ATM is required for the cellular response to thymidine induced replication fork stress

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Genetically distinct checkpoints, activated as a consequence of either DNA replication arrest or ionizing radiation-induced DNA damage, integrate DNA repair responses into the cell cycle programme. The ataxia-telangiectasia mutated (ATM) protein kinase blocks cell cycle progression in response to DNA double strand breaks, whereas the related ATR is important in maintaining the integrity of the DNA replication apparatus. Here, we show that thymidine, which slows the progression of replication forks by depleting cellular pools of dCTP, induces a novel DNA damage response that, uniquely, depends on both ATM and ATR. Thymidine induces ATM-mediated phosphorylation of Chk2 and NBS1 and an ATM-independent phosphorylation of Chk1 and SMC1. AT cells exposed to thymidine showed decreased viability and failed to induce homologous recombination repair (HRR). Taken together, our results implicate ATM in the HRR-mediated rescue of replication forks impaired by thymidine treatment.

INTRODUCTION

Cells respond to DNA damage by initiating cell cycle checkpoints, activating DNA repair or triggering cell death (1). The related ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases play key roles in the coordination of these events (2,3). Inherited mutations of the ATM gene (occurring in patients with ataxia-telangiectasia or AT) cause a genome instability disorder characterized by neurodegeneration, immunodeficiency, radiation sensitivity and cancer predisposition. ATM initiates a complex cellular response to DNA double strand breaks (DSBs) induced by agents such as ionizing radiation (IR) that results in the delay of DNA synthesis or the onset of mitosis. As an early event in the induction of the S-phase checkpoint following IR, ATM phosphorylates NBS1 and Chk2 (4–8). NBS1 is a component of the MRE11/NBS1/RAD50 complex that is required for the repair of DSBs as well as the induction of DNA synthesis delay (9). Cells defective in NBS1 or MRE11 are defective in the IR-induced DNA synthesis delay, although the effect is not as severe as that seen in AT cells (7,8,10). A tumour cell line defective in Chk2 function also appears to be defective in the IR-induced DNA synthesis delay (8), however, primary mouse fibroblasts obtained from Chk2−/− knockout mice retain this S-phase checkpoint (11). Chk2 activation by ATM stimulates the phosphorylation of the cell cycle regulator Cdc25A leading to its degradation through the polyubiquitination-mediated proteolysis pathway (6). There is also evidence that ATM is required for the activation of DNA repair following exposure to IR. ATM defective chicken DT40 cells show altered kinetics of the organization of homologous recombination repair (HRR) proteins such as RAD51 and RAD54 into foci following IR exposure (12). This may potentially lead to the suboptimal function of this repair pathway and reliance on error prone repair mechanisms to correct chromosome breaks.

ATR responds to a wider range of DNA lesions and appears to be important in maintaining the integrity of the DNA replication apparatus following damage that arrests the progression of this complex (1,13,14) ATR phosphorylates Chk1 (on Ser345) in response to agents that arrest DNA replication (15). Because Chk1 is essential for viability much like ATR, this protein is thought to be a key effector of the ATR pathway. ATR also phosphorylates a number of other proteins in response to DNA damage at replication forks induced by the ribonucleotide reductase inhibitor hydroxyurea (HU) including RAD17 (which loads the PCNA-like Rad1–Rad9–Hus1 onto damaged chromatin), (16,17) and SMC-1 (which is required for sister chromatid cohesion and recombination) (18–20).

Here, we present evidence for a novel S-phase response to the slowing of DNA replication by excess thymidine that
appears to include both ATM and ATR. The motivation for this work came from our observation that mismatch repair (MMR) deficient human tumour cell lines are acutely sensitive to the S-phase block triggered by thymidine (21). Thymidine is phosphorylated to dTTP, which is an allosteric inhibitor of the reduction of CDP by ribonucleotide reductase (22).

