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Bloom's syndrome protein response to ultraviolet-C radiation and hydroxyurea-mediated DNA synthesis inhibition

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Bloom's syndrome (BS) arises through mutations in both copies of the *BLM* gene that encodes a RecQ 3′-5′ DNA helicase. BS patients are predisposed to developing all the cancers that affect the general population, and BS cells exhibit marked genetic instability. We showed recently that BLM protein contributes to the cellular response to ionizing radiation by acting as downstream ATM kinase effector. We now show that following UVC treatment, BLM-deficient cells exhibit a reduction in the number of replicative cells, a partial escape from the G2/M cell cycle checkpoint, and have an altered p21 response. Surprisingly, we found that hydroxyurea-treated BLM-deficient cells exhibit an intact S phase arrest, proper recovery from the S phase arrest, and intact p53 and p21 responses. We also show that the level of BLM falls sharply in response to UVC radiation. This UVC-induced reduction in BLM does not require a functional ATM gene and does not result from a subcellular compartment change. Finally, we demonstrate that exposure to UVC and hydroxyurea treatment both induce BLM phosphorylation via an ATM-independent pathway. These results are discussed in the light of their potential physiological significance with regard to the role of BLM in the cellular pathways activated by UVC radiation or HU-mediated inhibition of DNA synthesis.
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Introduction

BLM is of particular interest among the genes implicated in maintaining genomic integrity and linked to genetic diseases predisposing to cancer, because mutations in both copies result in Bloom's syndrome (BS), an autosomal recessive disorder that displays one of the closest known correlations between chromosomal instability and an increased risk of malignancy at an early age (reviewed in German, 1993). Cytogenetic abnormalities associated with Bloom's syndrome include increased chromosome breaks, symmetric quadriradial chromatid interchanges between homologous chromosomes and sister chromatid exchanges (SCE) (reviewed in German, 1993). The *BLM* gene is located on chromosome 15 at 15q26.1, and encodes the BLM protein which belongs to the DExH box-containing the RecQ helicase subfamily (Ellis *et al*., 1995) and displays ATP- and Mg^{2+} dependent 3′-5′-DNA helicase activity (Karow *et al*., 1997). We had previously shown that BLM expression is regulated during the cell cycle, accumulating to high levels during the S phase, and that BLM accumulates in cells treated with the DNA replication inhibitors hydroxyurea and aphidicolin to reach the levels detected in S phase cells (Dutertre *et al*., 2000). This is in agreement with recent data showing that in aphidicolin-treated cells BLM co-immunoprecipitates *in vivo* with hRAD51, a key protein in homologous recombination (Wu *et al*., 2001). We also demonstrated that following ionizing irradiation, endogenous BLM protein is modified by phosphorylation and accumulates via an ATM-dependent pathway (Ababou *et al*., 2000). This is consistent with data showing that BLM co-immunoprecipitates with ATM within the supercomplex of BRCA1-associated proteins known as BASC (BRCA1-Associated genome Surveillance Complex) (Wang *et al*., 2000b), and assembles with PML (promyelocytic leukaemia protein) at sites of single-stranded DNA after γ-irradiation (Bischof *et al*., 2001). Overall, these data are consistent with a role for BLM in the maintenance of genome stability during DNA replication and in response to genotoxic stresses. To further investigate the signalling pathway(s) in which BLM acts, we tested whether BLM could be involved in the cellular response to short-wavelength light exposure (UVC) and to inhibition of DNA synthesis by hydroxyurea (HU) treatment, which are known to stimulate response pathways different from those induced by ionizing radiation.

In this study we show that UVC-treated BLM-deficient cells display a reduction in the number of replicating cells and partially escape from the G2/M cell cycle checkpoint. We also show that a BLM-deficient cell line that does not express BLM protein

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has a delayed and decreased p53 response to UVC exposure, whereas the accumulation of p53 in a BS cell line expressing a full length mutated BLM protein is similar to that in wild type cells. However, both BS cell lines exhibit an altered p21 response to UVC exposure. We found that when DNA synthesis is inhibited by HU treatment, BLM-deficient cells display an intact S phase arrest, exhibit a proper recovery from S phase arrest and present intact p53 and p21 responses. Finally, we present data showing that UVC radiation results in a strong decrease of BLM expression which does not require a functional ATM gene and does not result from a change of subcellular compartment, and that both UVC exposure and HU treatment induce BLM phosphorylation via an ATM-independent pathway.

Results

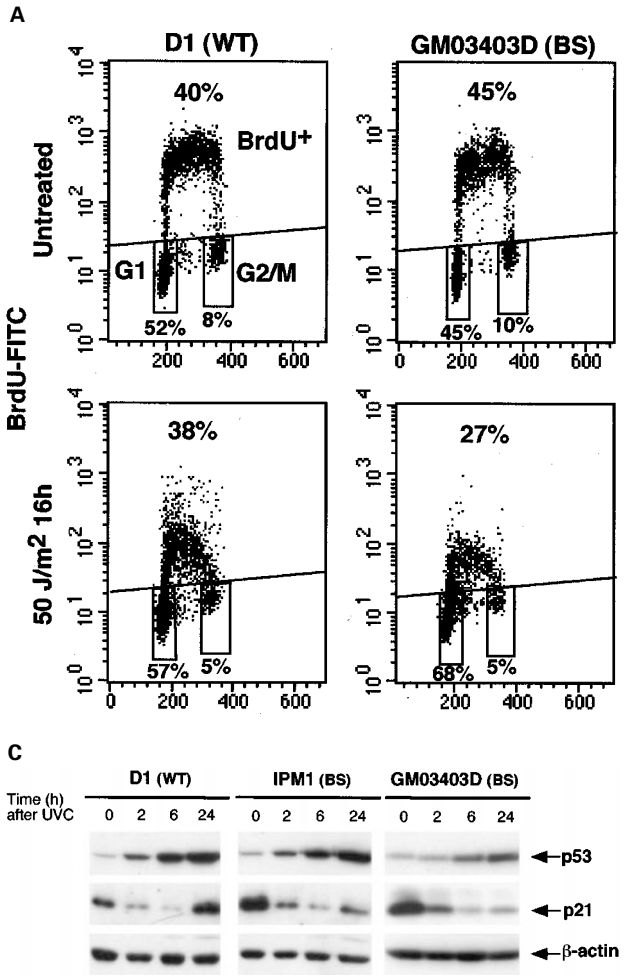
BLM-deficient cells display a reduction in the number of replicating cells and a partial escape from the G2/M cell cycle checkpoint in response to UVC

Altered p53 and p21 responses to UVC radiation have been reported in BS fibroblasts (Lu and Lane, 1993; Collister *et al*., 1998). UVC radiation is known to activate p53-dependent G1/S and G2/M checkpoints (for reviews, Dasika *et al*., 1999; Taylor and Stark, 2001). These data prompted us to analyse the UVC-induced G1/S and G2/M checkpoints in BS cells.

