The controlling role of ATM in homologous recombinational repair of DNA damage

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The human genetic disorder ataxia telangiectasia (A-T), caused by mutation in the *ATM* gene, is characterized by chromosomal instability, radiosensitivity and defective cell cycle checkpoint activation. DNA double-strand breaks (dsbs) persist in A-T cells after irradiation, but the underlying defect is unclear. To investigate ATM's interactions with dsb repair pathways, we disrupted *ATM* along with other genes involved in the principal, complementary dsb repair pathways of homologous recombination (HR) or non-homologous end-joining (NHEJ) in chicken DT40 cells. *ATM*$^{-/-}$ cells show altered kinetics of radiation-induced Rad51 and Rad54 focus formation. Ku70-deficient (NHEJ$^-$) *ATM*$^{-/-}$ chicken DT40 cells show radiosensitivity and high radiation-induced chromosomal aberration frequencies, while Rad54-defective (HR$^-$) *ATM*$^{-/-}$ cells show only slightly elevated aberration levels after irradiation, placing ATM and HR on the same pathway. These results reveal that ATM defects impair HR-mediated dsb repair and may link cell cycle checkpoints to HR activation.
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Introduction

Genetic stability requires that any damage sustained by the genome be repaired before the cell divides. Checkpoint signals resulting from the detection of DNA damage arrest the cell cycle to allow time for the cell's repair systems to act, with apoptosis as an alternative outcome in metazoans (Elledge, 1996; Paulovich *et al.*, 1997). Double strand breaks (dsbs) in DNA are an especially hazardous lesion caused by ionizing radiation and are repaired by two major repair pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR) (Weaver, 1995). NHEJ, involving the Ku proteins and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), acts predominantly in the G_1–early S phases of the cell cycle, while HR works in late S–G_2 (Takata *et al.*, 1998). Proteins encoded by the metazoan homologues of the *Saccharomyces cerevisiae RAD52* epistasis group genes are intimately involved in HR. Among them, the loss of Rad54 leads to recombinational deficiencies and dsb repair defects (Bezzubova *et al.*, 1997; Essers *et al.*, 1997), while Rad51's absence causes the accumulation of chromosomal abnormalities and cell death (Sonoda *et al.*, 1998). Both proteins mediate sister chromatid exchange (SCE), which reflects the post-replicational repair of spontaneous DNA damage by recombination with the intact sister chromatid (Sonoda *et al.*, 1999).

Genetic instability and abnormalities of the nervous, immune and reproductive systems are among the complex clinical features of the autosomal recessive disorder ataxia telangiectasia (A-T; recently reviewed in Lavin and Shiloh, 1997; Meyn, 1997), which also include a predisposition to lymphoid malignancy and extreme radiosensitivity. Cells derived from A-T patients show high levels of chromosomal aberrations, greatly potentiated by irradiation, and hypersensitivity to ionizing radiation (Taylor *et al.*, 1975; Thacker, 1994; Meyn, 1995). In A-T cells, ionizing radiation damage does not induce an arrest in DNA synthesis (causing the phenomenon of radioresistant DNA synthesis) or an appropriate arrest at the G_1–S or G_2–M cell cycle checkpoints (Painter and Young, 1980; Beamish and Lavin, 1994; Beamish *et al.*, 1996; Xie *et al.*, 1998; reviewed in Westphal, 1997; Jeggo *et al.*, 1998), suggesting that anomalous cell cycle regulation is a major underlying cause of the disease. After mapping (Gatti *et al.*, 1988) and cloning (Savitsky *et al.*, 1995a) of the gene responsible (designated *ATM*, for *A-T mutated*) the *ATM* locus has been shown to comprise 66 exons over ~150 kb of genomic DNA, encoding a widely expressed 13 kb mRNA transcript (Savitsky *et al.*, 1995b; Uziel *et al.*, 1996). The open reading frame of this transcript codes for a 350 kDa protein, which is absent or inactive in A-T. ATM is a member of a family of large proteins characterized by a C-terminal phosphatidylinositol (PI)-3 kinase-like domain (Jackson, 1995; Zakian, 1995). Recent work has shown that ATM acts on a number of cell cycle-regulating proteins following ionizing radiation, notably Gadd45 (Kastan *et al.*, 1992), p53 (Banin *et al.*, 1998; Canman *et al.*, 1998; Khanna *et al.*, 1998), replication protein A (Liu and Weaver, 1993), Chk2 (Matsuoka *et al.*, 1998) and c-Abl (Baskaran *et al.*, 1997; Shafman *et al.*, 1997), consistent with it being a major regulator of the cell cycle reaction to genome damage (recently reviewed in Brown *et al.*, 1999). Nevertheless, the cause of the

radiosensitivity in A-T remains controversial, being attributed to checkpoint defects, abnormal apoptosis and repair abnormalities (Painter and Young, 1980; Cornforth and Bedford, 1985; Thacker, 1994; Meyn, 1995, 1997; Jeggo *et al.*, 1998). However, despite the sequence homologies between ATM and the NHEJ component DNA-PKcs (Jackson, 1995; Zakian, 1995; Smith and Jackson, 1999), and the accumulation of much recent evidence describing biochemical links between HR proteins and ATM (Baskaran *et al.*, 1997; Shafman *et al.*, 1997; Yuan *et al.*, 1998; Chen *et al.*, 1999), the involvement of ATM in DNA damage recognition and repair remains unclear.