The increase of dTTP following thymidine treatment specifically leads to the depletion of dCTP and an accumulation of cells in the S-phase of the cell cycle (an effect known as thymidine block, 23). Surprisingly, little is known about the role of ATM and ATR in the response to thymidine although HU is known to trigger an ATR-mediated response (15). Here, we show that the cell survival following thymidine exposure is uniquely dependent upon both ATR and ATM protein kinases and that loss of either of these leads to thymidine sensitivity. We further show that thymidine induces ATM and ATR protein kinase cascades and that ATM is required for the induction of homologous recombination by thymidine in the absence of detectable DSBs. These data provide evidence for a novel role for ATM in the HRR-mediated rescue of DNA replication forks impaired by thymidine.

RESULTS

ATR- and ATM-defective cells are sensitive to thymidine

To determine whether the ATR and ATM protein kinases play a role in the cellular response to thymidine, we first examined the sensitivity of cells defective in ATR or ATM to its toxic effects. To determine the effect of ATR dysfunction, we used U2OS cells which inducibly express a dominant negative, kinase dead ATR allele (24). Both induced and uninduced U2OS cells were exposed to either thymidine or HU for varying lengths of time and cells surviving this treatment were allowed to form colonies. Cells induced for the expression of the ATRkd cDNA were more sensitive to both thymidine (Fig. 1A) and HU (Fig. 1B) than uninduced cells.

ATM deficient cells were next investigated for their ability to form colonies in the presence of thymidine. Three different immortalized AT fibroblast cell lines (TAT2SF, TAT5BIVA and AT221JE-T/pEBS) were significantly more sensitive to thymidine relative to uncorrected (MRC5VA) fibroblast line in colony forming assays (Fig. 1C). Thymidine sensitivity was the result of the loss of ATM function as a derivative of AT221JE-T/pEBS corrected for the AT phenotype by the introduction of the wild-type ATM cDNA (pEBS-YZ5) (25) was significantly more resistant to thymidine than the uncorrected cells. In contrast, AT cells did not show increased sensitivity to HU (Fig. 1D) although this agent also induces dNTP pool imbalances. In addition, pEBS-YZ5 showed no significant difference in sensitivity to HU relative to uncorrected cells. Thus, the ability of cells to survive the cytotoxic effects of thymidine requires both ATM and ATR, whereas survival in HU appears to depend only upon ATR.

Thymidine triggers both the ATM- and ATR-mediated protein kinase cascades

ATM undergoes a rapid autophosphorylation at serine 1981 following exposure to IR (26). Given the sensitivity of AT cells to thymidine, we next determined whether thymidine triggered a similar autophosphorylation of ATM. MRC5VA cells were treated with thymidine for varying lengths of time and cell free extracts prepared from these cultures were assayed for the presence of pSer1981-ATM by western blotting using an antibody specific for the activated protein (27). This analysis revealed that ATM was rapidly phosphorylated (within 15 min) following thymidine treatment (Fig. 2A). Interestingly, HU also triggered autophosphorylation of ATM although AT cells are not sensitive to this agent (Fig. 2B).

Chk2 and NBS1 are phosphorylated by ATM following its activation by IR exposure. To determine whether thymidine induced a similar response, the MRC5VA extracts prepared in the earlier-mentioned experiment were analyzed for phosphorylated forms of these proteins by western blotting (Fig. 2A). Like the autophosphorylation of ATM, phosphorylation of Chk2 on threonine 68 and NBS1 on serine 343 occurred within 15 min of treatment of wild-type cells with thymidine, although the phosphorylated forms of these proteins were much stronger at 24 h post-treatment.

AT cells, on the other hand, were defective in this response. After a 24 h treatment of AT cells with 10 mM thymidine or exposure to 10 Gy of IR, the autophosphorylated form of ATM was not detected and the phosphorylation of Chk2 and...
the phosphorylation of Chk2 and NBS1 were restored in the AT line corrected for the ATM defect (pEBS-YZ5), providing further evidence for the ATM dependence of this thymidine induced protein kinase cascade (Fig. 2B). Taken together, these results indicate that thymidine rapidly triggers the phosphorylation of both Chk2 and NBS1 through an ATM-mediated signalling pathway. HU has a similar effect, but the ATM-mediated response to this agent does not appear to be essential for cell survival.