Thus, in order to find out whether the G1/S checkpoint is affected in UVC-exposed BS cells, we measured DNA synthesis by labelling asynchronous BS (GM03403D) and wild type (D1) EBV-transformed lymphoblastoid cells, UVC-irradiated (50 J/m^2) or not, with bromodeoxyuridine (BrdU) 2 h before sampling. The labelling was carried out 16 h after irradiation. BrdU incorporation and cellular DNA content were determined simultaneously by flow cytometry. As shown in Figure 1a, we did not find any significant decrease in the number of DNA-replicating (BrdU positive) wild type cells (2%), whereas there was an apparent reduction of about 18% in the number of BrdU positive BS cells. We also observed a decrease in BrdU incorporation by both UVC-irradiated wild type cells and BS cells with S phase DNA content; this is very similar to the changes reported for U2OS cells (Allan and Fried, 1999). This could reflect the stalling of DNA replication at the bulky cyclobutane pyrimidine dimers generated by UVC radiation, as proposed by Allan and Fried (1999). These results were reproduced in three independent experiments, and similar results were also obtained in IPM1 EBV-transformed lymphoblastoid BS cells (data not shown). These results indicate that although replicating DNA synthesis is inhibited by UVC-irradiation, neither wild type cells nor BLM-deficient cells elicit a G1/S arrest since the proportion of S phase cells remained largely unchanged, particularly in wild type cells. However, these results also indicate that the synthesis of replicating DNA is affected by UVC to a greater extent in BS cells than in wild type cells.

The UVC-induced G2/M checkpoint was assessed in BLM-deficient cells by labelling mitotic cells using an antibody specific for the mitotic phosphorylated form of histone H3, and then scoring them by flow cytometry 1 and 2 h post-irradiation (so as to count only mitotic cells derived from irradiated G2 cells) (Ababou *et al*., 2000). As shown in Figure 1b (a, b), the wild type cells were arrested in G2 in response to UVC irradiation, as expected. Indeed, when compared to non-irradiated cells, the percentage of mitotic cells decreased 6.5-fold in average (mitotic index: 15%) 1 h after UVC irradiation and 4.3-fold in average (mitotic index: 23%) 2 h after UVC-irradiation. In contrast, in BLM-deficient cells the decrease was only twofold (mitotic index: 50%) 1 and 2 h following UVC irradiation, and this indicates that BS cells had a mitotic index two to three-times higher than wild type cells. These results were reproduced in three independent experiments, and similar results were also obtained for IPM1 BS cells (data not shown). These findings indicate that the BLM defect is associated with a partial escape of cells from the UVC-induced G2/M cell cycle checkpoint.

We also analysed the p53 and p21 responses to UVC in the two BLM-deficient lymphoblastoid cells. Thus, p53 protein expression was analysed by Western blot over a 24 h period following 50 J/m^2 of UVC-irradiation, in BS cells (GM03403D and IPM1) and in wild type cells (D1). As shown in Figure 1c (upper panels), p53 accumulated in IPM1 BS cells following a kinetic pattern similar to that seen in wild type cells, whereas the induction of p53 was decreased and delayed in GM03403D BS cells. Membranes were reprobed with an antibody specific for p21, and we observed that in both wild type and BS cells, the levels of p21 protein fell sharply in response to UVC (Figure 1c, middle panels), as previously described in U2OS cells (Allan and Fried, 1999) and in various cancer cells (Wang *et al*., 1999). However, as expected, by 24 h the p21 protein level had risen above its pre-damage level in wild type cells (Allan and Fried, 1999; Wang *et al*., 1999), whereas in BLM-deficient cells, it did not increase and remained well below its pre-damage level (Figure 1c, middle panels). This very low level of p21 was still observed in BLM-deficient cells 48 h after UVC exposure (data not shown). We should note that the basal level of p21 was higher in BLM-deficient cells than in normal cells. Reprobing the membranes with an antibody specific for β-actin (Figure 1c, lower panels) normalized amounts of protein in the different cell extracts. These results show that in response to UVC, the accumulation of p53 was reduced and delayed in BS cells that do not express BLM (GM03403D; Dutertre *et al*., 2000), but not in BS cells that express a full length mutated BLM protein (IPM1; Barakat *et al*., 2000). However, an alteration in the induction of p21 protein is observed in both types of BS cells 24 h after UVC exposure, independently of the rate and the kinetics of the accumulation of p53.



B

a

Cell lines	D1 (WT)			GM03403D (BS)		
Time after Irradiation (min)	0	60	120	0	60	120
% of mitotic cells	1,3	0,2	0,3	0,6	0,3	0,3
(± STDEV)	0,01	0,01	0,01	0,13	0,01	0,04
Mitotic index *	—	**15**	**23**	—	**50**	**50**

