

# Combined Haploinsufficiency for ATM and RAD9 as a Factor in Cell Transformation, Apoptosis, and DNA Lesion Repair Dynamics

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## Abstract

**Loss of function of oncogenes, tumor suppressor genes and DNA damage processing genes has been implicated in the development of many types of cancer, but for the vast majority of cases, there is no link to specific germ line mutations. In the last several years, heterozygosity leading to haploinsufficiency for proteins involved in DNA repair pathways was shown to play a role in genomic instability and carcinogenesis after DNA damage is induced. Because the effect of haploinsufficiency for one protein is relatively small, we hypothesize that predisposition to cancer could be a result of the additive effect of heterozygosity for two or more genes, critical for pathways that control DNA damage signaling, repair or apoptosis. To address this issue, primary mouse cells, haploinsufficient for one or two proteins, ATM and RAD9, related to the cellular response to DNA damage were examined. The results show that cells having low levels of both ATM and RAD9 proteins are more sensitive to transformation by radiation, have different DNA double-strand break repair dynamics and are less apoptotic when compared with wild-type controls or those cells haploinsufficient for only one of these proteins. Our conclusions are that under stress conditions, the efficiency and capacity for DNA repair mediated by the ATM/RAD9 cell signaling network depend on the abundance of both proteins and that, in general, DNA repair network efficiencies are genotype-dependent and can vary within a specific range. (Cancer Res 2005; 65(3): 933-8)**

## Introduction

In the last few years, mounting evidence suggests that heterozygosity leading to haploinsufficiency for proteins involved in DNA repair pathways plays a role in genomic instability and carcinogenesis. Haploinsufficiency for p53, PTEN, BubR1, NBS1, H2AX, p18(INK4c), BLM, Rb, APS, and ATM has been shown to be an important factor in carcinogen-induced tumors (1–11). Most of these genes code for tumor suppressor proteins. A major conclusion from these data is that, contrary to one of the current views on tumorigenesis, inactivation of one allele of a tumor suppressor gene is enough to contribute to tumor progression. Another conclusion from most of the cases is that animals or cells haploinsufficient for the specified proteins have higher transformation rates after DNA damage is induced, but when their DNA is not significantly damaged by exogenous sources, tumor development rates are the same as for their wild-type counterparts.

These data as well as the fact that most mice heterozygous for DNA repair genes have the same life span as the wild-types when not challenged with mutagens, strongly suggest that haploinsufficiency is a critical factor in the cellular response to stress conditions, and even more importantly, individuals with different genotypes respond differently to the same environmental challenges.

In the case of heterozygosity when one allele of a gene is inactivated, predisposition to transformation is based on a more probable frequency of mutations than on the complete inactivation of both alleles of cancer-related genes such as *RBI*, *p53*, *BRCA1*, or *BRCA2*. Epidemiologic studies indicate that only 15% to 20% of familial breast cancer cases, for example, are a result of mutation in *BRCA1* or *BRCA2* (12, 13). The rest are most probably due to genetic factors unlikely to involve a mutation in a highly related tumorigenesis gene (14). Evidence suggests that the risk might be based on the additive contribution of several factors, each individually having a small effect (15–17) difficult to determine when present alone.

We hypothesize that predisposition to cancer could be a result of the additive effect of heterozygosity for two or more genes, critical for pathways that control DNA damage signaling, repair, or apoptosis. Because in many cases heterozygosity leads to haploinsufficiency (18), we suggest that the function of signaling networks that impact on maintaining genomic integrity depends on the proper amounts of key proteins and that haploinsufficiency can lead to conditions where network efficiency under stress is suboptimal. This might result in a decrease in the effectiveness of processes related to suppression of tumor initiation, such as apoptosis and the efficient processing of DNA damage.