As cell survival following thymidine treatment requires both ATM and ATR, we reasoned that some substrates of these PIK kinases should be phosphorylated in an ATM-independent manner in response to thymidine treatment. Chk1 may be phosphorylated by either ATM (28) or ATR (15); therefore, we next determined the phosphorylation state of Chk1 following thymidine treatment. Western blot analysis of cell extracts obtained from thymidine treated wild-type cells revealed phosphorylation of Chk1, however, the appearance of pSer343-Chk1 was significantly delayed (detectable only at 4–24 h post-treatment, Fig. 2A). SMC1 was phosphorylated on serine 966 in MRC5VA cells following thymidine, HU or IR treatment (Fig. 2C). Phosphorylation of both Chk1 and SMC1 was retained in AT cells following treatment with thymidine or HU (Fig. 2C) and AT cells showed the same delayed appearance of pSer345-Chk1 (data not shown). Chk1 was phosphorylated in AT cells exposed to IR, but the phosphorylation of SMC1 was not detected in irradiated AT cells as reported by other laboratories (18).

**Figure 2.** Thymidine induces autophosphorylation of ATM, the ATM-dependent phosphorylation of Chk2 and NBS1 and the ATM-independent phosphorylation of Chk1 and SMC1. (A) Wild-type MRC5VA cells were treated with thymidine for varying lengths of time before preparation of cell free extracts for western blotting. Blots were probed with antibodies for pSer1981-ATM, pSer345-Chk1, pThr68-Chk2 or pSer343-NBS1. Phosphorylated forms of ATM, Chk2 and NBS1 are evident at very early times (within 15 min) after treatment with 10 mM thymidine. pSer345-Chk1 showed a weak increase in intensity at 4 h and a stronger response at 24 h. Immunoblots were also probed with antibodies against actin as loading controls. The relative intensity of each band as determined by densitometry is presented under the lanes. (B) Wild-type MRC5VA, ATM deficient AT221JE-T/pEBS and TAT5BIVA, and AT-corrected pEBS-YZ5 fibroblast lines were treated (+) with 10 mM thymidine or 2 mM HU for 24 h or exposed to 10 Gy of IR. Cell extracts prepared from treated or untreated cells were immunoblotted and probed with antibodies against the phosphorylated forms of ATM (pSer1981), Chk2 (pThr68) or NBS1 (pSer343). Blots were also probed with antibodies against total ATM. The autophosphorylated forms of ATM are evident in wild-type and AT corrected cells but not AT cells. Phosphorylated forms of Chk2 and NBS1 are much lower in AT cells relative to wild-type or AT corrected cells. (C) Western blots prepared using extracts of MRC5VA or TAT5 cells treated with thymidine, HU or IR as described earlier in (B) were probed with antibodies against pSer345-Chk1 or pSer966-SMC1.

NBS1 was markedly depressed (Fig. 2B). There appeared to be little effect on the phosphorylation of NBS1 in AT cells following treatment with HU, consistent with a previous report (27), but the level of pThr68-Chk2 was much lower following this treatment (Fig. 2B). The autophosphorylation of ATM and the phosphorylation of Chk2 and NBS1 were restored in the AT line corrected for the ATM defect (pEBS-YZ5), providing further evidence for the ATM dependence of this thymidine induced protein kinase cascade (Fig. 2B). Taken together, these results indicate that thymidine rapidly triggers the phosphorylation of both Chk2 and NBS1 through an ATM-mediated signalling pathway. HU has a similar effect, but the ATM-mediated response to this agent does not appear to be essential for cell survival.