* : ((%irradiated mitotic cells/ %untreated mitotic cells) X 100)

b

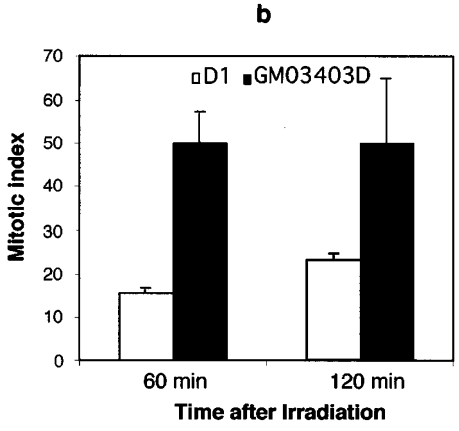


Figure 1 Analysis of the BS lymphoblast response to UVC radiation. (**A**) G1/S arrest analysis: Asynchronous populations of wild type cells (D1), and BS cells (GM03403D) were UVC-irradiated with 50 J/m^2, pulse-labelled with BrdU, fixed 16 h after exposure to UVC-irradiation, and analysed by flow cytometry (for details see Results and Material and methods sections). Cells above the diagonal line are BrdU positive and correspond to the percentage of cells in the S phase of the cell cycle. Cell cycle populations are characterized as G1 (2N DNA content with no incorporation of BrdU), S phase (variable DNA content with BrdU incorporation), or G2/M (4N DNA content with no incorporation BrdU). (**B**) G2/M arrest analysis: (a) The percentage of mitotic cells was quantified by flow cytometry (for details see Results and Material and methods sections) 60 and 120 min after exposure to 50 J/m^2 of UVC light. The percentage of mitotic cells in the corresponding non-irradiated cells were used as controls (0 min). The values correspond to the mean of two independent experiments and standard deviations are shown (STDEV). (b) The white (wild type D1 cells), and black (BS GM03403D cells) bars and error bars represent the means and standard deviations, respectively, of the percentage of mitotic cells from the irradiated population relative to the percentage of mitotic cells from the corresponding nonirradiated population (values of mitotic index shown in (a)). (**C**) p53 and p21 responses analysis: D1 wild type cells, IPM1 and GM03403D BS cells were subjected to 50 J/m^2 of UVC-irradiation. Cells were lysed at the indicated times after irradiation and protein extracts were separated on 12% polyacrylamide gels. Blot membranes were probed with anti-p53 antibody (upper panels), reprobed with anti-p21 antibody (middle panels) and reprobed again with anti-β-actin to ensure equal loading (lower panels)

In response to the HU-mediated inhibition of DNA synthesis, BLM-deficient cells exhibit an intact S phase arrest, and recover from the S phase arrest in the same way as normal cells

The above results indicate a reduction in the number of BrdU positive cells in UVC-irradiated BS cells, compared to wild type cells. BLM deficiency is known to result in abnormalities associated with replication (Hand and German, 1977; Gianneli *et al*., 1977; Lonn *et al*., 1990), and several lines of evidences suggest that a BLM defect would result in an accumulation of Holliday junctions that would be mainly resolved by sister chromatid exchanges (Karow *et al*., 2000a; Wang *et al*., 2000a). Furthermore, we previously showed that BLM accumulates in cells treated with the DNA replication inhibitors hydroxyurea and aphidicolin, and reaches the levels detected in S phase cells, indicating that BLM is involved in the S phase of the cell cycle (Dutertre *et al*., 2000). The combination of these data prompted us to analyse the S phase arrest and the recovery from the S phase arrest in BLM-deficient cells treated with hydroxyurea (HU) (2 mM at the times indicated), an inhibitor of DNA synthesis which blocks ribonuclotide reductase. As shown in Figure 2a, we found that the number of cells in G2/M

in the HU-treated populations was lower than in the HU-free controls, which indicates that an HU-induced S phase arrest takes place in both wild type and BS cells (Levenson and Hamlin, 1993). These results clearly show that BLM defect does not affect HU-induced S phase arrest.

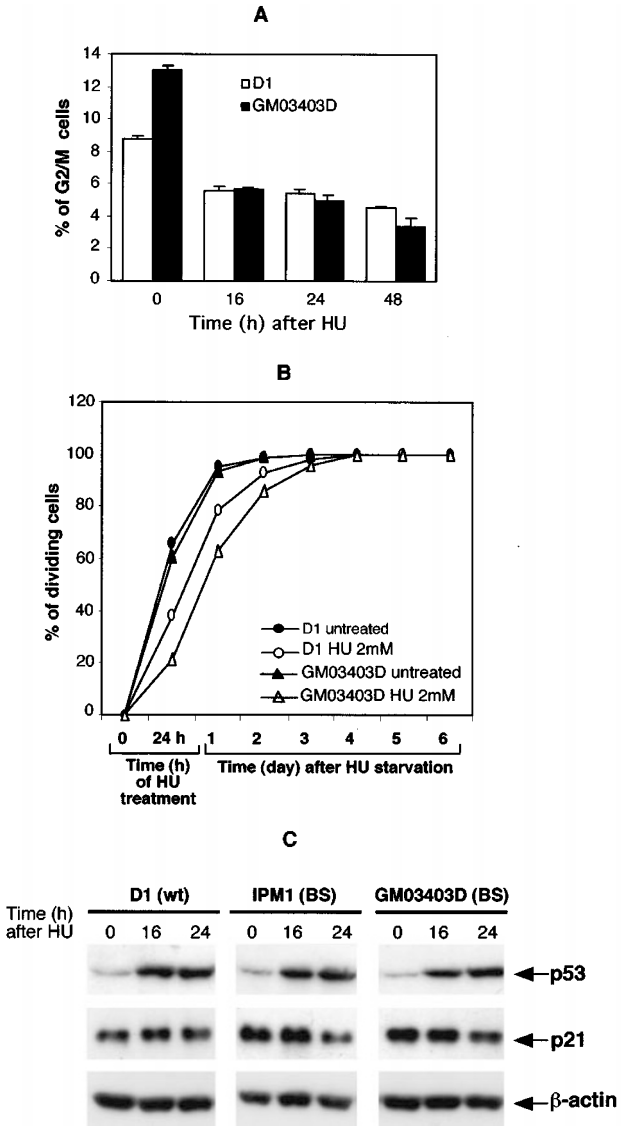


Figure 2 Analysis of the BS lymphoblast response to HU treatment. (**A**) S phase arrest analysis: Kinetics of progression of cells in G2/M cells after HU treatment: D1 wild type cells and GM03403D BS cells were treated with 2 mM of HU. Cells were fixed 0, 16, 24 and 48 h after treatment, then stained with propidium iodide and their DNA content determined by flow cytometry. (**B**) Analysis of the recovery from the S phase arrest: D1 wild type cells and GM03403D BS cells were pulse labelled with CFSE and then treated or not with 2 mM of HU for 24 h. After removing the HU, the cells were harvested and the intensity of CFSE fluorescence, indicating the percentage of dividing cells at different numbers of days before and after HU starvation, was measured by flow cytometry. (**C**) p53 and p21 responses analysis: D1 wild type cells and GM03403D BS cells were treated with 2 mM of HU. Cells were lysed at the indicated times after treatment and protein extracts were separated on 12% polyacrylamide gels. Blot membranes were probed with anti-p53 antibody (upper panels), reprobed with anti-p21 antibody (middle panels) and reprobed again with anti-β-actin to ensure equal loading (lower panels)

