig, F.W. Alt, Genetic interactions between ATM and the nonhomologous end-joining factors in genomic stability and development, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 3243–3248.
[42] S. Sakamoto, K. Iijima, D. Mochizuki, K. Nakamura, K. Teshigawara, J. Kobayashi, S. Matsuura, H. Tauchi, K. Komatsu, Homologous recombination repair is regulated by domains at the N- and C-terminus of NBS1 and is dissociated with ATM functions, Oncogene (2007).
[43] S. Ziv, O. Brenner, N. Amariglio, N.I. Smorodinsky, R. Galron, D.V. Carrion, W. Zhang, G.G. Sharma, R.K. Pandita, M. Agarwal, R. Elkon, N. Katzin, I. Bar-Am, T.K. Pandita, R. Kucherlapati, G. Rechavi, Y. Shiloh, A. Barzilai, Impaired genomic stability and increased oxidative stress exacerbate different features of Ataxia-telangiectasia, Hum. Mol. Genet. 14 (2005) 2929–2943.
[44] G. Petukhova, P. Sung, H. Klein, Promotion of Rad51-dependent D-loop formation by yeast recombination factor Rdh54/Tid1, Genes Dev. 14 (2000) 2206–2215.
[45] D. Ristic, C. Wyman, C. Paulusma, R. Kanaar, The architecture of the human Rad54-DNA complex provides evidence for protein translocation along DNA, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8454–8460.
[46] T.L. Tan, J. Essers, E. Citterio, S.M. Swagemakers, J. de Wit, F.E. Benson, J.H. Hoeijmakers, R. Kanaar, Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation, Curr. Biol. 9 (1999) 325–328.
[47] K.H. Herzog, M.J. Chong, M. Kapsetaki, J.I. Morgan, P.J. McKinnon, Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system, Science 280 (1998) 1089–1091.
[48] I. Kamer, R. Sarig, Y. Zaltsman, H. Niv, G. Oberkovitz, L. Regev, G. Haimovich, Y. Lerenthal, R.C. Marcellus, A. Gross, Gross proapoptotic BID is an ATM effector in the DNA-damage response, Cell 122 (2005) 593–603.
[49] S.S. Zinkel, K.E. Hurov, C. Ong, F.M. Abtahi, A. Gross, S.J. Korsmeyer, A role for proapoptotic BID in the DNA-damage response, Cell 122 (2005) 579–591.
[50] L.J. Niedernhofer, H. Odijk, M. Budzowska, E. van Drunen, A. Maas, A.F. Theil, J. de Wit, N.G. Jaspers, H.B. Beverloo, J.H. Hoeijmakers, R. Kanaar, The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks, Mol. Cell. Biol. 24 (2004) 5776–5787.
[51] G.A. Cromie, J.C. Connelly, D.R. Leach, Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans, Mol. Cell 8 (2001) 1163–1174.
[52] E. Mladenov, I. Tsaneva, B. Anachkova, Activation of the S phase DNA damage checkpoint by mitomycin C, J. Cell Physiol. 211 (2007) 468–476.
[53] G. Chen, S.S. Yuan, W. Liu, Y. Xu, K. Trujillo, B. Song, F. Cong, S.P. Goff, Y. Wu, R. Arlinghaus, D. Baltimore, P.J. Gasser, M.S. Park, P. Sung, E.Y. Lee, Radiation-induced assembly of Rad51 and Rad52 recombination complex requires ATM and c-Abl, J. Biol. Chem. 274 (1999) 12748–12752.
[54] S.S. Yuan, H.L. Chang, E.Y. Lee, Ionizing radiation-induced Rad51 nuclear focus formation is cell cycle-regulated and defective in both ATM(−/−) and c-Abl(−/−) cells, Mutat. Res. 525 (2003) 85–92.
[55] E.I. Golub, O.V. Kovalenko, R.C. Gupta, D.C. Ward, C.M. Radding, Interaction of human recombination proteins Rad51 and Rad54, Nucleic Acids Res. 25 (1997) 4106–4110.
[56] A.V. Mazin, C.J. Bornarth, J.A. Solinger, W.D. Heyer, S.C. Kowalczykowski, Rad54 protein is targeted to pairing loci by the Rad51 nucleoprotein filament, Mol. Cell 6 (2000) 583–592.
[57] S. Van Komen, G. Petukhova, S. Sigurdsson, S. Stratton, P. Sung, Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54, Mol. Cell 6 (2000) 563–572.