Cellular sensitivity to thymidine is governed by parallel pathways

The experiments described earlier show that thymidine sensitivity is dependent on both ATM and ATR and that thymidine treatment induces a protein kinase cascade characteristic of both ATM- and ATR-mediated damage responses. To determine whether survival in thymidine was dependent on parallel pathways triggered by these two protein kinases or a common pathway involving ATM and ATR, we measured the survival of MRC5VA and AT cells in thymidine when the levels of the ATM or Chk1 proteins were knocked down by siRNA treatment. MRC5VA and AT cells were treated with siRNA for ATM or Chk1 for 96 h. Protein extracts of the treated cells were then prepared at various times and the levels of these proteins were determined in the two cell lines by western blotting.

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This siRNA reduced the $D_{10}$ (the concentration required to reduce the plating efficiency to 10% of control) for thymidine 2.4-fold for both MRC5VA and TAT5 relative to cells treated with the scrambled siRNA. Again the increased sensitivity of the Chk1-depleted MRC5VA cells provided further evidence that the ATR-Chk1 pathway protects cells from the cytotoxic effects of thymidine. The increased sensitivity of AT cells to thymidine after Chk1 depletion demonstrates that the depletion of both ATM and Chk1 gives greater sensitivity to thymidine than the disruption of either protein alone, suggesting that the two proteins function in parallel pathways that govern the survival of cells treated with this agent.

**AT cells are defective in thymidine induced recombination**

Previous work has shown that cells defective in HRR become sensitive to thymidine and that thymidine is a potent inducer of homologous recombination (29). These observations indicate that thymidine induces the accumulation of lesions at DNA replication forks that must be resolved by HRR for cell survival. Given the increased thymidine sensitivity of AT cells, we next determined whether ATM defects affected the induction of recombination by thymidine. Recombination was assayed using the SCneo recombination reporter, which measures homology-based recombination events between two defective neo resistance genes (30). Replica cultures of MRC5VA and two AT cell lines carrying a single copy of the recombination reporter were treated with increasing concentrations of thymidine and then plated in G418 to determine the frequency of recombinants induced by the treatment. To more accurately determine the effect of thymidine on the induction of recombination, we inoculated replica cultures with 1000 cells to dilute out pre-existing neo+ cells and then grew them to $1 \times 10^5$ cells before a 24 h treatment with thymidine. Treated cultures were then left for 2 days to allow recovery before plating in selective medium containing G418. Using this approach, we found that the frequency of neo+ recombinants was increased 2.7-fold following treatment of MRC5VA cells with 2 mM thymidine and 7-fold following treatment with 10 mM (Fig. 4). In contrast, thymidine treated AT cells did not show an increase in the frequency of neo+ recombinants following treatment with up to 4 mM thymidine. AT cells were also treated with 10 mM thymidine, however, the low level of survival resulting from such treatment of the sensitive AT cells meant that we were unable to obtain enough cells to make a meaningful measurement.

**AT cells remain proficient in DSB induced HRR**

We next determined whether the thymidine-sensitive AT cells were deficient in the homology-directed repair of a site-specific DSB induced in the recombination reporter construct. The SCneo recombination reporter contained in our cell lines has a site for the I-SceI endonuclease in one of the defective neo genes. This makes it possible to introduce a site-specific DSB into this defective neo gene by transfection of an I-SceI expression construct into the cells and to determine the frequency of its repair by a homology-based recombination pathway (30). Two days following transfection of I-SceI, treated cells were plated on G418 to determine the frequency of neo+ recombinants. Wild-type and AT cells containing this recombination reporter showed an 84- to 258-fold induction of neo+ colonies (with two AT lines showing the extremes of induction, Fig. 5). Thus, cells defective in the ATM signalling pathway triggered by thymidine are defective in the recombinogenic response to thymidine but remain proficient in the response to a site-specific DSB.