Intrachromosomal recombination is significantly elevated in A-T cells, with frequent sequence alterations accompanying this recombination (Meyn, 1993; Luo *et al.*, 1996). Extrachromosomal recombination levels in A-T cells have been reported to be elevated (Luo *et al.*, 1996), but such enhancement has not been observed consistently (Debenham *et al.*, 1987; Thacker, 1989; Meyn, 1993; Powell *et al.*, 1993; Morrison and Wagner, 1996). However, mis-repair of the recombining DNA appears to be a feature of recombination in A-T (Shiloh, 1997). Taken together, these observations suggest generally aberrant recombinational repair in A-T. To test the hypothesis that ATM deficiency has an impact on recombination, we investigated recombinational repair in *ATM*-deficient mutants of the hyper-recombinogenic chicken DT40 cell line. The phenotype of these $ATM^{-/-}$ mutants recapitulates that of the equivalent mammalian mutants (Barlow *et al.*, 1996; Elson *et al.*, 1996; Xu and Baltimore, 1996), namely radiosensitivity and increased chromosomal aberrations; in addition, these cells show reduced gene targeting efficiencies, suggesting recombination defects (Takao *et al.*, 1999). However, they lack p53, so that the principal defects in $ATM^{-/-}$ DT40 cells arise from p53-independent ATM-mediated processes.

We based the work in this paper on the conjecture that ATM has a role in controlling recombinational repair. This has been hinted at by the various links found recently between HR and ATM (Baskaran *et al.*, 1997; Shafman *et al.*, 1997; Yuan *et al.*, 1998; Chen *et al.*, 1999), and by the recombinational abnormalities associated with A-T (Meyn, 1993; Luo *et al.*, 1996). This paper describes experiments using reverse genetics to ablate NHEJ (by disruption of *KU70*) or HR (by disruption of *RAD54*) on an $ATM^{-/-}$ background, to see whether the loss of either pathway exacerbates the $ATM^{-/-}$ phenotype. Comparison with the parental $ATM^{-/-}$, HR$^-$ ($RAD54^{-/-}$) (Bezzubova *et al.*, 1997) and NHEJ$^-$ ($KU70^{-/-}$) (Takata *et al.*, 1998) single mutants revealed a synergistic accumulation of spontaneous chromosomal aberrations and radiosensitivity in $ATM^{-/-}KU70^{-/-}$ cells, with a far less severe phenotype arising from the conditional loss of *RAD54* in $ATM^{-/-}$ cells. These findings demonstrate that ATM acts in concert with NHEJ to preserve the genome, through HR pathways. We suggest that this may explain the elevated radiosensitivity and deficiencies in the repair of spontaneous DNA damage in the absence of functional ATM and, given the importance of accurate recombination in maintaining the genome, clearly illustrates another tumour-suppressing role of ATM.

Results

Defective recombinational activity in ATM$^{-/-}$ DT40 cells as assessed by Rad51 and Rad54 focus formation
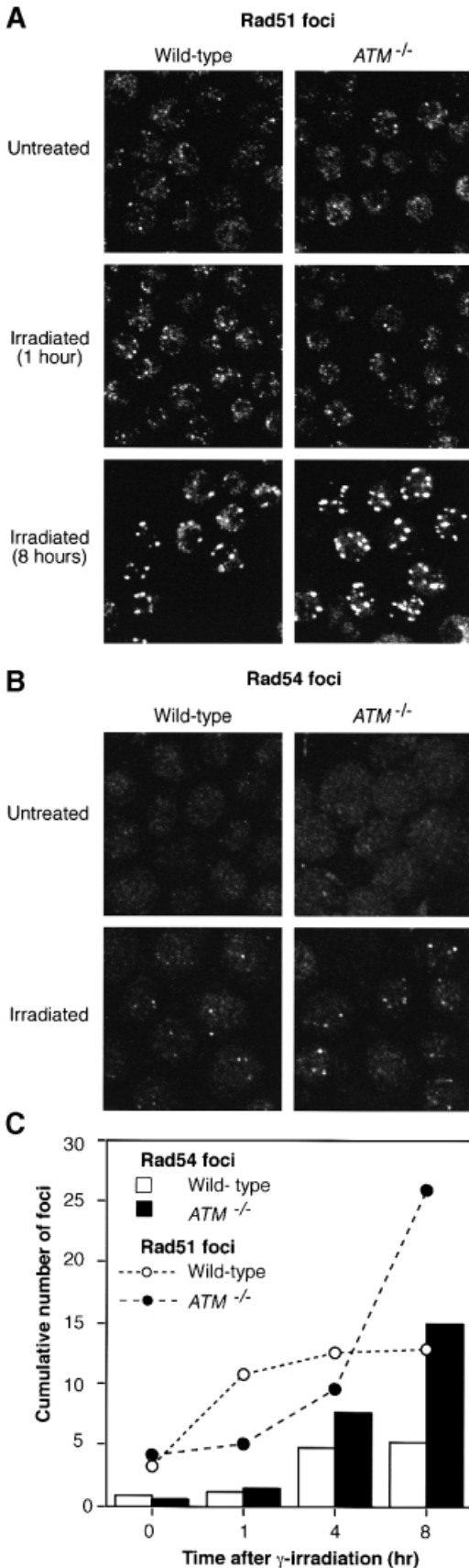
In order to examine recombinational repair before any checkpoint deficiencies might manifest themselves, we monitored the short-term ability of ATM-deficient cells to respond to DNA damage. The assay we used was the radiation-induced formation of nuclear foci of Rad51 and Rad54, which associate closely in the eukaryotic response to DNA damage (Clever *et al.*, 1997; Golub *et al.*, 1997; Tan *et al.*, 1999). Figure 1A shows the formation of distinct nuclear foci of Rad51 in response to ionizing radiation in both wild-type and ATM$^{-/-}$ DT40 cells. Figure 1B shows the formation of Rad54 foci after similar treatment. As shown in Figure 1C, the formation of Rad54 foci in $ATM^{-/-}$ cells continued to increase after such formation had plateaued in wild-type cells; so did the formation of Rad51 foci (Figure 1C), as others have noted previously (Maser *et al.*, 1997). It is perhaps significant, given the interactions described between Rad51 and Rad54 (Clever *et al.*, 1997; Petukhova *et al.*, 1998; Tan *et al.*, 1999), that the initial kinetics of Rad54 focus formation were very similar between wild-type and *ATM*-null DT40 cells, while that of Rad51 was markedly different, there being a much slower activation phase in cells lacking ATM. The altered induction pattern of recombination protein foci shows that HR proceeds abnormally in ATM-deficient cells.