To address this issue, cells haploinsufficient for one or two proteins related to the cellular response to DNA damage—ATM and RAD9—were examined. Both proteins are important factors in DNA double-strand break repair (19, 20), they rapidly colocalize to regions containing DNA double-strand breaks after DNA damage (21, 22), and ATM can phosphorylate RAD9 (23). We generated mice haploinsufficient for each or both proteins, and analyzed isolated mouse embryo fibroblasts (MEFs) and thymocytes to monitor three end points related to tumorigenesis—cell transformation, apoptosis and DNA double-strand break repair. The results show that cells having lower amounts of both ATM and MRAD9 are more sensitive to transformation induced by radiation, have different dynamics of DNA double-strand break repair, retain more double-strand breaks after radiation exposure and are less apoptotic than the wild-type control, or cells haploinsufficient for only one of these proteins. Our conclusions are that, under stress conditions, the efficiency and capacity for DNA repair mediated by the ATM/RAD9 cell-signaling network depends on the expression levels of both proteins and that, in general, DNA repair network efficiencies are genotype-dependent and can vary within a specific range. These findings point to the possibility of estimating an

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individual's susceptibility to health risks associated with carcinogen exposure based on genotype and levels of specific proteins.

## Materials and Methods

**Mice.** *Atm* and *Rad9* wild-type (wt), heterozygous (hz), and knockout (ko) mice used in the experiments were described previously (24, 25). Both mouse mutant heterozygous genotypes caused haploinsufficiency for the corresponding proteins (11, 25). The two groups of mice were mated and only F1 littermates were used. Genotypes were determined by PCR (24, 25). Mice heterozygous for *Atm* or *Rad9*, or the double heterozygous animals, displayed no detectable abnormalities through 12 months of age.

**Embryo Cell Preparation.** Pregnant females were sacrificed on day 14 of gestation. The embryos were surgically removed and embryonic tissue prepared in culture. Each embryo was cultured separately, and during the 4 days necessary to amplify MEF cells in mass culture, they were genotyped.

**Cell Transformation Assay.** Exponentially growing MEFs received a dose of 2 Gy of  $\gamma$ -rays in an acute exposure, and controls were sham-irradiated. MEFs were then plated in 100-mm plates at a density of 6,000 cells/plate over a feeder layer of 70,000 cells prepared from the same embryo but irradiated previously with a supralethal dose. After 2 weeks of growth in DMEM medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> air-humidified incubator, cells were fixed, stained, and yields of transformed clones scored. The scoring criteria were developed and examined by preliminary experiments, where embryo cells were irradiated and plated with the same density. The clones which seemed dense and had stellate-shaped piled cells were isolated with cloning cylinders. These clones were expanded and injected into nude mice. Those that caused the development of tumors were designated as transformed. Clones that matched their shape and dimensions were scored as transformed in later experiments. Plating efficiency, cell surviving fractions, and the spontaneous and radiation-induced frequency of morphologic transformation were determined.

**Clonogenic Survival.** To assess clonogenic survival, MEFs were exposed to different doses of  $\gamma$ -rays. Following irradiation, sufficient numbers of cells were plated into 100 mm culture dishes so that accounting for plating efficiency and surviving fraction following radiation, ~100 viable cells would be present in each dish. The total number of cells (viable plus feeder) was 70,000 per dish. Dishes were incubated for 2 weeks without a medium change, and the resulting colonies were stained with Giemsa to determine both the plating efficiencies and surviving fractions of control and irradiated cells. Data from a minimum of three independent experiments were pooled. All data for clonogenic survival were presented as a mean together with SE.

**Apoptosis and Cell Survival Assays.** Wild-type and heterozygous mice, at the age of 2 months, were sacrificed, and thymocytes were isolated after careful thymus homogenization. Thymocytes were seeded into 24-well plates (in RPMI/10% fetal bovine serum) at a density of  $5 \times 10^6$ /mL and exposed to  $\gamma$ -radiation at doses of 1, 2, 4, and 8 Gy. Six hours later

(or 24 hours where indicated), the proportion of apoptotic cells was measured by staining with Annexin V-PE and 7-AAD according to the manufacturer's instructions (PharMingen, San Diego, CA).