**Thymidine does not induce detectable DSBs in either wild-type or AT cells**

As ATM is thought to respond largely to agents that induce DNA DSBs, we next determined whether thymidine induced...
such lesions in wild-type or AT cells. Cultures of each cell type treated with thymidine, HU or IR were harvested for analysis of DSB induction by pulsed-field gel electrophoresis. When the pulsed field gel was stained with ethidium bromide, no lower molecular weight DNA generated as a result of DSB accumulation was detectable in thymidine treated cells (Fig. 6A). In contrast, DSBs were readily detectable in all the cells treated with 10 Gy of IR. As ethidium bromide staining may not be sensitive enough to detect a low level of DSBs, we also Southern blotted gels on which DNAs from AT cells were fractionated and probed them with labelled whole cellular DNA. We compared the pattern obtained with these samples to patterns obtained from wild-type cells exposed to low levels of IR (0.1 or 0.2 Gy, Fig. 6B). A low level of fragmentation was detected in DNA obtained from the wild-type cells treated with 0.1 or 0.2 Gy of IR. However, patterns characteristic of DNA containing DSBs were not evident in AT cells treated with as much as 10 mM thymidine. Some lower molecular weight DNA is detectable in both treated and untreated AT cells. However, this DNA does not migrate similarly to the DNA from IR treated cells and is likely to represent cells undergoing apoptosis. This argument is supported by measurements of apoptotic cells by the Annexin V assay, which indicate a higher level of cell death in treated or untreated AT cells relative to the wild-type MRC5VA (data not shown). Given that 0.1 Gy IR produces about four DSB per cell (26), these experiments suggest that even highly toxic doses of thymidine induce very few, if any, DSBs although the dNTP pool imbalances generated by thymidine are likely to affect all replication forks.

DISCUSSION

Here, we show that thymidine triggers both the ATM- and ATR-mediated protein kinase cascades. Both of these pathways appear to be required for cell survival following thymidine treatment and our data suggest that the pathways work in parallel as disruption of both signalling cascades increases sensitivity to this agent. Thymidine-induced auto-phosphorylation of ATM and the ATM-mediated phosphorylation of Chk2 and NBS1 can be detected soon after treatment (within 15 min), whereas the ATM-independent response through Chk1 appears to be delayed (detectable at 4–24 h post-treatment). These observations suggest that the signal triggering ATM is generated soon after thymidine treatment, whereas that triggering ATR may arise as a downstream event. Finally, we show that ATM is required for HRR induced by thymidine, suggesting that ATM plays a role in the HRR-mediated rescue of DNA replication forks whose progression has been impaired by thymidine treatment.

The thymidine sensitivity of cells in which ATR function has been disrupted was not surprising, considering that such disruption confers sensitivity to HU (24,31). The requirement for ATM, on the other hand, was unexpected. ATM responds primarily to agents that induce DSBs (2,3). The data presented here, as well as previously published work (29,32), suggest that thymidine induces few, if any, DSBs in wild-type, AT or HRR defective cells. Considering that ATM may be activated by as few as two DSBs per cell (26), we cannot eliminate the possibility that transient DSBs generated at replication forks impaired by thymidine treatment may induce activation. However, Bakkenist and Kastan (26) have provided evidence that alterations of chromatin structure may be sufficient to activate ATM. Given that thymidine treatment is likely to affect all replication forks, some alteration of chromatin structure is possible. Furthermore, there is evidence for the generation of other types of lesions at replication forks slowed or stalled by agents such as thymidine that could trigger ATM. These include the so-called ‘chicken foot’ structure that may be formed by the reversal of impaired replication forks (33–35). Such intermediates have been visualized by electron microscopy in a Rad53 defective yeast strain treated with
ATM defective cells are unable to induce recombination at a rate that ATM is required for HRR at DNA replication forks as a default of HR. The data presented here provide the first evidence that ATM and RAD54 lie on the same HRR pathway. Mutations relative to ATM mutants have only slightly higher levels of chromosome aberrations compared to ATM-deficient cells, suggesting an effect of ATM on HRR proficiency.