Generation of ATM$^{-/-}$KU70$^{-/-}$ and conditionally Rad54-deficient ATM$^{-/-}$ DT40 cells

Mitotically detectable, spontaneous chromosomal aberrations reflect dysfunctional repair of DNA damage. Cells that lack Ku70, a key component of NHEJ through its interaction with the DNA-dependent protein kinase (Jeggo *et al.*, 1995; Weaver, 1995; Smith and Jackson, 1999), show no such chromosomal instability, although there does exist a deficit in repairing radiation-induced lesions in G_1–early S phase. HR-impaired *RAD51*- or *RAD54*-null cells show high levels of spontaneous chromosomal anomalies and a repair deficiency in late S–G_2 phase (Kemp and Jeggo, 1986; Sonoda *et al.*, 1998; Takata *et al.*, 1998). These findings indicate that the majority of such lesions are normally repaired by HR during S phase, although the complementary NHEJ pathway acts as an alternative, so that the loss of both pathways increases the incidence of chromosomal aberrations (Takata *et al.*, 1998). This overlapping function provided the basis for our experiment: if ATM controls HR, the disruption of HR in $ATM^{-/-}$ cells should be without significant effects, while the loss of NHEJ should have a marked effect. If ATM controls NHEJ, then the converse would be expected.

To test this idea, we disrupted *ATM* in $KU70^{-/-}$ cells and sought to generate $RAD54^{-/-}ATM^{-/-}$ cells, with the expectation of a relatively mild phenotype. However, despite repeated targeting attempts in $ATM^{-/-}RAD54^{+/-}$ cells, we were unable to obtain any double mutants, so we generated conditionally Rad54-null cells instead by placing a human *RAD54* cDNA under the control of a tetracyclin-repressible promoter and transfecting this construct into $RAD54^{-/-}$ DT40 cells. After selecting a

clone that showed wild-type levels of γ-sensitivity and good tet-responsive repression of *hRAD54* (i.e. to *RAD54*$^{-/-}$ levels of radiosensitivity; Figure 2D), we tar-

geted the *ATM* locus. Figure 2 verifies the generation of these *ATM*$^{-/-}$*KU70*$^{-/-}$ and conditionally Rad54-null *RAD54*$^{-/-}$*ATM*$^{-/-}$ mutants; Figure 2A shows a control experiment where we confirm that the disruption of *KU70* results in the absence of Ku70. This immunoblot was stripped and reblotted with antisera raised against Rad54 as a control for protein loading. *RAD54* disruption (Bezzubova *et al.*, 1997) results in the absence of Rad54 protein, so this experiment also confirms the specificity of our Rad54 antisera. Figure 2B shows the results of an RT–PCR experiment used to confirm the disruption of the kinase domain of ATM (Takao *et al.*, 1999). Primers directed against an exon 3′ to the disrupted sequence (ATM k.d.) do not amplify any target, showing that the *ATM* locus has been disrupted, while a control amplification, using primers directed against an ATM sequence closely upstream of the targeted integration site (ATM 5′), confirms the quality of the RNA used in this experiment. Figure 2C shows the strong repression of Rad54 in the *ATM*$^{-/-}$*RAD54*$^{-/-}$ clone that we obtained (lanes 2–5), despite the high initial expression levels of the transgene (lane 1). Figure 2D confirms that the *RAD54* transgene confers a conditionally Rad54-deficient phenotype upon the *RAD54*$^{-/-}$ cells in which we targeted the *ATM* gene.

Proliferative properties of NHEJ- or HR-deficient *ATM*$^{-/-}$ cells

As shown in Figure 3A, the disruption of *ATM* markedly reduced the proliferation rate of the mutant cells, although this reduction is less pronounced in *ATM*$^{-/-}$ DT40 cells than in mammalian *ATM* mutants. This appeared to be due to elevated levels of cell death during each cell cycle, rather than to any marked elongation of cell cycle duration (data not shown). The conditionally Rad54-deficient *ATM* null clone grew rather slowly (Figure 3B) and had a low plating efficiency (<10%) in methylcellulose medium, perhaps reflecting either small incompatibilities between the human and chicken *RAD52* group genes becoming more pronounced in the absence of ATM or some negative effects of the tags carried on the Rad54 construct used (Swagemakers *et al.*, 1998). Perhaps surprisingly, the repression of *hRAD54* resulted in only a minor proliferative retardation (Figure 3B) up to 5 days.

Genomic instability in NHEJ- or HR-deficient *ATM*$^{-/-}$ cells

Next, we monitored the occurrence of spontaneous chromosomal aberrations in *ATM*-null cells lacking Ku70 or Rad54. While ATM-deficient cells show slightly elevated

