

Bloom's syndrome protein is required for correct relocalization of RAD50/MRE11/NBS1 complex after replication fork arrest

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Bloom's syndrome (BS) is a rare genetic disorder characterized by a broad range of symptoms and, most importantly, a predisposition to many types of cancers. Cells derived from patients with BS exhibit an elevated rate of somatic recombination and hypermutability, supporting a role for bleomycin (BLM) in the maintenance of genomic integrity. BLM is thought to participate in several DNA transactions, the failure of which could give rise to genomic instability, and to interact with many proteins involved in replication, recombination, and repair. In this study, we show that BLM function is specifically required to properly relocalize the RAD50/MRE11/NBS1 (RMN) complex at sites of replication arrest, but is not essential in

the activation of BRCA1 either after stalled replication forks or γ -rays. We also provide evidence that BLM is phosphorylated after replication arrest in an Ataxia and RAD3-related protein (ATR)-dependent manner and that phosphorylation is not required for subnuclear relocalization. Therefore, in ATR dominant negative mutant cells, the assembly of the RMN complex in nuclear foci after replication blockage is almost completely abolished. Together, these results suggest a relationship between BLM, ATR, and the RMN complex in the response to replication arrest, proposing a role for BLM protein and RMN complex in the resolution of stalled replication forks.

Introduction

Cells have evolved several genes to maintain genomic integrity and stability. In mammals, the failure of the complex molecular pathways ensuring genomic stability almost inevitably leads to cancer. Despite the importance of the precise knowledge of these pathways, little is known about the precise mechanism by which genomic integrity is maintained in mammalian cells, and in particular in human cells. Recently, it has been proposed that a class of DNA helicases, related to the bacterial RecQ helicase, plays an important role in the molecular pathways leading to genetic stability (Chakraverty and Hickson, 1999). Whereas *Escherichia coli* and yeast genomes encode only one RecQ helicase, which is essentially involved in the control of recombinational processes (Watt et al., 1996; Hanada et al., 1997; Myung et al., 2001) and also in yeast in chromosome segregation (Watt et al., 1995), human cells have multiple RecQ-class helicases. Five human RECQ

genes have been cloned and, among these, three are correlated to genetic diseases: WRN, mutated in Werner's syndrome; RTS, mutated in Rothmund-Thomson syndrome; and BLM, found mutated in Bloom's syndrome (BS)* (Mohaghegh and Hickson, 2001).

Bloom's syndrome is a rare genetic disorder characterized by a broad range of symptoms and, most importantly, a predisposition to many types of cancers (German, 1995). Cells derived from patients with BS exhibit elevated frequency of chromosome and chromatid breaks, chromatid exchanges, and sister chromatid exchanges (SCEs) (McDaniel and Schultz, 1992; Neff et al., 1999), in addition to increased levels of locus-specific mutations (German, 1995). Such hypermutability strongly supports a role for BLM in the maintenance of genomic integrity. In fact, BLM is thought to participate in several DNA transactions, the failure of which could give rise to genomic instability, such as recombination, replication, and repair (Hickson et al., 2001). Consistent with a proposed role in recom-

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*Abbreviations used in this paper: ATR, Ataxia and RAD3-related protein; BLM, BS protein; BrdUrd, 5'-bromo-2-deoxyuridine; BS, Bloom's syndrome; HU, hydroxyurea; RMN, RAD50/MRE11/NBS1; SCE, sister chromatid exchange; WS, Werner's syndrome.

bination in somatic cells, BLM can bind Holliday junctions (Karow et al., 2000; Mohaghegh et al., 2001) and D-loops (van Brabant et al., 2000), and physically interact with the RAD51 recombinase (Bischof et al., 2001; Wu et al., 2001). In addition, BLM can also interact with one topoisomerase III isoform, TOPOIII α , and this interaction could be important for BLM's role in controlling recombination (Johnson et al., 2000; Wu et al., 2000b). Recently, it has also been reported that BLM interacts with several proteins involved in either DNA repair or DNA damage signalling, such as BRCA1, MRE11, and ATM to form a surveillance complex, called BASC, that could function as a sensor for various types of DNA lesions or aberrant structures (Wang et al., 2000). BLM protein seems to relocalize to nuclear structures containing BRCA1 and/or the complex formed by RAD50/MRE11/NBS1 (RMN) complex, either after hydroxyurea (HU)-induced replication arrest or ionizing radiation-induced DNA damage (Wang et al., 2000). Furthermore, BLM itself is able to relocalize after DNA damage (Bischof et al., 2001; Wu et al., 2001), also interacting with another protein possible involved in genomic stability, PML (Ishov et al., 1999; Bischof et al., 2001). However, despite the possible crucial role for BLM in the pathways controlling genetic stability the knowledge of its functions is still incomplete.