Figure 6. DSBs are not detectable in wild-type cells or AT cells treated with thymidine. (A) DSBs were visualized by pulsed field gel electrophoresis of DNA obtained by gentle lysis of wild-type (MRC5VA), AT (AT221JE-T/pEBS) and AT corrected (pEBS-YZ5) cells in agarose plugs after the indicated treatment with thymidine, hydroxyurea or IR followed by ethidium bromide staining of the gel. Two other AT-lines (TAT2SF and TAT5BIVA) showed patterns identical to those displayed (data not shown). (B) A pulsed-field gel containing samples of TAT5BIVA cells treated with thymidine and wild-type cells treated with low levels of IR that produce few DSBs was Southern blotted and probed with labelled whole cell DNA. DNA entering the gel as a result of DSBs was detectable in wild-type cells treated with 0.1 or 0.2 Gy IR but not in AT cells treated with as much as 10 mGy thymidine.

HU (34). We speculate that such a structure could activate ATM as it would present a double stranded DNA end in the absence of a DSB. The ability of DNA ends in the form of uncapped telomeres to activate an ATM-mediated damage response has been documented (36).

A role for ATM in HRR has long been surmised, but the previous evidence supporting this was largely indirect (37). ATM−/− DT40 chicken cells show altered kinetics of RAD51 foci formation and ATM−/− RAD54−/− double mutants have only slightly higher levels of chromosome aberrations relative to ATM−/− cells (12). These observations suggest that ATM and RAD54 lie on the same HRR pathway. The data presented here provide the first evidence that ATM is required for HRR at DNA replication forks as ATM defective cells are unable to induce recombination at a reporter substrate after thymidine treatment. In contrast, the response of AT cells to a site specific DSB in the recombination reporter appears to be unaffected. We have previously reported (32) that a tumour-derived mutation of the Rad51 parologue XRCC2 has a similar effect on HRR. Thymidine induced HRR was acutely sensitive to the presence of the mutant XRCC2, whereas HRR triggered by a site specific DSB in the recombination reporter was only affected in cells depleted of the wild-type gene as well as expressing the mutant gene. Thus, the thymidine induced HRR appears to be highly sensitive to subtle changes in HRR proficiency. This may be the result of the level of lesions induced by thymidine treatment. Thymidine can potentially induce lesions at all replication forks, thus saturating HRR capacity. In contrast, only a single DSB is introduced into the recombination reporter by I-SceI. How ATM is involved in HRR in thymidine treated cells is not clear. Classically, ATM has been viewed as playing a central role in the induction of checkpoints that allow repair to occur. It may also play a more direct role by activating proteins (e.g. the MRE11–Rad50–NBS1 complex) involved in the processing of structures generated at the impaired replication forks.

Another striking feature of the ATM-mediated response to thymidine is its rapidity. Thymidine has an indirect effect on DNA replication in cells in that it must be phosphorylated to dTTP before it inhibits ribonucleotide reductase. Furthermore, the dTTP accumulating in cells following thymidine treatment inhibits reductase activity by an allosteric mechanism that does not completely shut-off the supply of dCTP (22). However, early work showed that dNTP pools in cells and DNA replication rates responded to exogenous thymidine within minutes (23). DNA synthesis is substantially slowed in such cells but not completely arrested. This slow progression of the replication fork in the presence of dNTP precursor imbalances that do not provide for high fidelity DNA synthesis generates DNA replication errors (38). These are likely to be processed by MMR or exonucleolytic proofreading that could stimulate replication fork reversal and generate lesions that require HRR for resolution and trigger the ATM-mediated protein kinase cascade. It is interesting to note that HU, which also inhibits ribonucleotide reductase, triggered a similar protein kinase cascade including the autophosphorylation of ATM, however, AT cells are not sensitive to HU (1). Thus, the ATM-mediated response triggered by HU does not appear to be essential for cell survival. However, there are significant differences in the cellular effects of HU. HU inactivates the iron-free radical complex essential for the activity of the enzyme and depletes cells of the dNTPs required for DNA synthesis (39). DNA synthesis is rapidly shut down in HU treated cells (38) and, as a result, fewer replication abnormalities (or a different set of them) may be generated.