Fig. 1. Radiation-induced nuclear foci of Rad51 and Rad54 proteins. (**A**) Immunofluorescence visualization of Rad51 foci in cells of the genotypes indicated before and 1 or 8 h after 4 Gy γ-irradiation. (**B**) Immunofluorescence visualization of Rad54 foci in cells of the genotypes indicated before and 8 h after 4 Gy γ-irradiation. The control frames are slightly overexposed relative to the experimental frames to emphasize that few foci are present. (**C**) Quantitation of focus formation in wild-type and *ATM*$^{-/-}$ DT40 cells following irradiation. Data points for Rad54 foci show the cumulative number of foci per 100 cells and were counted in at least 100 cells in randomly selected frames in two separate experiments. A third experiment gave similar results. The cumulative numbers of Rad51 foci are presented per 50 cells and show representative results from two separate experiments.
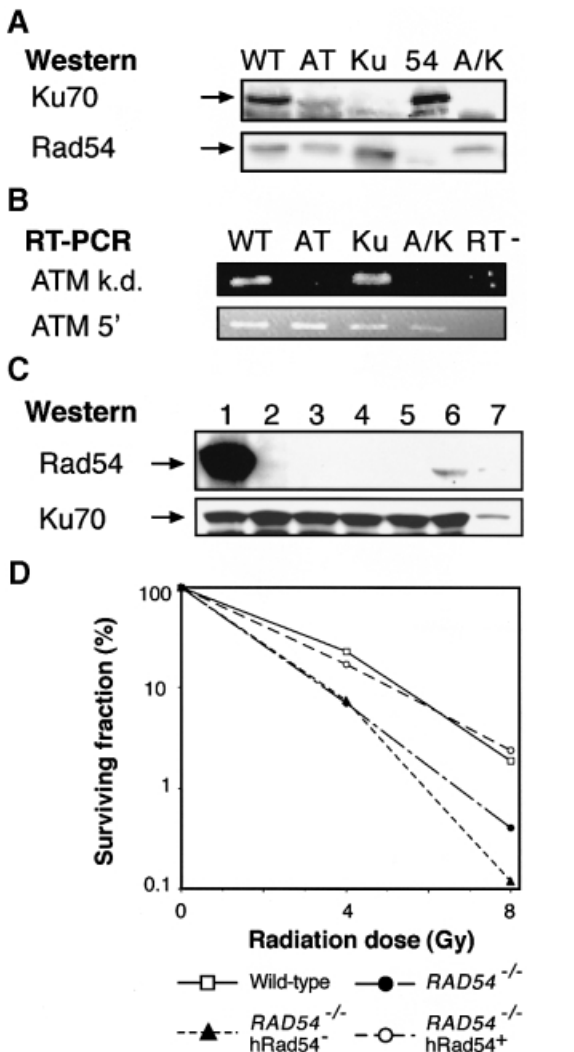
465

Fig. 2. Phenotypic controls of DT40 mutants used in this paper.
(**A**) Immunoblot of total protein from wild-type (WT), $ATM^{-/-}$ (AT),
$KU70^{-/-}$ (Ku), $RAD54^{-/-}$ (54) and $ATM^{-/-}KU70^{-/-}$ (A/K) DT40 cells.
The same blot was hybridized with antisera against Ku70 (upper
panel) and Rad54 (lower panel). (**B**) Ethidium bromide-stained agarose
gel showing the products of RT–PCR of the ATM kinase domain
(ATM k.d.) or of the region 5′ to the disrupted kinase motif (ATM 5′).
Lane designations are as for (A), with RT⁻ indicating the lane
showing RNA subjected to PCR without the addition of reverse
transcriptase. (**C**) Immunoblot showing Rad54 suppression in $ATM^{-/-}$
$RAD54^{-/-}$ tet-hRAD54⁺ cells. Lanes show protein prepared from
$ATM^{-/-}RAD54^{-/-}$ tet-hRAD54⁺ cells prior to (lane 1) and at 1, 2, 3 and
5 days after Rad54 repression (lanes 2–5, respectively). Lane 6 shows
protein from wild-type cells, lane 7 a 5-fold dilution of the sample in
lane 6. Hybridization with anti-Ku70 antibody was used to control for
protein loading. (**D**) Radiosensitivity of $RAD54$ mutants as assessed by
clonogenic survival of the indicated clones following γ-irradiation.
Data points show the average of duplicate experiments for each clone.
The conditionally Rad54-deficient cells were irradiated on day 4 of
$RAD54$ repression.

Fig. 3. Proliferative characteristics of $ATM^{-/-}KU70^{-/-}$ and conditionally
Rad54⁻$ATM^{-/-}$ cells. (**A**) Proliferation analysis of the clones is
indicated at the bottom. Data points on the growth curves shown are
the means + SD of values obtained in two separate duplicate
experiments per clone. A second $ATM^{-/-}KU70^{-/-}$ clone gave similar
results. (**B**) Proliferation analysis of $ATM^{-/-}RAD54^{-/-}$ tet-hRAD54⁺
cells prior to and after Rad54 repression, as shown at the bottom. Data
points represent the means + SD of values obtained from two separate
duplicate experiments with three different $ATM^{-/-}RAD54^{-/-}$ tet-
hRAD54⁺ subclones. Analysis of the pool of $ATM^{-/-}RAD54^{-/-}$ tet-
hRAD54⁺ cells gave the same results.