In this study, we investigated whether the absence of an active BLM protein could give rise to abnormal response of the BRCA1 protein and the RMN complex, two of the proposed molecular partners of BLM, either after HU-induced replication fork stall or ionizing radiation-induced DNA damage. In addition, we examined the possibility that BLM phosphorylation by the ATM/Ataxia and RAD3-related

protein (ATR) kinases could be important for subnuclear relocalization after replication arrest or DNA damage.

We found that BLM was essential to correctly relocalize and activate the RMN complex after replication arrest, but not after γ -rays. On the contrary, an active BLM protein was not crucial in BRCA1 phosphorylation and focus-forming activity, either after replication arrest or γ -rays. We also found that BLM phosphorylation after HU was essentially ATR dependent, but that it is unrelated to BLM subnuclear relocalization. Furthermore, lack of BLM phosphorylation determined a dramatic decrease of the RMN foci after HU, suggesting a possible role for BLM protein in organizing RMN foci after replication arrest.

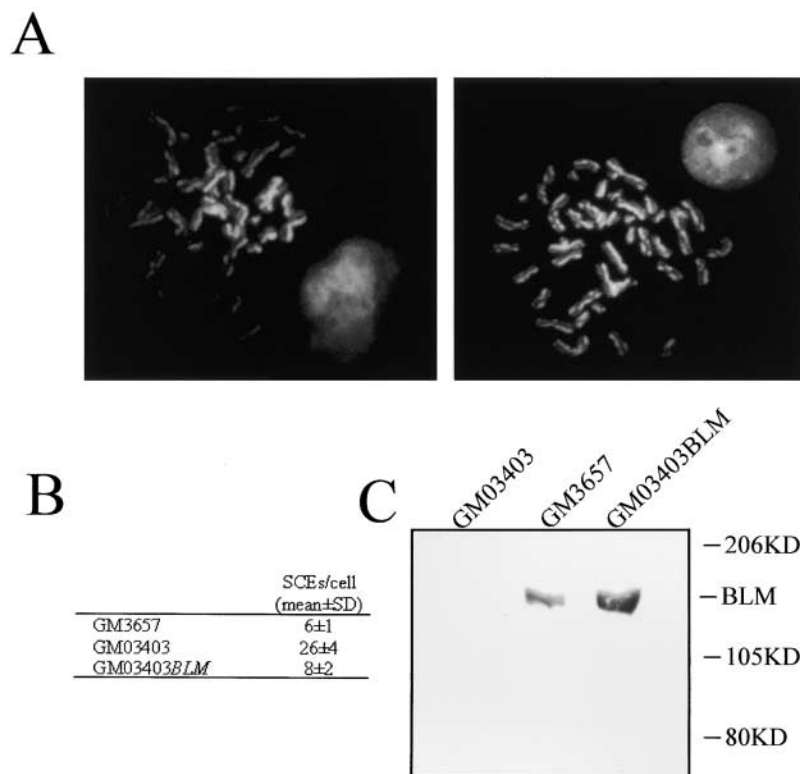
Results

BS cells show defective formation of RMN complex foci and absence of NBS1 phosphorylation upon HU treatment, but not after γ -irradiation

It has been recently reported that BLM interacts and colocalizes with the RMN complex (Wang et al., 2000) and redistributes at sites of replication fork arrest (Wang et al., 2000; Bischof et al., 2001). Thus, we sought to determine whether BS cells properly activated the RMN complex after ionizing radiation-induced DNA damage or HU-induced replication arrest. Focal localization of the RMN complex was analyzed in wild-type, BS cells, and in cells from BS in which the wild-type BLM-coding sequence was introduced by transfection (Fig. 1).