**DNA Lesion Visualization.** MEFs were isolated, cultured close to the stage of senescence, and then plated in two-well slide chambers (Lab-Tek, Naperville, IL) at a density of  $3 \times 10^4$ /well. After 2 days, the cells were irradiated with 0.5 Gy of  $\gamma$ -rays, fixed for 10 minutes in 2% paraformaldehyde, permeabilized for 20 minutes in methanol at -20°C, blocked for 1 hour in 5% goat serum, and stained with rabbit anti- $\gamma$ -H2AX antibody (a gift from Dr. W. Bonner) for 2 hours. The bound antibody was visualized using Alexa Fluor goat anti-rabbit antibody (Molecular Probes, Eugene, OR), and cell nuclei were stained with PI/RNase solution (PharMingen). Slides were viewed on a laser scanning confocal microscope (Nikon Co., Tokyo, Japan). At least 100 cells were scored, and the average number of foci per cell was calculated.

## Results

**Cell Transformation Assay.** Radiation-induced transformation of MEFs was examined to begin to access the impact of genotype on this end point. A total of 21 embryos from five litters were used and included five for genotypes *Atm*wt/*Mrad*9wt, *Atm*hz/*Mrad*9wt, and *Atm*wt/*Mrad*9hz and six for *Atm*hz/*Mrad*9hz. Yields of transformed clones were measured both for unexposed controls and after a dose of 2 Gy. The results shown in Tables 1 and 2 indicate a statistically significant higher transformation frequency for the double heterozygous cells. Transformation frequencies for these cells are more than double that of the wild-type population. The *Mrad*9 heterozygous cells show a transformation frequency close to that of the wild-type cells, and the frequency for the ATM heterozygous cells is between the wild-type and double heterozygous cells. There were small differences in the clonogenic survival for all populations after irradiation (Fig. 1).

**Apoptosis of Thymocytes.** We examined thymocytes from single and double heterozygous animals for radiation-induced apoptosis. The number of animals and the genotypes used were, respectively: *Atm*wt/*Rad*9wt (five), *Atm*hz/*Rad*9hz (seven), *Atm*wt/*Rad*9hz (five), *Atm*hz/*Rad*9wt (six), *Atm*ko/*Rad*9wt (three). The mice were from four different litters. The results show differences in apoptotic frequencies related to genotype (Fig. 2). Wild-type cells display the highest apoptotic frequencies after irradiation, whereas ATM-deficient cells show the lowest. The differences between apoptotic frequencies in *Atm* wild-type and heterozygous cells were small but statistically significant and show that *Atm* heterozygosity could be a factor influencing programmed cell death. *Mrad*9 heterozygous cells show the same apoptotic rates as the wild-type

**Table 1.** Transformation frequencies of unirradiated or irradiated cells differing in the status of *Atm* and *Mrad*9

Genotype	Dose (Gy)	Total number of clones scored	Number of transformed clones	Transformed clones (%)
<i>Atm</i> wt/ <i>Mrad</i> 9wt	0	31,240	5	0.02
	2	22,800	24	0.11
<i>Atm</i> wt/ <i>Mrad</i> 9hz	0	28,450	5	0.02
	2	16,470	27	0.16
<i>Atm</i> hz/ <i>Mrad</i> 9wt	0	35,170	5	0.01
	2	16,720	35	0.21
<i>Atm</i> hz/ <i>Mrad</i> 9hz	0	27,830	13	0.05
	2	18,900	63	0.33

**Table 2.** Comparisons of radiation-induced transformation between MEFs of different genotypes versus wild-type MEFs

	<i>Atm</i> hz/ <i>Mrad9</i> wt	<i>Atm</i> wt/ <i>Mrad9</i> hz	<i>Atm</i> hz/ <i>Mrad9</i> hz
Relative transformation (2 Gy)	1.91	1.45	3.10
<i>t</i> test (2 Gy)	<i>P</i> = 0.03	<i>P</i> = 0.31	<i>P</i> = 0.0001

NOTE: Relative transformation is defined as the ratio of the number of transformed clones per surviving heterozygous cells relative to the number of transformed clones per surviving wild-type cells. The statistical significance of differences in transformation frequency between the various cells with heterozygous genotypes and wild-type cells was analyzed by Student's *t* test.

control. Remarkably, the apoptotic frequencies were significantly reduced in the double heterozygous cells. The results for this genotype are closer to those obtained for the *Atm* null cells than for the wild-type, showing that haploinsufficiency for two functionally related proteins may have an additive negative effect on pathways where both proteins are normally involved.