levels of spontaneous chromosomal damage, the additional
loss of Ku70 greatly potentiates the occurrence of both
single chromatid and isochromatid (both chromatids) gaps
and breaks (Table I). These results suggest that the
aberrations resulting from the absence of ATM are no
longer properly repaired in the absence of Ku70, which
acts as a backup for HR (Takata *et al.*, 1998). Karyotypic
analysis of conditionally Rad54-deficient $ATM^{-/-}$ cells
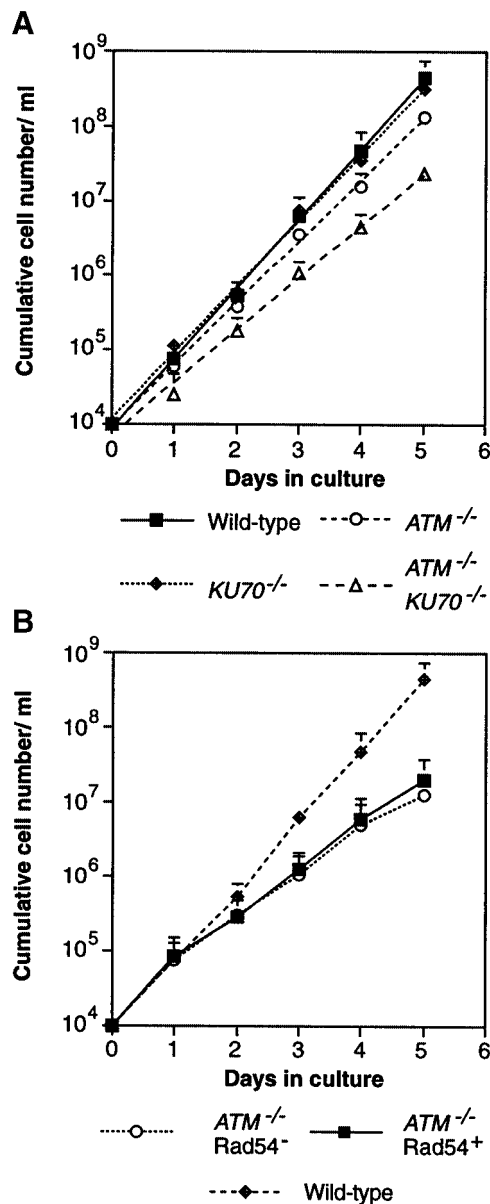after $hRAD54$ repression also revealed an increase in

the level of chromosomal aberrations (Table II). This
accumulation was accompanied by a slight decrease in
the proliferation of the Rad54⁻$ATM^{-/-}$ cells (Figure 3B),
although this minor decrease was the extent of the prolifer-
ative reduction after up to 7 days of $hRAD54$ repression,
with no continued accumulation of chromosomal abnor-
malities (data not shown). If we compare the extent of
the synergistic increase in chromosomal aberrations in the
absence of ATM and either repair pathway [i.e. the increase
beyond the additive effects of checkpoint deficiency (in
$ATM^{-/-}$ cells) and dsb repair deficiency (in $KU70^{-/-}$ or

Table I. Spontaneous chromosomal aberrations in ATM/Ku70-deficient cells

Genotype	Chromatid		Isochromatid		Exchanges	Total aberrations ($\pm$ SE)
	Breaks	Gaps	Breaks	Gaps		
wild type	0	2	0	0	0	2 $\pm$ 1.4
$ATM^{-/-}$	0	3	2	1	0	6 $\pm$ 2.4
$KU70^{-/-}$	3	1	0	0	0	4 $\pm$ 2.0
$ATM^{-/-}KU70^{-/-}$ #1	3.5	4.5	4	4	0	16 $\pm$ 2.8
$ATM^{-/-}KU70^{-/-}$ #2	3	1.5	5	5.5	0	15 $\pm$ 3.9

Data are presented as macrochromosomal (1–5 and Z) aberrations per 100 metaphase spreads, the SE being calculated as ($\sqrt{}$ the number of aberrations)/the number of spreads analysed. One hundred wild-type or single mutant metaphases and 200 from both $ATM^{-/-}KU70^{-/-}$ clones were analysed.

Table II. Spontaneous chromosomal aberrations in ATM/Rad54-deficient cells

Genotype	Chromatid		Isochromatid		Exchanges	Total aberrations ($\pm$ SE)
	Breaks	Gaps	Breaks	Gaps		
$RAD54^{-/-}$	3	1.9	0.7	0.8	0.3	7 $\pm$ 1
$ATM^{-/-}RAD54^{-/-}$Rad54$^+$	2.5	2.5	1	0.5	0.5	7 $\pm$ 2
$ATM^{-/-}RAD54^{-/-}$Rad54$^-$ d1	2	4.5	4.5	3.5	0.5	15 $\pm$ 3
$ATM^{-/-}RAD54^{-/-}$Rad54$^-$ d2	2.5	3.5	2.5	4	0	13 $\pm$ 3
$ATM^{-/-}RAD54^{-/-}$Rad54$^-$ d3	2	4.5	3.5	2.5	0	13 $\pm$ 3
$ATM^{-/-}RAD54^{-/-}$Rad54$^-$ d4	3.5	5.5	3	5	1	18 $\pm$ 3
$ATM^{-/-}RAD54^{-/-}$Rad54$^-$ d5	5.5	5.5	8	8.5	0	27 $\pm$ 4

Data for $RAD54^{-/-}$ cells are taken from Takata *et al.* (1998). Two hundred metaphases were analysed in each other case, from 1–5 days after $RAD54$ repression (d1–d5). Data were calculated and are presented as described for Table I.

Table III. Chromosomal aberrations induced by irradiation during G_2 and late S–G_2 phase

Genotype	1.5 h				3 h			
	Chromatid	Isochromatid	Exchanges	Total	Chromatid	Isochromatid	Exchanges	Total
wild type	28	6	0	34 $\pm$ 6	11	4	0	15 $\pm$ 4
$ATM^{-/-}$	40	9	1	50 $\pm$ 6	19	11	0	30 $\pm$ 5
$KU70^{-/-}$	58	7	4	69 $\pm$ 8	10	7	1	18 $\pm$ 4
$RAD54^{-/-}$	37	10	1	48 $\pm$ 7	19	5	1	25 $\pm$ 5
$ATM^{-/-}/KU70^{-/-}$ #1	131	13	0	144 $\pm$ 12	113	17	1	131 $\pm$ 11
$ATM^{-/-}/KU70^{-/-}$ #3	nd	nd	nd	nd	77	71	0	148 $\pm$ 9
$ATM^{-/-}/RAD54^{-/-}$ Rad54$^+$	28	9	0	37 $\pm$ 6	32	4	0	36 $\pm$ 8
$ATM^{-/-}/RAD54^{-/-}$ Rad54$^-$	43	8	0	51 $\pm$ 7	26	8	0	34 $\pm$ 8

Cells were subjected to 0.3 Gy γ-irradiation, then incubated with colcemid for 1.5 or 3 h, as indicated, and harvested. At least 100 metaphases at 1.5 h and at least 50 at 3 h were analysed. The data from $ATM^{-/-}RAD54^{-/-}$Rad54$^-$ cells were obtained after 5 days of Rad54 repression. Data were calculated and are presented as described for Table I. nd, not determined.