Relocalization of the RMN complex, here visualized as MRE11 and NBS1 focus-forming activity, was observed either after HU or γ -rays in normal cells (Fig. 2, A and B). As

Figure 1. Phenotypic reversion of BS cells through introduction of a wild-type copy of the *BLM* cDNA. (A) Transfection of the BS lymphoblasts GM03403 with a BLM-expressing plasmid resulted in the recovery of a normal SCE yield (a) compared with the unverted cell line (b). (B) Table showing the SCE yield in the wild-type, BS and BS reverted cell lines. (C) Transfection of the BS lymphoblasts GM03403 with a *BLM*-expressing plasmid resulted in the recovery of the expression of the BLM protein.



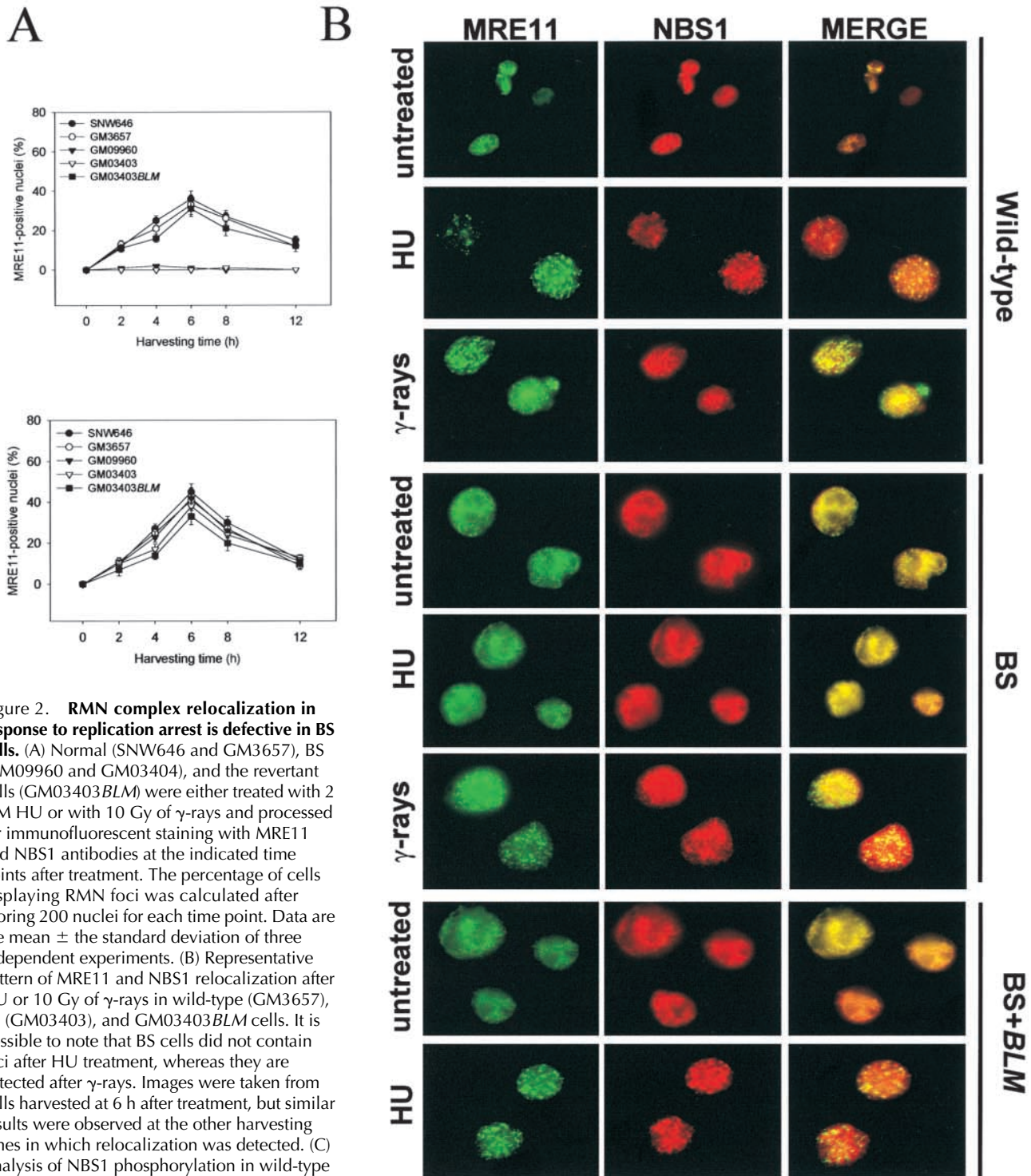
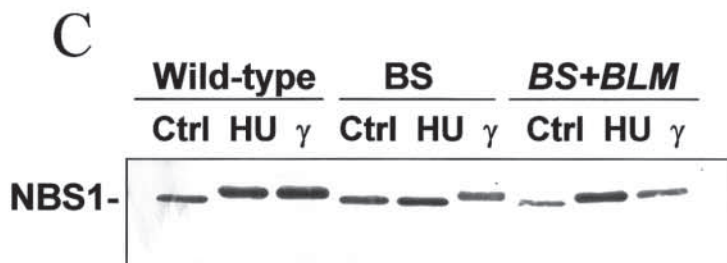