To assess the recovery from the S phase arrest, normal and BLM-deficient cells were pulse labelled using CFSE (carboxyfluorescein succinimidyl ester), and then treated with HU (2 mM) for 24 h. CFSE-labelled cells were then released from the block by removing the HU, harvested and subjected to flow cytometry analysis at the times indicated. As shown in Figure 2b, a threefold decrease in the percentage of dividing cells was observed among the population of BLM-deficient cells treated for 24 h with HU, which confirms their arrest in S phase. Furthermore, both normal cells and BLM-deficient cells resumed similar division after release from the HU block. In parallel, cells were fixed and stained with propidium iodide for flow cytometry, and we did not observe any abnormality (no polyploidization) in the DNA contents of either type of cells (data not shown). These findings indicate that BLM is not required for a proper recovery from S phase.

We also analysed the expression of the p53 protein by Western blot in both HU-treated normal cells and BS cells (2 mM, at the times indicated). As shown in Figure 2c, p53 accumulates in BLM-deficient cells, following a kinetic pattern similar to that seen in wild type cells (Figure 2c, upper panels). Membranes were reprobed with an antibody specific for p21, and we did not detect any accumulation of p21 in either normal or BLM-deficient cells. This confirms recent data showing that p53 accumulates but is impaired in inducing many of its target genes, including p21, when DNA synthesis is blocked by HU treatment (Gottifredi *et al*., 2001). Reprobing the membranes with an antibody specific for β-actin (Figure 2c, lower panel) normalized the amounts of protein in the different cell extracts.

The BLM protein level is reduced in response to UVC-exposure

We had previously shown that BLM accumulates in response to hydroxyurea-mediated inhibition of DNA synthesis (Dutertre *et al*., 2000) or ionizing radiation (Ababou *et al*., 2000). To further investigate the possible involvement of BLM in the cellular response to UVC radiation, we analysed BLM protein expression following exposure of the cells to UVC. Thus, wild type D1 cells were left untreated or exposed to UVC (50 J/m^2, harvested 2 to 16 h later). Cell extracts were fractionated in 7.5% SDS–PAGE and subjected to Immunoblot analysis with the BLM specific antibody 1343 (Dutertre *et al*., 2000). As shown in Figure 3a (upper panel), we observed a marked fall in BLM level 2 h after UVC-exposure, and further decreases 6 and 16 h after exposure. Similar results were obtained using wild type EBV-transformed lymphoblastoid cells, Priess (data not shown).

Western blots presented in Figure 3a were performed using protein extracts solubilized in 300 mM NaCl. In order to find out whether the apparent decrease of

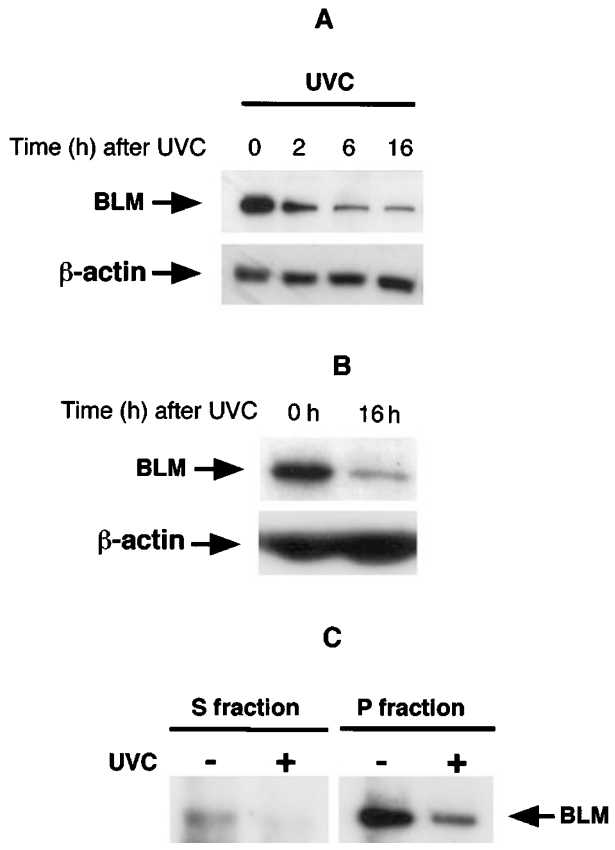


Figure 3 Analysis of BLM expression in response to UVC-irradiation. (**A**) Wild type cells (D1) were left untreated or exposed to 50 J/m^2 of UVC. Cells were harvested and lysed at the indicated times after treatment and protein extracts were separated on 7.5% polyacrylamide gel. The blot membrane was probed with 1343 anti-BLM antibody (upper panel) and reprobed with anti-β-actin to ensure equal loading (lower panel). (**b**) Wild type cells (D1) were left untreated or exposed to 50 J/m^2 of UVC. Sixteen hours after exposure, 10^6 cells were lysed in hot SDS and separated on 7.5% polyacrylamide gel. The blot membrane was probed with 1343 anti-BLM antibody (upper panel) and reprobed with anti-β-actin to ensure equal loading (lower panel). (**c**) Wild type cells (D1) were left untreated or exposed to 50 J/m^2 of UVC. Sixteen hours later, 10^6 cells were extracted with a buffer containing NP-40 (S fraction). Pellets were solubilized in P buffer (P fraction) and sonicated. Samples were run on 7.5% polyacrylamide gel. The membrane was probed with 1343 anti-BLM antibody

BLM level that we observed could result from a subcellular compartment change, we performed Western blot analysis of the expression of the BLM protein in crude protein extracts prepared by lysing cells in hot SDS under the same conditions as described above. We also permeabilized cells with the non-ionic detergent Nonidet P40 (NP-40), which leaves in place proteins bound to chromatin and to the nuclear scaffold whereas other proteins are solubilized (Martinez-Balbas *et al*., 1995; Muchardt *et al*., 1996; Frei and Gasser, 2000). As shown in Figure 3b,c, 16 h after UVC exposure, we still observed a marked fall in the level of the BLM protein in the crude extract proteins (Figure 3b, upper panel), as well as in the NP-40 insoluble fraction (Figure 3c). These findings indicate that the fall in BLM protein level in response to UVC radiation does not result from a change in subcellular localization.