$RAD54^{-/-}$ cells)], it appears that ATM acts together with either HR or NHEJ during repair of replication-associated DNA damage.

Recombinational repair of radiation-induced DNA damage

Since HR is used to repair induced DNA damage in S–G_2, with NHEJ acting as a backup (Takata *et al.*, 1998), we reasoned that an increase in induced chromosomal aberrations observed within a short time after irradiation (i.e. irradiation in G_2 or S–G_2) would reflect a defect in HR, and such a defect should be exacerbated by the loss of NHEJ. Therefore, to test whether ATM has a role in HR, we examined the karyotypes of $ATM^{-/-}$ and $ATM^{-/-}KU70^{-/-}$ cells after γ-irradiation. As shown in Table III, $ATM^{-/-}$ cells showed slightly higher levels of chromosomal aberrations than wild-type cells following treatment with 0.3 Gy. Since radiation principally induces chromatid-type damage (i.e. breaks in single chromatids), the conversion of such lesions to isochromatid-type damage may suggest a failure during the process of recombinational repair. Therefore, the slight increase of isochromatid abnormalities in $ATM^{-/-}$ cells (similar to that observed in $RAD54^{-/-}$ cells) may reflect a HR defect. $ATM^{-/-}KU70^{-/-}$ cells showed a dramatically elevated inability to repair chromosome damage. The different pattern of chromosomal aberrations observed in the two $ATM^{-/-}/KU70^{-/-}$ clones examined is likely to be due to clonal variation (as might be expected in mutants with such frequent spontaneous aberrations), rather than to any technical problem. The strongly increased radiosensitivity in $ATM^{-/-}KU70^{-/-}$ cells compared with either single mutant (Figure 4) confirms the significance of our cytogenetic analysis. However, ATM deficiency does not disrupt NHEJ drastically, since the radioresistance of $ATM^{-/-}$ cells is higher than that of $KU70^{-/-}$ cells at doses ≤2 Gy.
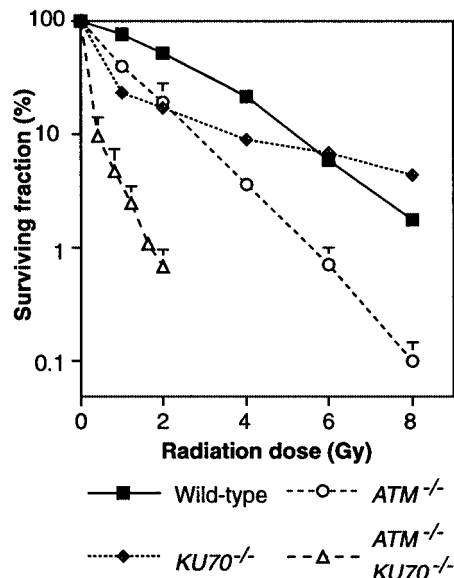
Fig. 4. Radiosensitivity of $ATM^{-/-}KU70^{-/-}$ cells. Clonogenic survival of the indicated clones following γ-irradiation. Data points show the means + SD for at least three separate experiments per clone.

To examine the extent to which ATM and Rad54's roles overlap in recombinational repair of induced damage, we monitored cytogenetic abnormalities induced by irradiation of Rad54-deficient *ATM*-null cells in G_2 phase (1.5 h after irradiation) or in late S–G_2 (3 h after irradiation) (Table III); the poor plating efficiency of these cells did not permit us to assess radiosensitivity by survival. The conditionally Rad54$^-$*ATM*$^{-/-}$ cells showed rather high levels of chromosomal aberrations prior to repression of the *hRAD54* transgene, perhaps reflecting the inter-species incompatibilities between the *RAD52* group genes suggested above. However, after repression of Rad54, they showed only a slightly increased inability to repair their chromosomes by HR, showing the mild phenotype expected if the ATM and HR involving Rad54 lie on the same pathway of dsb repair. In contrast to our findings in *RAD54*$^{-/-}$*KU70*$^{-/-}$ cells (Takata *et al.*, 1998), it is noteworthy that no major increase in radiation-induced damage occurred, further demonstrating that ATM deficiency does not significantly disrupt NHEJ.

Discussion

The major evidence for a DNA dsb repair defect additional to the cell cycle checkpoint deficiencies caused by ATM deficiency was reviewed recently (Jeggo *et al.*, 1998). This consists of the inability of experimentally imposed cell cycle delay to compensate for the absence of the ATM-imposed arrest (since time for DNA repair is considered to be a major reason for checkpoint arrest) (Weichselbaum *et al.*, 1978), high levels of radiation-induced chromosomal damage in A-T cells without passage through the cell cycle (Taylor *et al.*, 1976; Cornforth and Bedford, 1985), and the ability to distinguish separate ATM domains responsible for the radiosensitivity and cell cycle defects (Morgan *et al.*, 1997). To investigate whether HR might be directly affected by the absence of ATM, we monitored the formation of nuclear foci of Rad54 and Rad51 in response to irradiation. The alteration of subnuclear local-