How the loss of this pathway contributes to the genetic instability seen in AT cells has not been investigated here. An inability to process aberrant structures at stalled replication forks may lead to more error prone processing of such lesions. The thymidine sensitivity of MMR deficient tumour cells that we recently reported (21) may reflect the presence of a defect in this DNA damage response pathway in this subset of tumours. Work is currently underway to test this hypothesis.
MATERIALS AND METHODS

Cell lines and culture

The human immortalized fibroblast cell lines used in these experiments were obtained from Coriell (MRC5VA), and the ECACC (TAT2SF and TAT5BIVA). Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum. Derivatives of the immortalized AT fibroblast line AT221JE-T carrying either an empty vector (AT221JE-T/pEBS) or a construct containing the ATM cDNA that corrects the AT-phenotype (pEBS-YZ5), (25) were a kind gift from Yoshi Shiloh, Tel Aviv University, Israel. These cells were maintained as described earlier, in medium supplemented with 100 μg/ml hygromycin. The U2OS cell lines (24) were also maintained as described earlier, in medium supplemented with 200 μg/ml G418 and 50 μg/ml hygromycin.

Cytotoxicity assays

The cytotoxic response to thymidine or HU was measured in DMEM supplemented with dialysed serum (to remove exogenous sources of deoxynucleosides). A total of 500 cells were plated on 10 cm tissue culture dishes and treated with varying concentrations of thymidine or HU in duplicate. Cells were left for 10–14 days to allow colony formation before staining with 0.4% methylene blue/50% methanol (Fisher Scientific). Colonies of >50 cells were scored. The surviving fraction was determined by dividing the average number of colonies at each dose by the average number of colonies on the control plates.

For the U2OS ATRkd cytotoxicity assays 500 cells were treated in duplicate for 3 days with 1.5 μg/ml doxycycline (the inducer of the ATRkd allele) prior to thymidine or HU treatment. Both induced and uninduced cells were then exposed to 0.5 mM HU or 4 mM thymidine for 24–96 h after which the agent was removed and cells were allowed to grow for another 8–12 days before staining. The surviving fraction was calculated as mentioned earlier. All cytotoxicity experiments were performed independently three to five times.

Western blot analysis

Cells were plated at a density of 2 × 10^6 and left to grow for 24 h. Cells were either left untreated, treated for 24 h with 10 mM thymidine or 2 mM HU, or exposed to 10 Gy IR prior to being lysed with RIPA buffer in the presence of the proteinase inhibitor PMSF (100 μg/ml) and the phosphatase inhibitor microcystin (1 mM). An aliquot of 40 μg of each protein sample was fractionated on 8–12% sodium dodecyl sulphate polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia) using a semi-dry transfer cell (BioRad). This membrane was blocked in 5% milk for 1 h and immunoblotted with rabbit polyclonal antibodies: phospo-Ser1981-ATM (Rockland 1:1000), ATM (Oncogene 1:500), phospho-Thr68-CHK2 (Cell Signalling; 1:800 dilution), phospho-Ser345-CHK1 (Cell Signalling 1:1000 dilution), phospho-Ser343-NBS1 (Cell Signalling 1:1000 dilution), phospho-Ser966-SMC1 (Upstate Technology 1:1000) or β-actin (Sigma; 1:2500 dilution) proteins in 5% milk overnight. Anti-rabbit peroxidase conjugates (Cell Signalling) were used as a secondary antibody at a dilution of 1:1000. Immunoreactive protein was visualized using ECL reagents (Amersham Pharmacia) following the manufacturer’s instructions.