BLM protein is phosphorylated via an ATM-independent pathway in response to exposure to UVC or HU treatment

Our previous results, showing that BLM is phosphorylated in response to ionizing radiation via an ATM-dependent pathway (Ababou *et al*., 2000), led us to check the presence of a possible BLM post-translational modification following exposure of cells to UVC or HU. Extracts from UVC-exposed (50 J/m^2, 2 h) or HU-treated (2 mM, 24 h) D1 cells were fractionated in 5.5% SDS–PAGE and subjected to Immunoblot analysis with 1343 antibody. In parallel, the same experiments were performed using ATM-deficient cells (EBV-transformed lymphoblastoid B cells GM03189B). As shown in Figure 4, we observed that in protein extracts from both wild type D1 cells (Figure 4a) and ATM-deficient GM03189B cells (Figure 4b), BLM from cells exposed to UVC for 2 h and from cells exposed to HU treatment for 24 h, migrated more slowly than BLM from untreated cells (left and right panels, respectively). It should be noted that these upshifts of BLM were not detectable on 7.5% polyacrylamide gels.

To determine whether the increase in the apparent molecular weight of the BLM protein following UVC or HU treatment was due to specific phosphorylation, protein extracts from untreated, UVC-exposed and HU-treated D1 cells were treated with λ protein phosphatase. As shown in Figure 4c, λ phosphatase treatment of protein extracts both from UVC-treated cells (left panels) and from HU-treated cells (right panels) resulted in the recovery of a band that migrated similarly to BLM from untreated cells. These results indicate that the BLM upshift observed in response to UVC and HU is indeed due to phosphorylation. Similar results were obtained using Priess cells (data not shown).

These findings therefore demonstrate that BLM is phosphorylated via an ATM-independent pathway in response to either UVC exposure or HU treatment.

To determine whether the UVC-induced decrease of BLM or the HU-induced accumulation of BLM (that we described in Dutertre *et al*., 2000) are dependent on the presence of a functional ATM, cell extracts from UVC-exposed (50 J/m^2, 2 h) or HU-treated (2 mM, 24 h) ATM-deficient cells were fractionated in 7.5% SDS–PAGE and subjected to Immunoblot analysis with 1343 antibody. The same experiments were also performed using D1 cells, as control. The membranes were reprobed with anti-β-actin antibody to standardize the amounts of protein in the different cell extracts. As shown in Figure 4d, the same pattern of BLM expression was observed in the ATM-deficient cells and in the control cells. These findings show that the UVC-induced decrease in BLM and HU-induced