ization of repair proteins in response to DNA damage is an established motif in the cell biology of DNA repair (Haaf *et al.*, 1995; Maser *et al.*, 1997; Tan *et al.*, 1999). Rad51 foci were formed far more rapidly in wild-type than in *ATM*$^{-/-}$ cells, although many more accumulated in the *ATM*$^{-/-}$ cells at later timepoints (Figure 1A and C), probably reflecting increased numbers of unrepaired breaks due to cells carrying unrepaired lesions proceeding through the cell cycle. Conversely, Rad54 foci formed with similar initial kinetics in wild-type and ATM-deficient cells (Figure 1B and C), and then accumulated in the *ATM* null cells, as did Rad51 foci. Since Rad51 and Rad54 interact closely (Clever *et al.*, 1997; Golub *et al.*, 1997; Petukhova *et al.*, 1998; Tan *et al.*, 1999), we interpret these findings to mean that the later stages of recombinational repair, including Rad54 focus formation, proceed normally, but the initial stages, mediated principally by Rad51 (but with assistance from other proteins including Rad54) (Petukhova *et al.*, 1998), are disrupted by ATM deficiency. This model implies that ATM lies upstream in focus formation of Rad51, but not of Rad54. The reduction of targeting efficiency in *ATM*$^{-/-}$ cells (Takao *et al.*, 1999) presents further evidence for an HR deficiency in the absence of ATM function.

We next used reverse genetics to test the hypothesis that HR is affected by ATM deficiency. Our examination of *ATM*$^{-/-}$*KU70*$^{-/-}$ cells revealed the accumulation of spontaneous chromosomal abnormalities, with an associated proliferative defect (Table I and Figure 3). HR is able to repair almost all the additional lesions incurred by Ku70 deficiency, so that *KU70*$^{-/-}$ cells have a phenotype indistinguishable from wild-type cells with regard to spontaneous lesions (Table I; and Kemp and Jeggo, 1986; Takata *et al.*, 1998). However, the loss of ATM leads to a low level of chromosome aberrations (Table I), suggesting that HR is not capable of completely repairing spontaneous damage without it. Significantly, frequent isochromatid gaps and breaks occur in *ATM*$^{-/-}$*KU70*$^{-/-}$ cells, suggesting that Ku70 deficiency may exacerbate the *ATM*$^{-/-}$ phenotype by removing a back-up system for HR, as is seen with Ku70 deletion in HR-deficient *RAD54*$^{-/-}$ cells (Takata *et al.*, 1998). Stronger evidence for a HR defect comes from our karyotypic analysis of γ-irradiated *ATM*$^{-/-}$*KU70*$^{-/-}$ cells, which show a greatly elevated incidence of chromosomal aberrations. A possible link between ATM and the HR apparatus is c-Abl, which associates with and is activated by ATM (Baskaran *et al.*, 1997; Shafman *et al.*, 1997) and has been shown to phosphorylate Rad51 in response to DNA damage (Yuan *et al.*, 1998; Chen *et al.*, 1999). However, the biological significance of these findings is not yet clear, as c-Abl-mediated phosphorylation negatively affected Rad51's activity in one set of experiments reported (Yuan *et al.*, 1998), but enhanced its association with Rad52, a putative modulator of Rad51 activity, in the other (Chen *et al.*, 1999).

From the lethality of Rad51 deficiency (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996; Sonoda *et al.*, 1998) and the requirement of HR for repair of replication-associated DNA damage (Sonoda *et al.*, 1999), it appears that HR is essential in vertebrate cells. Since neither ATM nor Rad54 deficiency alone results in lethality, based on the assumption that ATM activates HR as well as the DNA damage checkpoint, the generation of *RAD54*$^{-/-}$*ATM*$^{-/-}$ double

mutants had two possible outcomes: lethality, showing that an ATM-controlled homologue of Rad54 compensates for RAD54 deficiency; or survival, showing that the compensating protein(s) are independent of ATM. Analysis of survivors' phenotype would indicate the importance of Rad54-linked HR as a target of ATM. The observation of relatively few additional radiation-induced chromosomal aberrations (Table III) strongly suggests that ATM interacts with HR involving Rad54 during the repair of induced damage. It is possible that low levels of Rad54 are still present in these cells and permit their survival, although the $RAD54^{-/-}$ levels of radiosensitivity in the parental conditionally null cell line upon Rad54 repression (Figure 2D) argue that the putative leaky Rad54 expression is not sufficient for efficient dsb repair after ionizing radiation. Another possibility is that homologous proteins may mediate the HR activity known to be essential from the lethality of Rad51 deficiency (Sonoda *et al.*, 1998). However, the rather gradual increase in spontaneous chromosome abnormalities (Table II) in the absence of Rad54 and ATM demonstrates that their interaction is less important for the repair of spontaneous DNA damage, although Rad54 is not necessarily the only major target of ATM in the recombinational repair of replication-associated DNA damage.

While the possible synergistic effects of the dsb repair and checkpoint defects in $ATM^{-/-}$ cells in spontaneous chromosomal aberrations or in the clonogenic survival assay are unclear, our karyotypic analysis after irradiation addresses this issue. By irradiating the cells a short time before analysis, we limit the DNA repair to that which can occur in late S–G$_2$. Since HR is the predominant activity in that period, its loss would be expected to compound the effects of checkpoint deficiency, if such checkpoint deficiency contributes significantly to the level of aberrations observed. Because the Rad54$^-ATM^{-/-}$ cells show a minor effect following irradiation, the loss of the ATM-mediated checkpoint does not lead to a major accumulation of chromosomal damage after irradiation. Therefore, aberrant repair, rather than aberrant checkpoint activation, is the predominant cause of the severe effects seen in $KU70^{-/-}ATM^{-/-}$ cells after irradiation.

Taken together, our results show that ATM controls recombinational repair of DNA damage. A direct link between cell cycle checkpoint proteins and dsb repair is a conceptually satisfying motif and has recently been suggested for NHEJ and ATM based on yeast data (Martin *et al.*, 1999; Mills *et al.*, 1999). Defective HR is consistent with all the known aspects of A-T and ATM-deficient cells, from cellular assays revealing abnormal recombination (Meyn, 1993; Luo *et al.*, 1996), slightly-reduced SCE levels (Galloway, 1977) and radiosensitivity (Taylor *et al.*, 1975), to chromosomal instability and predisposition to malignancy (Lavin and Shiloh, 1997; Meyn, 1997; Shiloh, 1997). Investigation of the interactions of the recombinational repair machinery and the DNA damage checkpoint apparatus will shed further light on this aspect of the disease.