Figure 2. RMN complex relocalization in response to replication arrest is defective in BS cells. (A) Normal (SNW646 and GM3657), BS (GM09960 and GM03404), and the revertant cells (GM03403BLM) were either treated with 2 mM HU or with 10 Gy of γ -rays and processed for immunofluorescent staining with MRE11 and NBS1 antibodies at the indicated time points after treatment. The percentage of cells displaying RMN foci was calculated after scoring 200 nuclei for each time point. Data are the mean \pm the standard deviation of three independent experiments. (B) Representative pattern of MRE11 and NBS1 relocalization after HU or 10 Gy of γ -rays in wild-type (GM3657), BS (GM03403), and GM03403BLM cells. It is possible to note that BS cells did not contain foci after HU treatment, whereas they are detected after γ -rays. Images were taken from cells harvested at 6 h after treatment, but similar results were observed at the other harvesting times in which relocalization was detected. (C) Analysis of NBS1 phosphorylation in wild-type (GM3657), BS (GM09960), and revertant GM03403 cells in response to 2 mM HU or 10 Gy of γ -rays. Cells were lysed 2 h after treatments, and protein extracts were separated on 7.5% SDS-PAGE gel. Blot membrane was probed with NBS1 antibody (Novus Biologicals). Ponceau Red staining of the blot assessed equal loading and transfer.