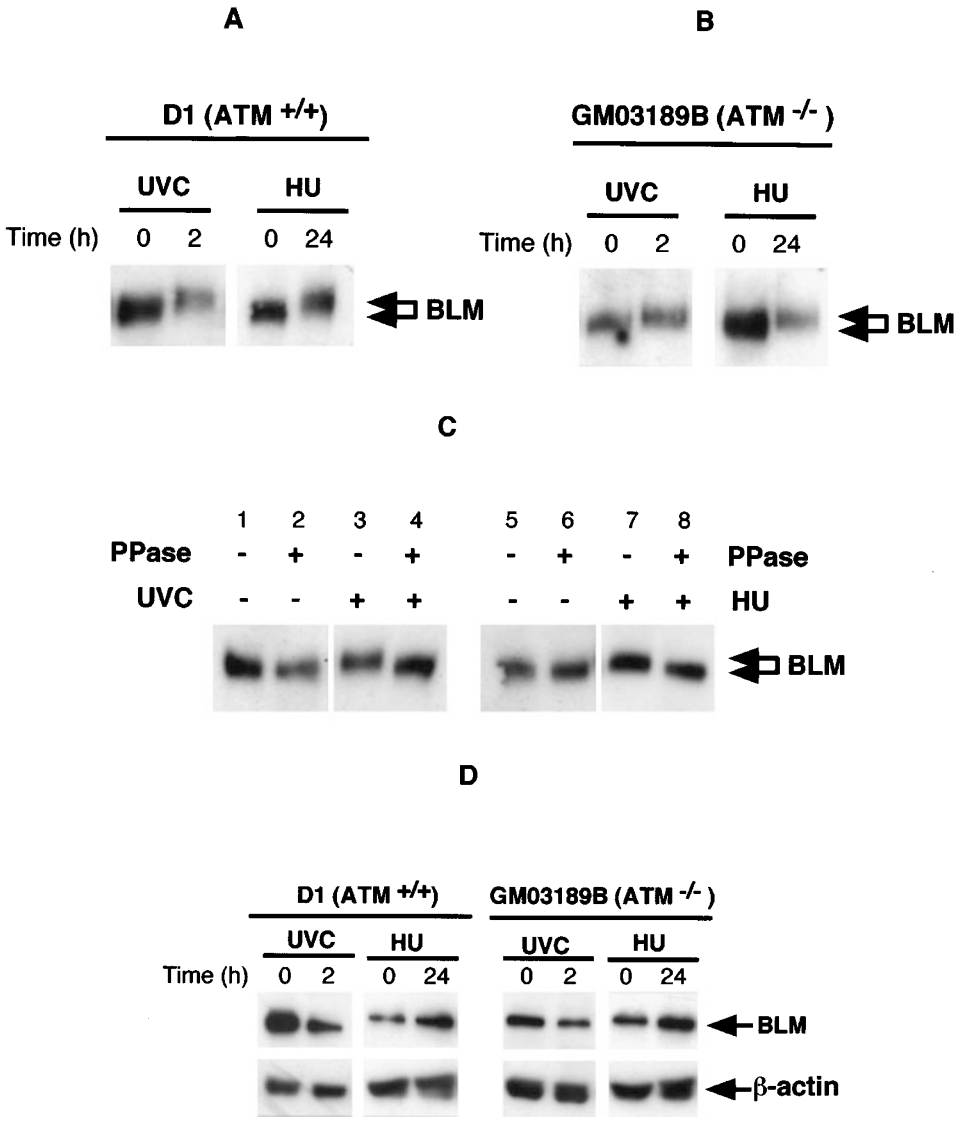


Figure 4 Analysis of BLM phosphorylation in D1 wild type cells and ATM-deficient GM03189B cells in response to UVC-irradiation and HU treatment. (**a**) Wild type cells (D1) were left untreated or exposed to 50 J/m^2 of UVC or treated with 2 mM of HU. Cells were harvested and lysed at the indicated times after treatment, and protein extracts were separated on 5.5% polyacrylamide gels. The blot membranes were probed with 1343 anti-BLM antibody. (**b**) ATM-deficient cells (GM03189B) were left untreated, exposed to 50 J/m^2 of UVC or treated with 2 mM of HU. Cells were harvested and lysed at the indicated times after treatment and protein extracts were separated on 5.5% polyacrylamide gels. The blot membranes were probed with 1343 anti-BLM antibody. (**c**) Protein extracts from (**a**) were incubated in the absence (lanes 1, 3, 5 and 7) or the presence (lanes 2, 4, 6 and 8) of 1200 U of λ phosphatase at 30°C for 2 h. Controls (lanes 1, 3, 5 and 7) were incubated with equivalent amounts of λ phosphatase buffer without enzyme. All extracts were separated on 5.5% polyacrylamide gels. The blot membranes were probed with 1343 antibody. (**d**) Wild type (D1) cells (left panels) and ATM-deficient cells (GM03189B) (right panels) were left untreated or exposed to 50 J/m^2 of UVC or treated with 2 mM of HU. Cells were harvested and lysed at the indicated times after treatment, and protein extracts were separated on 7.5% polyacrylamide gels. The blot membranes were probed with 1343 anti-BLM antibody (upper panels) and reprobed with anti-β-actin to ensure equal loading (lower panels)

accumulation of BLM are independent of the presence of a functional ATM.

Discussion

In this study, we investigated the response of BLM to UVC radiation and to HU-mediated inhibition of DNA synthesis. We first showed that BLM-deficient cell population displays a reduction in the number of replicating cells in response to UVC radiation. This probably reflects a delay in the progression of replication forks. BS cells are characterized by having a rate of sister chromatid exchanges (SCE) 10 times higher than that in normal cells (reviewed in German, 1993), and ultraviolet-light has been shown to further increase the SCE rate in BS cells (Kurihara *et al*., 1987; Mamada *et al*., 1989). BLM has been shown to

promote an ATP-dependent branch migration of Holliday junctions, and it has therefore been proposed that BLM may act at sites of arrested replication forks to resolve Holliday junctions formed by the annealing of two newly synthesized daughter strands by means of reverse branch migration activity (Karow *et al*., 2000a). Lack of BLM activity would result in an accumulation of Hollyday junctions that are resolved mainly by SCE (Karow *et al*., 2000a; Wang *et al*., 2000a). Thus, it is likely that the increase of SCE in UVC-irradiated BS cells leads to a delayed replication rate, and thus to the very slow incorporation of BrdU by some of the UVC-irradiated BLM-deficient cells. Such cells would appear to be BrdU negatives, which could explain the apparent reduction in the number of DNA replicating BS cells.

The partial escape from the UVC-induced G2/M cell cycle checkpoint that we observed in BS cells is very similar to the one we previously described in response to ionizing radiation (Ababou *et al*., 2000). Thus, despite the major differences between these two stress pathways, a point of convergence in the stress responses of the BLM-deficient cells appears to be the partial escape from the G2/M cell cycle checkpoint. This suggests as discussed below, that BLM could be involved in the G2/M cell cycle checkpoint through a p53- and a p21-independent mechanism.

Surprisingly, we did not detect any obvious defect in the HU-induced S phase arrest, or in the recovery from the S phase arrest in BLM-deficient cells. Furthermore, BLM-deficient cells do not exhibit any particular sensitivity to HU, even when exposed to high doses of HU (data not shown). These results indicate that BLM differs from its homologues Rqh1 in *Schizosaccharomyces pombe*, and Sgs1p in *Saccharomyces cerevisiae*. Indeed, it has been reported that mutation of *Rqh1* or *Sgs1* results in an increased sensitivity to HU (Stewart *et al*., 1997; Yamagata *et al*., 1998; Frei and Gasser, 2000), and that BLM can restore the increased sensitivity of the sgs1 mutant to HU (Yamagata *et al*., 1998). Furthermore, Rqh1 has been shown to be crucial for S phase arrest recovery after HU treatment (Stewart *et al*., 1997; Davey *et al*., 1998). Only one RecQ helicase is present in fission yeast and in budding yeast (Rqh1 and Sgs1p, respectively), whereas five human RecQ helicases, namely RecQL, BLM, WRN, RecQ4, RecQ5, have been identified to date (for review, Karow *et al*., 2000b). Thus, yeast helicases are likely to cumulate several functions that are shared out among the five human RecQ helicases. This is probably why the response of BLM-deficient cells to HU differs from cells lacking Rqh1 or Sgs1p.

We present data showing that BS cells that do not express any detectable BLM protein (GM03403D; Dutertre *et al*., 2000) display a delayed and decreased p53 response to UVC radiation, whereas BS cells that express a full length mutated BLM protein (IPM1; Barakat *et al*., 2000) exhibit UVC-induced p53 accumulation similar to that seen in wild type cells. It is interesting to note that the four BS fibroblasts cell lines (GM1492, GM03510, GM02932 and GM03402; Catalog of cell lines, NIGMS Human Genetic Mutant Cell Repository) reported to display an altered UVC p53 response (Lu and Lane, 1993; Collister *et al*., 1998), as well as the BS lymphoblastoid cells that we used in the present study (GM03403D), were all derived from Ashkenazi BS patients, known to carry the same blm^{Ash} homozygous mutation, due to a founder effect (Ellis *et al*., 1995; Ellis and German, 1996; Li *et al*., 1998). Thus, it is tempting to postulate that in response to UVC radiation, the absence of BLM expression is associated with an alteration of p53 expression, suggesting that BLM may contribute to the UVC-induced accumulation of p53 through interaction(s) with protein(s). However, our results showing that in both BS cell lines, the p21 protein is not induced 24 h after UVC exposure such as in wild type cells, led us to postulate that this p21 defect could be common to all BS cells. These findings suggest that either p53 may be functionally impaired in both UVC-treated BS cell lines, independently of its accumulation pattern, or that p21 may be regulated via a BLM-dependent pathway in response to UVC. Further work is needed to clarify this point. These results, combined with our previous data showing that BS cells exhibit intact p53 and p21 responses to ionizing radiation, support the possibility that the partial escape from the UVC- or γ-radiation-induced G2/M cell cycle checkpoints in BS cells could be independent of p53 and p21. However, we cannot exclude the possibility that the BLM protein could be involved in the UVC- or the γ-radiation-induced G2/M cell cycle checkpoints via different pathways. Indeed, most of the cellular responses induced by UVC or ionizing radiation are independent, and mediated by different kinases, ATR and ATM, respectively (for review, Shiloh, 2001). Furthermore, the BRCA1 protein, shown to co-immunoprecipitate with BLM (Wang *et al*., 2000b), seems to be involved in the γ-radiation-induced G2/M checkpoint, but not in the UVC-induced G2/M checkpoint (Xu *et al*., 1999). In a previous work, we proposed that BLM could be involved in the γ-radiation induced G2/M checkpoint by interacting with BRCA1 (Ababou *et al*., 2000). It has been shown recently that the p21 protein is required for the cell cycle arrest at G2 upon DNA damage, via a p53 independent pathway, probably by blocking the interaction of Cdc25C with PCNA (Ando *et al*., 2001). Thus, BLM could contribute to the UVC-induced G2/M cell cycle checkpoint via a p21 dependent pathway.