We measured apoptosis in thymocytes 6 hours after irradiation to avoid high background apoptotic frequencies that are in the range of 25% to 30% for these cells, 24 hours after isolation. A concern was that results at 6 hours could represent only delayed apoptosis for the double heterozygous thymocytes relative to the wild-type because activation of ATM-dependent pathways is considered the first response to irradiation followed by activation of other compensatory DNA damage repair pathways. To address this, survival and apoptotic frequencies for *Atm*wt/*Mrad9*wt and *Atm*hz/*Mrad9*hz thymocytes were also measured 24 hours after irradiation. The results confirm the higher survival for double heterozygous cells in comparison to the wild-type counterparts, indicating that differences in apoptosis are maintained for longer periods of time (Fig. 3).

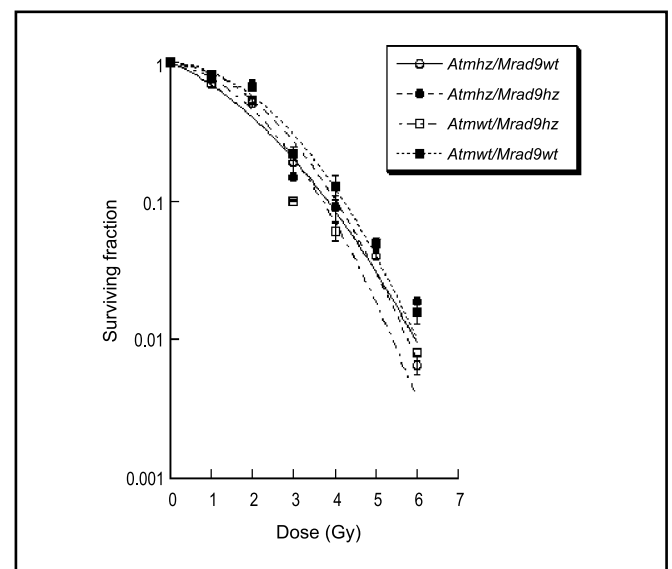
Because ATM is an important factor in T cell differentiation, thymocytes from all animals were examined for CD4/CD8 markers. No difference was found in double positive and single positive distributions except for the ATM-deficient thymocytes where, as expected, a partial block at the CD4/CD8 double positive stage and reduced numbers of single positive CD4 and CD8 cells were found (data not shown).

**DNA Double-Strand Break Repair Dynamics.** The appearance of DNA double-strand breaks and their repair in MEFs having different genotypes were examined. Cells were passaged until close to senescence because fast proliferating early passage MEFs show high background  $\gamma$ -H2AX staining. Changes in the number of foci formed in response to 0.5 Gy of  $\gamma$ -rays were followed for up to 24 hours (Fig. 4). The genotypes evaluated were *Atm*wt/*Mrad9*wt, *Atm*wt/*Mrad9*hz, *Atm*hz/*Mrad9*wt, *Atm*hz/*Mrad9*hz, and *Atm*ko/*Mrad9*wt. There were no significant differences between the number of foci in the wild-type and single heterozygous cells.  $\gamma$ -H2AX foci formation was slower in the *Atm*hz/*Mrad9*hz cells but after 2 hours were statistically equal to those shown by cells with the other genotypes. After 24 hours, the *Atm*hz/*Mrad9*hz cells show more residual double-strand breaks than wild-type and the single heterozygous cells. The *Atm*

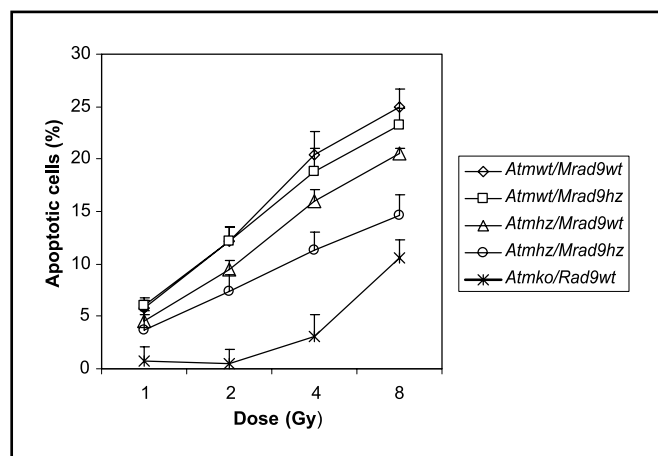
knockout cells had the highest background staining and showed slow foci formation, and high residual foci frequencies after 24 hours.