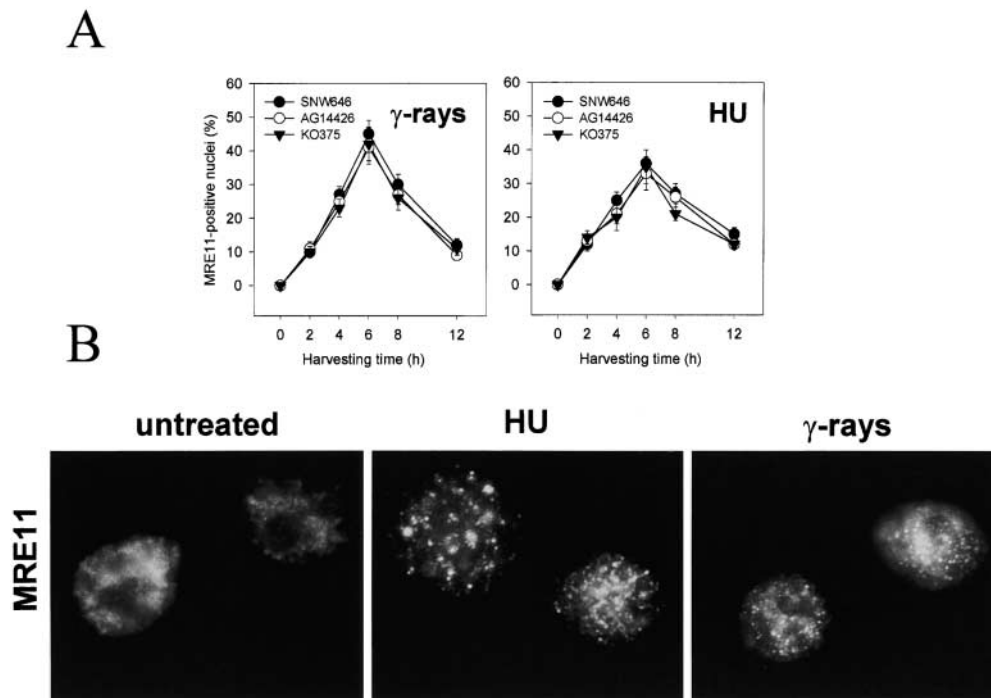
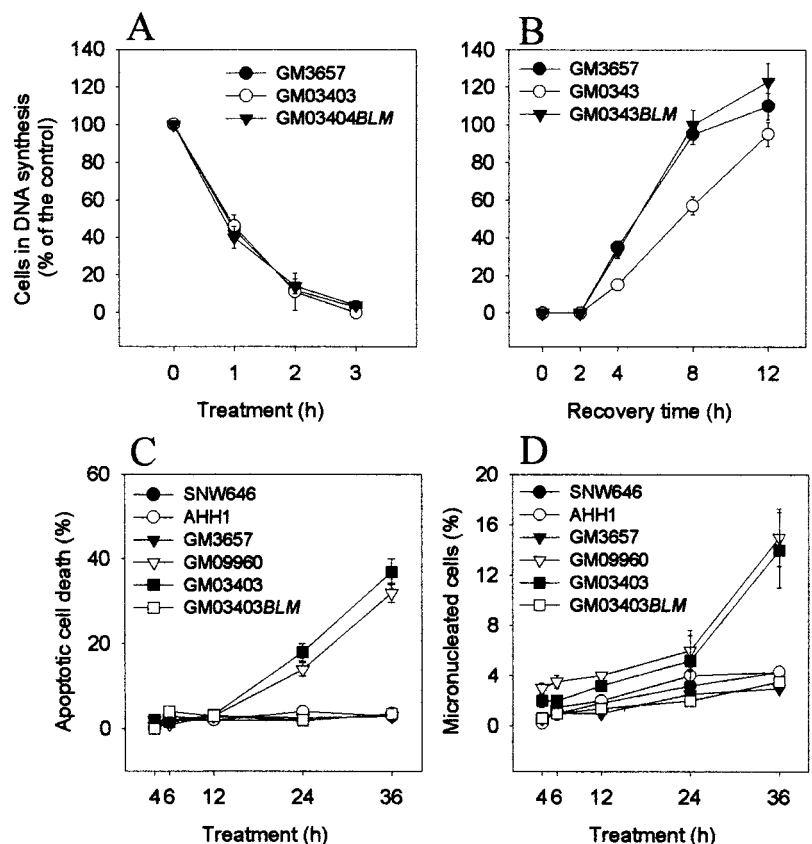


Figure 3. **MRE11 complex relocalization in response to replication arrest is normal in WS cells.** (A) Normal (SNW646) and WS (AG14426 and KO375) were either treated with 2 mM HU or with 10 Gy of γ -rays and processed for immunofluorescent staining with MRE11 antibody (GenTex; 1:500) at the indicated time points after treatment. The percentage of cells displaying MRE11 foci was calculated after scoring 200 nuclei for each time point. Data are the mean \pm the standard deviation of three independent experiments. (B) Representative pattern of MRE11 complex relocalization after HU or 10 Gy of γ -rays in WS cells (AG14426).

Figure 4. **BS cells arrest and resume correctly DNA synthesis after HU treatment.** (A) Normal (GM3657), BS (GM0343) and BS revertant cells (GM03403BLM) were exposed to 2 mM HU and harvested at the indicated times. Alternatively, Normal (GM3657), BS (GM0343), and BS revertant cells (GM03403BLM) were exposed to 2 mM HU for 2 h and recovered at the indicated times in drug-free medium (B). Percentage of S-phase cells was determined labeling DNA synthesis by adding BrdUrd in the last hour before harvesting. BrdUrd incorporation was assessed as described in Materials and methods. Similar results were obtained also with the other normal (GM3657 and AHH1) and BS (GM09960) cells. Points represent mean \pm SE from at least three experiments. Replication blockage by HU leads to the induction of apoptotic cell death (C) and micronuclei (D) in BS. Normal (SNW646, AHH1 and GM3657), BS (GM09960 and GM03404), and the BS revertant cells (GM03403BLM) were exposed to 2 mM of HU and harvested at different time points. The induction of apoptotic and micronucleated nuclei was evaluated at the indicated time by bis-benzimide staining of cells smeared onto microscopic slides as described in Materials and methods. For the analysis of the apoptotic cell death similar results were obtained by TdT-mediated end-labelling assay. Points represent mean \pm SE from at least three experiments.