We also showed that both BS cell lines display a normal p53 response to HU-induced DNA synthesis inhibition, and that p21 is not induced by HU treatment in either wild type or BS cells. Thus, all round, our experiments using EBV-transformed lymphoblastoid cells confirm the data from other cell systems. Indeed, it has been shown that in response to HU-mediated DNA synthesis inhibition, p53 is stabilized but fails to induce p21 (Gottifredi *et al*., 2001), and that in response to UVC radiation, p21 expression is repressed in various human cancer cell

lines (Allan and Fried, 1999; Wang *et al*., 1999). Our data (this study and Ababou *et al*., 2000) have also confirmed that p53 is stabilized via different pathways depending on the type of DNA damage, further supporting the finding that multiple mechanisms can result in p53 stabilization in response to various cellular stresses (Ashcroft *et al*., 2000).

Our results demonstrating that BLM protein level was markedly reduced after cells were irradiated with UVC are reminiscent of the recently reported UVC-irradiation-induced depletion of BRCA2 and BRCA1 (Wang *et al*., 2001). We found that UVC-mediated decrease in BLM does not require a functional ATM gene and does not result from any change in subcellular compartment change. It has been proposed that UVC-induced down regulation of BRCA1 and BRCA2 is mediated by an as yet unknown mechanism (Wang *et al*., 2001). However, whatever the mechanism involved, the rapid phosphorylation of BLM in response to UVC radiation, with the concomitant reduction in the number of replicating cells and the partial escape from the G2/M cell cycle checkpoint exhibited by UVC-irradiated BS cells, led us to propose that the UVC-induced decrease in BLM is a part of the cellular response activated by UVC.

Finally, we demonstrated that UVC radiation and HU-mediated DNA synthesis inhibition both induced BLM phosphorylation via an ATM-independent pathway, whereas we had previously showed that the γ-irradiation-induced BLM phosphorylation in response to ionizing radiation requires a functional ATM gene (Ababou *et al*., 2000). Among the members of the supercomplex of BRCA1-associated proteins to which BLM belongs (Wang *et al*., 2000b), Nijmegen breakage syndrome (NBS) protein and BRCA1 are both phosphorylated via an ATM-dependent pathway in response to ionizing radiation, and via an ATM-independent pathway in response to UVC and HU (Wu *et al*., 2000; Tibbetts *et al*., 2000). Several studies have demonstrated that ATM responds exclusively to DNA double-strand breaks, whereas the ATM-related kinase, ATR, also reacts to UV damage and replication arrest (for review, Shiloh, 2001). Moreover, ATR has been shown to exhibit similar substrate specificity to ATM (Kim *et al*., 1999), and the phosphorylation of BRCA1 mediated by UVC or HU has been shown to be controlled by ATR (Tibbetts *et al*., 2000). These findings have led us to suggest that BLM is a target of ATR kinase. Future studies will help to address this question.

Materials and methods

Cell lines and culture conditions

The EBV-transformed lymphoblastoid B cell line GM03403D derived from BS patient 9 (EmSh) on the Bloom's Syndrome registry, was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, USA). The EBV-transformed lymphoblastoid B cell line IPM1 was derived from a Moroccan BS patient (Barakat *et al*., 2000).

The EBV-transformed lymphoblastoid B cell line GM03189B was derived from an AT patient and kindly provided by Dr Filippo Rosselli (Institut André Lwoff, Villejuif, France).

The EBV-transformed lymphoblastoid B cell line D1, derived from a normal individual, is considered to be the wild type equivalent of the BLM-deficient cells and was kindly provided by Dr Françoise Praz (Institut André Lwoff, Villejuif, France).

The EBV-transformed lymphoblastoid B cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc) containing 10% heat-inactivated foetal calf serum (FCS).

Radiation treatment

Asynchronous cells were split 24 h prior to the experiment and then seeded in a glass Petri dish at a density of 4×10^5 cells/ml. The medium was removed and replaced with PBS, and then the cells were exposed to UVC light (254 nm) from a germicidal UV lamp (Fisher Bioblock Scientific). The distance between the lamp and the dish was adjusted to obtain a fluence of 1 J/m^2 per second measured using a UV radiometer (VLX-3W, Fisher Bioblock Scientific). The irradiation time was set as to obtain a dose of 50 J/m^2. After irradiation, growth medium was added and the cells were grown at 37°C for the times indicated. The untreated cells were not irradiated but otherwise were treated in the same way as the irradiated cultures.

Chemicals

Hydroxyurea (Sigma) was resuspended in water to a stock concentration of 1 M and added to a final concentration of 2 mM.