## Discussion

We showed that cells haploinsufficient for both *Atm* and *Mrad9* are more sensitive to transformation induced by radiation, show different dynamics of double-strand break repair, retain more double-strand breaks after radiation exposure, and are less apoptotic than wild-type cells or those haploinsufficient for only one of the encoded proteins. Haploinsufficiency as a result of heterozygosity for tumor suppressor genes in combination with carcinogens has been implicated in tumorigenesis (5–12). Our previous results show that heterozygosity for *Atm* modestly increased the transformation of MEFs after irradiation (11), and was also a factor in cataract formation (26). Heterozygosity for *Atm* has long been suspected as a contributing factor in familial breast cancer as well as other types of cancer (12). Theoretically, all familial cases of tumor development where one protein is haploinsufficient or deficient, points to other important factors characteristic for the family. Because usually no mutations in other genes have been identified in many of these cases, we hypothesize that some of these unknown factors could be a second haploinsufficiency for a protein related functionally to the first one. In this respect, we analyzed three cancer-related events: cell transformation, apoptosis, and double-strand break repair in cells single- or double-haploinsufficient for *Atm* and *Mrad9*. We found that haploinsufficiency for both proteins had an additive effect, noticeably increasing cell transformation after radiation-induced DNA damage, and decreasing apoptotic frequencies in irradiated thymocytes, bringing the apoptotic levels closer to that observed in the *Atm* null phenotype. Additionally, the double-haploinsufficient phenotype changed the dynamics of double-strand break repair and decreased the efficiency of removal of those lesions, as shown by staining for  $\gamma$ -H2AX. These data are consistent with a model indicating that low levels of ATM and MRAD9 proteins result in a relatively inefficient cellular response to excessive DNA damage,



**Figure 1.** Clonogenic survival of MEF cells irradiated with  $\gamma$ -rays. Points, means from at least three independent experiments; bars, SE. Surviving fractions measured at the doses tested were fitted with the linear-quadratic equation.



**Figure 2.** Apoptosis of thymocytes having different genetic backgrounds. Thymocytes from mice were irradiated with different doses of  $\gamma$ -rays and apoptosis was measured 6 hours after irradiation. The percentage of apoptotic cells at 0 Gy were subtracted from the rest of the data points for each genotype.

including DNA repair and apoptosis. The mechanisms behind these events could be related to specific functions of ATM and MRAD9 in DNA repair and signal transduction networks. ATM is a sensor/transducer protein involved in the initial response to DNA double-strand breaks. The protein is activated immediately after DNA double-strand break induction (27) and is subsequently involved in a large group of events, including downstream signaling by activating DNA repair, checkpoint, and apoptotic control proteins. *Atm* knockout organisms develop progressive cerebellar ataxia, lymphoma and leukemia. They are characterized by chromosomal instability and hypersensitivity to ionizing radiation (19, 28). The role of RAD9 is less well established but we know that it forms a heterotrimer with RAD1 and HUS1 (29, 30), a complex believed to be critical for cell cycle checkpoint control. Both ATM and the RAD9 complex colocalize at points of double-strand breaks minutes after DNA damage is incurred, as part of a large protein complex involving TopBP1, RAD50, RAD9, ATM, and BRCA1 (21, 22), and ATM can phosphorylate RAD9 (23). These data indicate strong cooperation between RAD9 and ATM proteins during the initial events of the cellular response to DNA damage.