Materials and methods

Plasmid construction

The construction of gene targeting vectors designed to disrupt the kinase domain of *ATM* with neomycin and puromycin as selection markers has

been described (Takao *et al.*, 1999). A vector carrying a blasticidin cassette (Takata and Kurosaki, 1996) was prepared in the same manner and was linearized with *Not*I for gene targeting. To generate a tet-repressible (Gossen *et al.*, 1995) *RAD54* expression vector, a *Sca*I–*Mlu*I sequence from pZeoSV-HishRad54HA, coding for human Rad54 bearing an N-terminal polyhistidine tag and a C-terminal haemagglutin tag (Swagemakers *et al.*, 1998), was Klenow-treated and inserted into the *Bam*HI site of pUHG10-3 (M.Gossen, ZMBH, Heidelberg, unpublished), yielding pUHG10-3HishRad54HA. ptTA-Hyg^R has been described (Sonoda *et al.*, 1998). For expression of chicken Rad54 in *Escherichia coli*, a PCR fragment coding for amino acids 332–515 of the chicken sequence (Bezzubova *et al.*, 1997) was cloned into pET15b (Novagen, Madison, WI), yielding pETchRad54(332–515). The sequences of the primers used were: GTGGATCCGTTGCTTGAATATTTCAGCC and GTGGATCCGCTTGTCGTTACTGGTGCT.

Cell culture and gene targeting

DT40 cells were maintained as described (Sonoda *et al.*, 1998). Electroporation of linearized targeting vectors and antibiotic selection conditions were as described previously (Sonoda *et al.*, 1998; Takata *et al.*, 1998). The *ATM* locus in $KU70^{-/-}$ cells (Takata *et al.*, 1998) was successively targeted with targeting vectors carrying a neomycin and subsequently a puromycin resistance cassette. $RAD54^{-/-}$ DT40 cells (Bezzubova *et al.*, 1997) were transfected with linearized pUHG10-3HishRad54HA and ptTA-Hyg^R and selected as described (Sonoda *et al.*, 1998), and then examined for Rad54 expression by Western blot analysis. A clone showing tet-repressible restoration of radioresistance was used for further experiments and was successively targeted with *ATM*-disrupting vectors carrying puromycin and then blasticidin resistance. Selection for both puromycin and blasticidin resistance was maintained during targeting of the second *ATM* allele.

Proliferation analyses and colony survival assay

Cells were counted daily by flow cytometric comparison with fixed numbers of 25 μm microspheres (Polysciences Inc., Warrington, PA) (Takata *et al.*, 1998). Cells were split each day to keep them sub-confluent. Radiosensitivity of cells was examined by their colony-forming ability in methylcellulose-containing medium 7–10 days after γ-irradiation with ^{137}Cs (Gammacell 40, Nordion, Kanata, Ontario, Canada) (Takata *et al.*, 1998).

Karyotypic analysis

For karyotype analysis, colcemid (Gibco-BRL, Grand Island, NY) was added to the growth medium to a concentration of 0.1 μg/ml for 1.5 or 3 h, after which metaphase spreads were placed on glass slides and Giemsa-stained as described (Sonoda *et al.*, 1998). Scoring of chromosomal aberrations was according to the standard criteria (ISCN, 1985) with breaks being distinguished from gaps by a diagnostic misalignment of the portion of the chromosome distal to the break.

Preparation of Rad54 antisera and immunoblotting

pETchRad54(332–515) was transformed into *E.coli* and the Rad54 fragment produced was purified by nickel column chromatography. Rabbit polyclonal antibodies were raised against this Rad54 fragment. For immunoblot analysis, total cellular proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with rabbit antisera raised against Rad54, Ku70 (Takata *et al.*, 1998) or Rad51 (Tashiro *et al.*, 1996) at room temperature and visualized using horseradish peroxidase-coupled secondary antibodies directed against rabbit immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA).

RT–PCR

Total RNA was isolated with TRIzol reagent (Gibco-BRL) and 2 μg oligo(dT)-primed, and reverse-transcribed using the Superscript cDNA synthesis system as described by the suppliers (Gibco-BRL). One-tenth of this reaction was denatured at 94°C for 180 s, then PCR amplified with 25 cycles of 94°C (45 s), 55°C (45 s) and 68°C (60 s) and a final 72°C step for 5 min. Primers were used at 200 nM and their sequences were: chicken *ATM* kinase domain, GTGGATCCCACAGGAAGATA and GTCAGCTTCATCCTCTGGTC; *ATM* 5′ region, GGAGTTTCTGAATGGCAACTAGAAGAAGCT and GATTATATTTCTAGCAGCCTC.

Immunofluorescent visualization of nuclear foci

Cells were spun out of culture on to glass slides using a Cytospin 3 (Shandon, Pittsburgh, PA) and fixed with 3.7% formalin solution. NP-40 solution (0.1%) was used to permeabilize the cells and then primary

antibody stainings were performed in 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20 using optimal dilutions of the Rad51 or Rad54 antisera described above. Fluorescein isothiocyanate-coupled anti-rabbit immunoglobulin secondary antibodies (Santa Cruz) were applied in the same buffer, with at least three washes in buffer between each step and before microscopy. Foci were visualized using confocal microscopy (MRC-1024; Bio-Rad, Hercules, CA). Analysis was of untreated cells or of cells γ-irradiated with 4 Gy at different times after treatment. Following microscopy and image processing with Adobe Photoshop version 4.0J, colour-inverted images were printed and distinct foci counted.

Acknowledgements

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