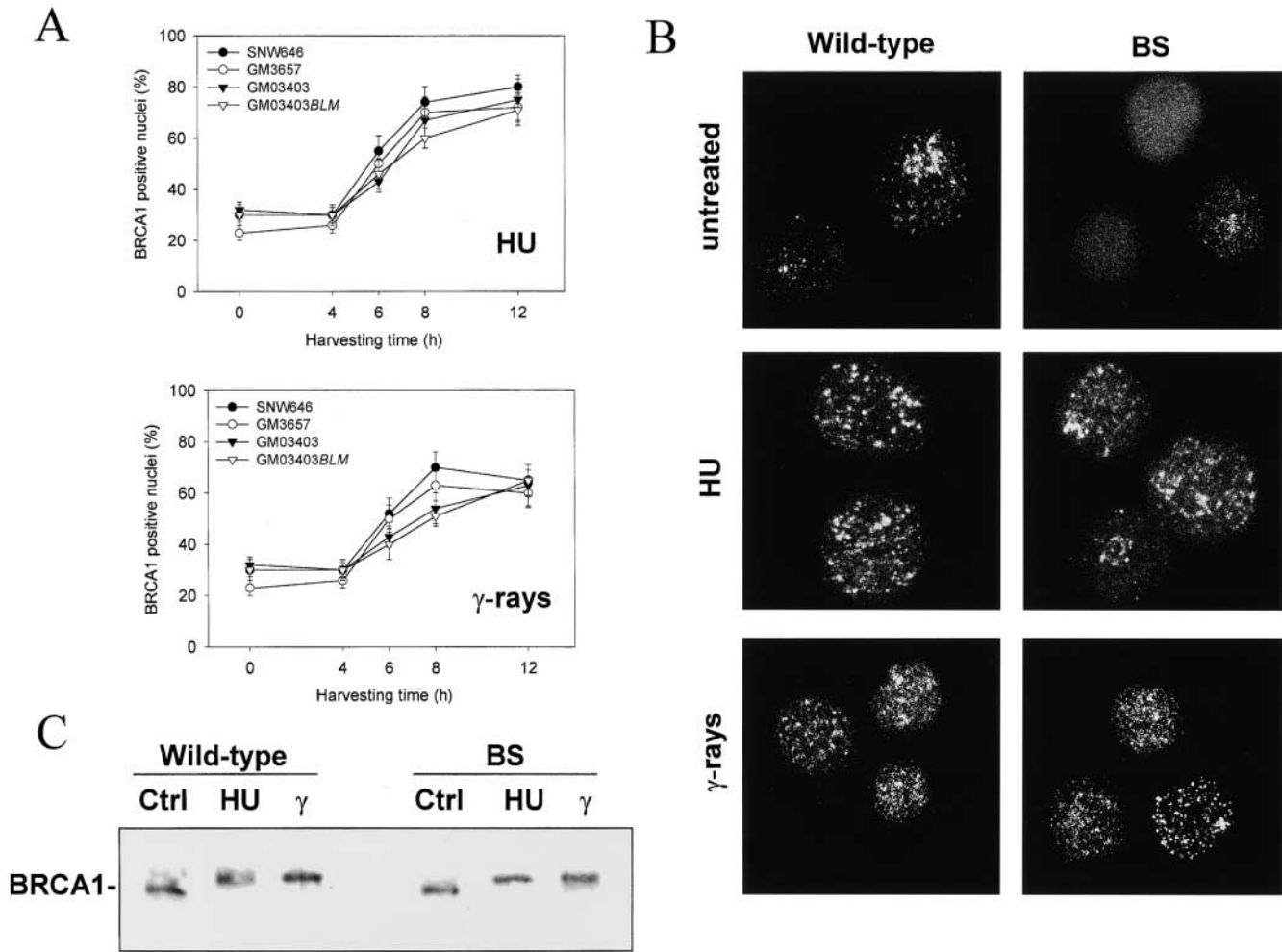


Figure 5. In BS cells, BRCA1 activation and relocalization is normal in response to replication arrest or ionizing radiation-induced DNA damage. Normal (SNW646 and GM3657), BS (GM03403), and the BS revertant cells (GM03403BLM) were either treated with 2 mM HU or with 10 Gy of γ -rays and processed for immunofluorescent staining with BRCA1 antibody at the indicated time points after treatment (A). The percentage of cells displaying BRCA1 foci was calculated after scoring 200 nuclei for each time point. Data are the mean \pm the standard deviation of three independent experiments. (B) Representative pattern of BRCA1 relocalization after HU or 10 Gy of γ -rays in wild-type (GM3657), BS (GM03403), and GM03403BLM cells. Images were taken from cells harvested at 8 h after treatment, but similar results were observed at the other harvesting times in which relocalization was detected. (C) Analysis of BRCA1 phosphorylation in wild-type (GM3657), BS (GM03403), and revertant GM03403 cells in response to 2 mM HU or 10 Gy of γ -rays. Cells were lysed 2 h after treatments and protein extracts were separated on 5% SDS-PAGE gel. Blot membrane was probed with BRCA1 antibody (Oncogene). Ponceau red staining of the blot assessed equal loading and transfer.

expected, MRE11 and NBS1 foci colocalized (Fig. 2 B), and each separately colocalized with RAD50 (unpublished data). Therefore, we feel confident that each of the three foci represents the activity of the whole complex. RMN complex focalization appeared time