Immunoblot analysis

Cells were lysed in 25 mM Tris pH 7.5, 300 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% Igepal Ca-630 (Sigma) and protease inhibitor cocktail (Roche) for 30 min on ice. Membrane debris was eliminated by centrifuging at 14 000 r.p.m. for 30 min. Protein concentrations were measured by the Coomassie protein assay according to the Manufacturer's instructions (Pierce). Aliquots of 50 μg proteins were diluted in Laemmli buffer, denatured by heating for 5 min at 95°C and subjected to electrophoresis in a 5.5% (BLM phosphorylation), 7.5% (BLM accumulation and β-actin) or 12% (p53, p21 and β-actin) SDS-polyacrylamide gel. Protein fractions from the gel were electrophoretically transferred to PVDF membranes (Immobilon P, Millipore) in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol and 0.1% SDS for 2 h at 800 mA. After 1 h saturation in PBS containing 5% dry non-fat milk and 0.5% Tween 20, the membranes were incubated for 1 h with primary antibody diluted in PBS containing 5% dry non-fat milk and 0.5% Tween 20. After four 15-min washes with PBS containing 0.5% Tween 20, the membranes were incubated for 45 min with a 1 : 10 000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum (Pierce) or with a 1 : 2000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin G antiserum (Zymed). Membranes were then washed four times with PBS containing 0.5% Tween 20, and the reaction was developed according to the manufacturer's specifications (ECL kit; Amersham). Membrane stripping was performed in 62.5 mM Tris pH 6.2 with 2% sodium dodecyl sulphate (SDS) and 100 mM β-mercaptoethanol for 30 min at 50°C.

Antibodies

The rabbit antiserum 1343 was generated against the carboxy-terminal peptide of human BLM as described (Dutertre *et al*., 2000), and used at 1 : 5000 dilution. Monoclonal antibody Ab6 specific for p53 (Oncogene) was used at a final concentration of 0.1 μg/ml, monoclonal antibody F-5 specific for p21 (Santa Cruz Biotechnology) was used at 1 : 250 dilution, polyclonal antibody A2066 against β-actin (Sigma) was used at 1 : 1000 dilution.

Phosphatase treatment

Cells were lysed by incubation in 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.5% Igepal Ca-630 (Sigma), and protease inhibitor cocktail (Roche), for 15 min at 4°C. Membrane debris was eliminated by centrifuging at 14 000 r.p.m. for 15 min. Protein concentrations were measured by the Coomassie protein assay according to the Manufacturer's instructions (Pierce). Aliquots of 50 μg of proteins were resuspended in phosphatase buffer and then incubated for 2 h at 30°C with 1200 U of lambda protein phosphatase in the presence of $MnCl_2$, according to the Manufacturer's instructions (Biolabs). Controls were incubated with an equivalent amount of buffer without phosphatase. Dephosphorylation was stopped by adding an equal volume of Laemmli buffer.

Flow cytometry analysis

For G1/S checkpoint analysis, cells exposed to UVC (50 J/m^2) or mock treated were pulse labeled with 10 μM of BrdU (Sigma) for 2 h. Sixteen hours after irradiation, cells were harvested, washed twice with PBS and fixed with 70% ethanol at −20°C for at least 30 min. BrdU incorporation was detected by monoclonal anti-BrdU antibody (Pharmingen) (1 h at room temperature) and subsequently by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson) (30 min at room temperature). Cells were then washed and incubated for 30 min at 37°C in PBS containing 100 μg/ml RNase A (Sigma) and 10 μg/ml propidium iodide (PI) (Sigma). DNA synthesis (FITC) and DNA content (PI) were analysed by FACScalibur (Becton Dickinson).

For G2/M checkpoint analysis, exponentially growing cells were mock treated or irradiated with 50 J/m^2 of UVC and then returned to the incubator. Sixty minutes and 120 min after irradiation, 10^6 cells were fixed with 85% methanol at −20°C for at least 30 min and permeabilized in 0.5% Triton X-100 with PBS for 5 min. They were then incubated for 2 h at room temperature with a polyclonal antibody specific for the phosphorylated Ser 10 of the histone H3 (Euromedex), at a final concentration of 5 μg/ml. This antibody labels mitotic cells with specifically increased intensity because all H3 molecules become phosphorylated at Ser 10 during mitosis, whereas few H3 molecules are phosphorylated in interphase (Gurley *et al*., 1978; Sauvé *et al*., 1999). Cells were then rinsed and incubated with an FITC-conjugated goat anti-rabbit antibody (Jackson) (45 min at room temperature), resuspended in PBS containing 100 μg/ml RNase A and 10 μg/ml propidium iodide, and mitotic cells were scored (on a total of 10 000 cells) by two-color FACScalibur (Becton Dickinson) analysis, gating for phospho-Histone H3 positive cell populations.

NP-40 extraction

Cells were UVC-irradiated or not, washed with PBS, and then resuspended in nuclear buffer (15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM Tris pH 7.4, 0.5 mM dithiothreitol (DTT), 300 mM sucrose) containing 0.3% NP-40. After incubating for 3 min at RT, samples were centrifuged, and the supernatant was kept as the NP-40 soluble fraction. The pellet, resuspended in solution P (20 mM HEPES, 10% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM DTT) and sonicated, constituted the NP-40 insoluble fraction.

Analysis of cell division

The percentages of cell division were estimated by labelling cells with CFSE (CarboxyFluorescein Succinimidyl Ester, Molecular Probes, Eugene, OR, USA). Briefly, CFSE was dissolved in dimethylsulphoxide (DMSO) at a concentration of 5 mM as a stock solution and kept at −20°C until further use. The cells to be labelled were washed and resuspended at 10^6 cells/ml in phosphate-buffered saline (PBS) containing 10% heat-inactivated foetal calf serum (FCS). The CFSE stock solution was used at 1 : 250 dilution. After incubating for 10 min at 37°C with CFSE, cells were centrifuged, washed three times, resuspended in fresh medium at 10^6 cells/ml, and then incubated overnight at 37°C. The CFSE-labelled cells were then treated or not with 2 mM of HU for 24 h. CFSE-labelled cells were then released from the block by removing HU, growth medium was added and the cells were grown at 37°C for the times indicated, harvested and fixed with 2% paraformaldehyde at 4°C. The intensity of CFSE fluorescence was measured by flow cytometry analysis.

Abbreviations

BrdU, Bromodeoxyuridine; BS, Bloom's syndrome; CFSE, Carboxyfluorescein succinimidyl ester; DSBs, Double-strand breaks; FITC, Fluorescein isothiocyanate; PI, Propidium iodide; SCE, Sister chromatid exchange

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