siRNA transfection

All siRNA duplexes were purchased from Dharmacon. The ATM siRNA was a Smartpool™ duplex. Control duplex RNA corresponding to unknown protein, with a G+C ratio of 42% was used in control experiments. The Chk1 siRNAs were designed to correspond to the Chk1 DNA sequence (J. Blackburn and C. Smythe, manuscript in preparation) and purchased from Dharmacon. siRNA duplexes were transfected into cells using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. To analyse gene silencing, total cell extracts were taken and western blotting carried out as described later. The Chk1 antibody was a kind gift from C. Smythe and the ATM antibody was obtained from Oncogene and used at a dilution of 1:500.

To determine the survival of cells transfected with siRNA, cells were replated 20 h after treatment in DMEM supplemented with dialysed serum. Four hours after replating various doses of thymidine were added and cells were allowed to grow up for 10–14 days. The surviving fraction calculated as before.

Recombination assay

The SCneo recombination reporter and pCMV3nls-I-SceI expression vector were kind gifts from Dr Maria Jasin, Memorial Sloan Kettering Cancer Center. Strains of the fibroblast line MRC5VA and the AT-fibroblast lines TAT5BIVA and TAT2SF carrying single copies of the SCneo recombination reporter were obtained as described previously (21). To measure recombination events induced by a DSB, isolates carrying SCneo were transfected with 10 ng of the pCMV3nls-I-SceI expression vector using Lipofectamine (Invitrogen) to a final concentration of 10 μg/ml in 5 h treatment. To measure recombination induced by thymidine, isolates carrying SCneo were treated for 24 h with various doses of thymidine. To minimize the background of recombinant cells for these experiments replica cultures of cells to be treated with thymidine were inoculated with 1000–10 000 cells. These replica cultures were then grown to 1–2 × 10^6 cells before they were treated with thymidine. Following thymidine treatment, cells were allowed to recover in complete medium for 2 days before plating. Recombinants were scored by plating treated cells in medium containing 1 mg/ml G418 (at a density of 13–25 cells/mm²). In addition, two dishes were plated with 500 cells each to measure cloning efficiency in the absence of selection. Neo+ colonies were allowed to form over 12–14 days before staining with 0.4% methylene blue/50% methanol (Fisher Scientific), colonies of >50 cells were scored. Recombination frequencies were calculated by dividing the number of colonies on the test plates by the total number of cells plated. This frequency was then corrected for differences in cell viability between treated and untreated cultures by dividing the frequency of recombinants by the plating efficiency obtained in the absence of neo selection.
All experiments were repeated independently two to four times. Dose response data was analysed by multiple linear regression analysis using STATA statistical software. Log, recombination frequency was the dependent variable and thymidine dose and cell line were the independent variables. The contribution of the cell line variable to recombination frequency was determined by likelihood ratio test for the comparison of the linear regression model with and without that variable. Plots of residuals and fitted values were used to check the assumptions of linearity and constant variance of the error term.

Detection of DSBs by pulsed field gel electrophoresis

In total, 1 x 10⁶ cells were inoculated into plates and were treated with various doses of thymidine. Following treatments 1 x 10⁶ cells were embedded into agarose inserts. For IR treatment 1 x 10⁶ cells were melted into an agarose insert and then treated with 10 Gy of IR. The agarose inserts were then incubated in 0.5 M EDTA, 1% N-laurylsarcosyl and proteinase K (1 mg/ml) for 48 h then washed four times in TE buffer, prior to loading onto a 1% agarose gel. Pulse-field gel electrophoresis (BioRad: 120° angle, 60–240 s switch time, 4 V/cm) was then carried out. The gel was subsequently stained with ethidium bromide and analysed using Image Gauge software. Gels that were to be transferred and probed for Southern blotting were transferred onto nitrocellulose using alkaline transfer methods. A 32P-labelled probe was prepared using sheared total DNA extracted from the cell line MRC5VA.

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