dependent, reaching the top value at 6 h after treatment with γ -rays or HU, and showing a similar kinetic in both the two normal cell lines tested. In contrast, MRE11 and NBS1 foci were not observed in BS cells after HU treatment. However, BS cells were able to correctly relocalize the RMN complex after γ -rays, suggesting that the absence of an active BLM protein impaired RMN focus-forming activity, specifically after replication fork arrest. Interestingly, introduction of a wild-type BLM-expressing plasmid completely restored the ability of BS cells to form RMN foci after HU treatment (Fig. 2, A and B). Focalization of the RMN complex has been correlated to

NBS1 phosphorylation, and such a modification is considered a prerequisite or a consequence of the complex subnuclear relocalization (Zhao et al., 2000; Gatei et al., 2001; Mirzoeva and Petrini, 2001). Consistent with the immunocytochemical data, we found that NBS1 dephosphorylated in BS cells after HU exposure, but not after γ -rays (Fig. 2 C). Interestingly, the MRE11 complex is properly relocalized after HU and γ -rays in cells mutated in another RecQ-like helicase, WRN (Fig. 3, A and B).

Because RMN complex has been correlated with checkpoint activation in the S-phase of the cell cycle after ionizing radiation-induced DNA damage (Petrini, 2000), and given that HU specifically interferes with the S-phase progression, we investigated whether in BS cells the absence of RMN focal localization could determine deficiency in the arrest of DNA synthesis after HU treatment. Analysis of the rate of