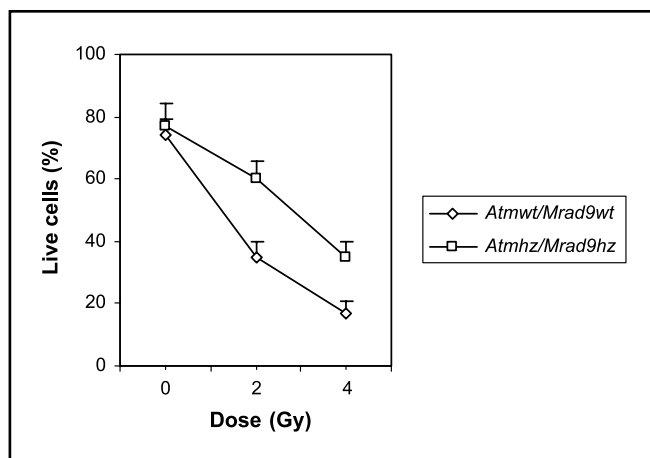
Our previous results (11) show increased sensitivity to radiation oncogenesis in MEFs haploinsufficient for the ATM protein. We used the same approach estimating the transformation frequency of MEFs haploinsufficient for both ATM and RAD9 proteins. Surprisingly, the double-haploinsufficient cells were significantly more sensitive to radiation oncogenesis than were the corresponding wild-type cells by a factor of about 3. The transformation frequency of the double heterozygous cells were also higher than for the cells haploinsufficient for only ATM or RAD9 proteins. Comparison of the RAD9-haploinsufficient MEFs and wild-type MEFs didn't show statistically significant differences. ATM-haploinsufficient cells were more sensitive in comparison with the wild-type MEFs by a factor of 2. Clonogenic survival results were similar in the range from 1 to 6 Gy for all genotypes tested.

The function of ATM in triggering apoptosis is well established. Our data shown here as well as that of others (31–33) indicate that thymocytes deficient for ATM are less apoptotic after irradiation in comparison to wild-type cells. This is a result of the lack of detection of double-strand breaks by ATM, and lack of activation of cell cycle checkpoints and apoptosis. The consequences are a

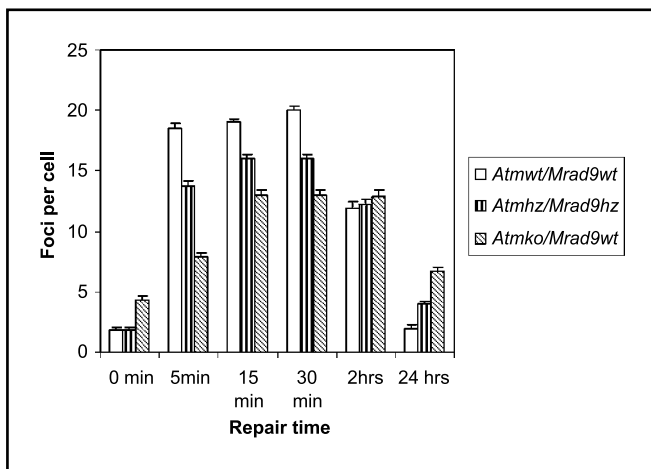
significant accumulation of mutations and retention of damage, which are the reasons for subsequent accelerated cell death by mitotic catastrophe. Depending on the magnitude of DNA damage, in some cases, a small fraction of cells are able to escape, continue to divide and can develop into tumors. This scenario is well supported by the inevitable development of thymic lymphomas in all *Atm* knockout mice. Although they develop in unirradiated as well as irradiated mice, the lack of proper end joining for V(D)J recombination is similar to the lack of DNA double-strand break repair generated by radiation, and illustrates the link between the accumulation of mutations, improper apoptosis, and tumor development. Our results show that double heterozygous thymocytes were much less apoptotic than wild-type and single heterozygous cells. Applying the same logic as to the *Atm* knockout thymocytes, we suggest that the rate of mutation accumulation in these cells should be higher than in the wild-type and single heterozygous cells, and their respective oncogenic transformation potentials should also be higher. These results could explain the higher transformation frequency we observe in the double-haploinsufficient MEFs. Indeed, transformation and apoptotic death seem to be inversely related.

In another approach, we estimated the dynamics of double-strand break repair in MEFs by visualizing the phosphorylated form of histone  $\gamma$ -H2AX. As was shown previously,  $\gamma$ -H2AX is an important marker for detection of double-strand breaks (34) and was used for evaluating DNA repair dynamics in different types of cells including MEFs (35). Our results demonstrate that MEFs haploinsufficient for ATM and RAD9 show different repair dynamics than wild-type cells and accumulate a higher number of residual double-strand breaks after irradiation. This could indicate higher frequencies of mutation accumulation for cells with that specific genotype. The mechanisms underlying these results could be related to less efficient DNA damage detection, repair, and apoptosis.

All of these results indicate that haploinsufficiency for two closely interacting proteins (i.e., ATM and MRAD9) functioning in related pathways could play a significant role in tumorigenesis by altering the mechanisms preventing it. Another conclusion is that the effectiveness of DNA repair networks may vary within a specific range depending on the genotype and levels of the proteins



**Figure 3.** Thymocyte survival 24 hours after irradiation. Thymocytes were isolated from mice, irradiated, and incubated overnight at a density of  $5 \times 10^6$  cells/mL. Twenty-four hours later, 100  $\mu$ L of the cell suspension was labeled with annexin V/7-AAD. Each sample was measured by flow cytometry for 30 seconds. Cells not stained by the annexin-V or 7AAD are shown as live nonapoptotic cells.



**Figure 4.** Double-strand break repair in different genetic backgrounds. MEFs having the indicated genotypes were irradiated with 0.5 Gy of  $\gamma$ -rays. The number of DNA double-strand breaks were revealed by staining with anti  $\gamma$ -H2AX antibody. Columns, average of at least 100 cells counted for each genotype at the specific time points indicated. Bars, SE from two independent experiments. The *Atmh/Mrad9wt* and *Atmw/Mrad9hz* MEFs showed results similar to the *Atmw/Mrad9wt* number of foci (results not shown).

involved. An additive effect of a second haploinsufficiency may significantly contribute to the destabilization of a particular cell signaling network. Therefore, it seems that cell signaling networks are finely tuned and are most effective only when the concentration and consequently the activity of the proteins involved are at optimal levels. As a result, the capacity for DNA repair under stress conditions will depend on an individual's genotype, and the degree of accumulation of mutations in the presence of the same dose of mutagens could vary from individual to individual.

Interpretation of these findings in the context of tumor initiation and progression leads to the suggestion that initial events in transformation could arise in a heterozygous background of partially destabilized networks and the presence of mutagens. Mutations where one copy of a gene is inactivated are much more probable than complete gene inactivation. Therefore, the additive effect of two or more haploinsufficiencies may be a decisive factor in the initial accumulation of damage leading to cancer.

Currently, there are two basic paradigms for the initial stage of tumorigenesis—mutator phenotype and aneuploidy (36, 37). According to the mutator phenotype hypothesis, the initial stages of carcinogenesis are a result of mutation of genetic stability genes which increase mutation rates for other genes, and eventually lead to cell transformation. The aneuploidy model suggests that mutation of a small number of genes required for cell division leads to chromosome breaks or unequal chromosome segregation. This results in genetic instability and the generation of mutations in multiple genes. The aneuploidy model explains well how small numbers of initial mutations could lead to the high subsequent number of mutations needed for cancer progression. A problem with both models is the inability to explain how the initial mutations occur because efficiency of DNA repair networks in normal cells is very high and the probability of complete inactivation of even three to five relevant genes in the course of the lifetime of a cell is very low (38). Our results suggest that DNA repair pathway effectiveness could be significantly lowered by a few mutations affecting one copy of related genes. This, especially when combined with the presence of environmental mutagens, may result in a high number of DNA lesions, mutation accumulation and tumor initiation. This model could support the conclusion that each individual has unique inherent sensitivity to mutagens, depending on the levels or activity of the proteins involved in DNA repair or related processes. This sensitivity could be measured *in vitro* by the analysis of genotype and levels of specific proteins. This could lead to the establishment of individual limits for mutagen exposure that will bear a health risk, and may thus have significant consequences for cancer prevention.

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