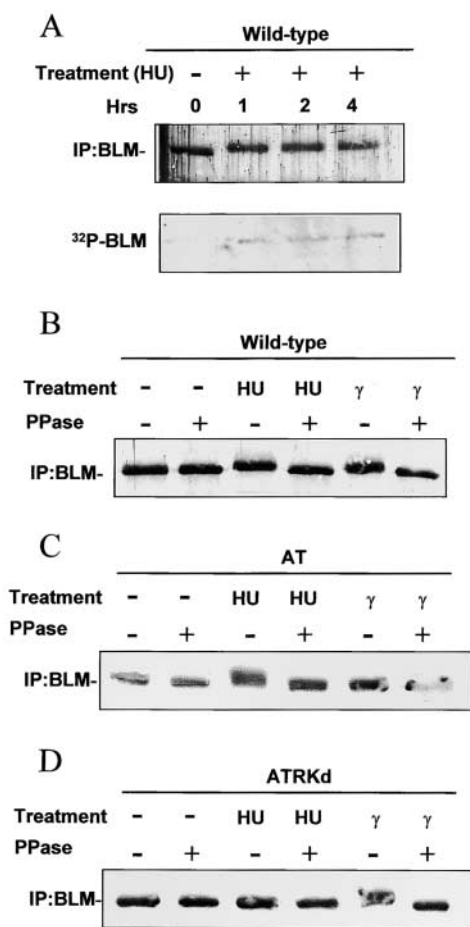


Figure 6. BLM phosphorylation in response to replication arrest is ATM-independent but ATR dependent. Wild-type cells (SNW646) were starved for phosphate for 30 min, and then labeled with [³²P]-orthophosphate and treated with 2 mM HU for the indicated time before extraction and immunoprecipitation. The protein levels and phosphorylation were analyzed by Western blotting and autoradiography, respectively. Immunoprecipitated were separated on 5% SDS-PAGE gels. Blot membranes were probed with BLM antiserum. Proteins extracted from mock-, HU- (2 mM, 2 h), or γ -rays-treated cells (10 Gy, 2 h), or wild-type (GM3657, Panel B), AT (GM2782, Panel C), or ATRKd cells (Panel D) were immunoprecipitated using BLM antisera. BLM immunoprecipitated from untreated and treated wild-type, AT, or ATRKd cells were incubated in the absence (-) or in the presence (+) of lambda phosphatase (PPase). All extracts were separated on 5% SDS-PAGE gels (30 V for 16 h at 4°C) and blot membranes were probed with BLM antiserum. Ponceau red staining of the blot assessed equal loading and transfer.

5'-bromo-2-deoxyuridine (BrdUrd) incorporation after HU treatment revealed that BS cells normally arrested and resumed DNA replication, even if with a slower rate (Fig. 4, A and B), suggesting that RMN complex is not crucial for the S-phase checkpoint activation after replication arrest. However, treatment with HU resulted in a higher apoptotic cell death in BS cells, as well as in a ~4-fold higher induction of micronuclei compared with normal cells. It is important to note that hypersensitivity to HU was corrected by transfection of wild-type BLM (Fig. 4, C and D).

These results suggest that BLM protein is needed to the correct subnuclear assembly of the RMN complex after rep-

lication fork stall, possibly resulting in a protection against cell death and chromosomal damage.

BLM protein is not required for correct formation of BRCA1 foci after both HU and γ -irradiation

Because BRCA1 interacts with both the RMN complex and BLM (Zhong et al., 1999; Wang et al., 2000), and given that there are conflicting results on its requirement in RMN focal localization after DNA damage (Zhong et al., 1999; Wang et al., 2000; Wu et al., 2000a), we investigated whether BRCA1 activation was normal in BS cells after HU or γ -rays treatments. In agreement with previously reported data (Scully et al., 1997; Wang et al., 2000), BRCA1 formed nuclear foci in normal cells either after HU or γ -rays (Fig. 5, A and B) and these foci correctly colocalized with components of the RMN complex or with RAD51, the strand-exchange protein involved in HR (unpublished data). Interestingly, despite the fact that BLM and BRCA1 colocalize (Wang et al., 2000), lack of BLM did not result in an impaired capability of BRCA1 to form foci. In fact, BRCA1 was normally relocalized after γ -rays or HU in BS cells (Fig. 5, A and B). It has been previously reported that BRCA1 subnuclear assembly requires phosphorylation by either the ATM and ATR kinases (Cortez et al., 1999; Tibbetts et al., 2000; Gatei et al., 2001). Thus, to expand our observations we also determined the phosphorylation state of BRCA1 in BS compared with normal cells. As expected, BRCA1 was found phosphorylated in normal cells after ionizing radiation or HU, and a similar result was observed in BS cells (Fig. 5 C).

These results show that absence of RMN complex formation in BS cells after HU is not attributable to a defect in BRCA1 phosphorylation/relocalization, and suggest that BLM is not required for proper subnuclear localization of the BRCA1 protein.

BLM protein is phosphorylated after HU exposure in an ATR-dependent manner

It has been reported that BLM is phosphorylated after γ -rays in a ATM-dependent manner (Ababou et al., 2000), therefore we investigated the possibility that BLM could be similarly modified after HU treatment, and whether such a modification could correlate with the subnuclear assembly of the RMN complex after replication fork arrest.

We found BLM modified in response to HU-induced replication arrest, resulting in the appearance of a slower-migrating form that was already detectable after 1 h of treatment and persisted up to 8h (Fig. 6 A; unpublished data). In vivo labeling experiments using [³²P]-orthophosphate showed that immunoprecipitated BLM was radiolabeled, clearly demonstrating that BLM was phosphorylated after HU. In addition, the slower-migrating form was easily converted into the faster-migrating form by phosphatase treatment, confirming that BLM was actually phosphorylated after HU (Fig. 6 B). Consistent with the data reported by others (Ababou et al., 2000), BLM was also found phosphorylated after γ -rays (Fig. 6 B). We then tested the possibility that the HU-dependent phosphorylation of BLM could also be under the control of ATM by analyzing BLM phosphorylation in AT

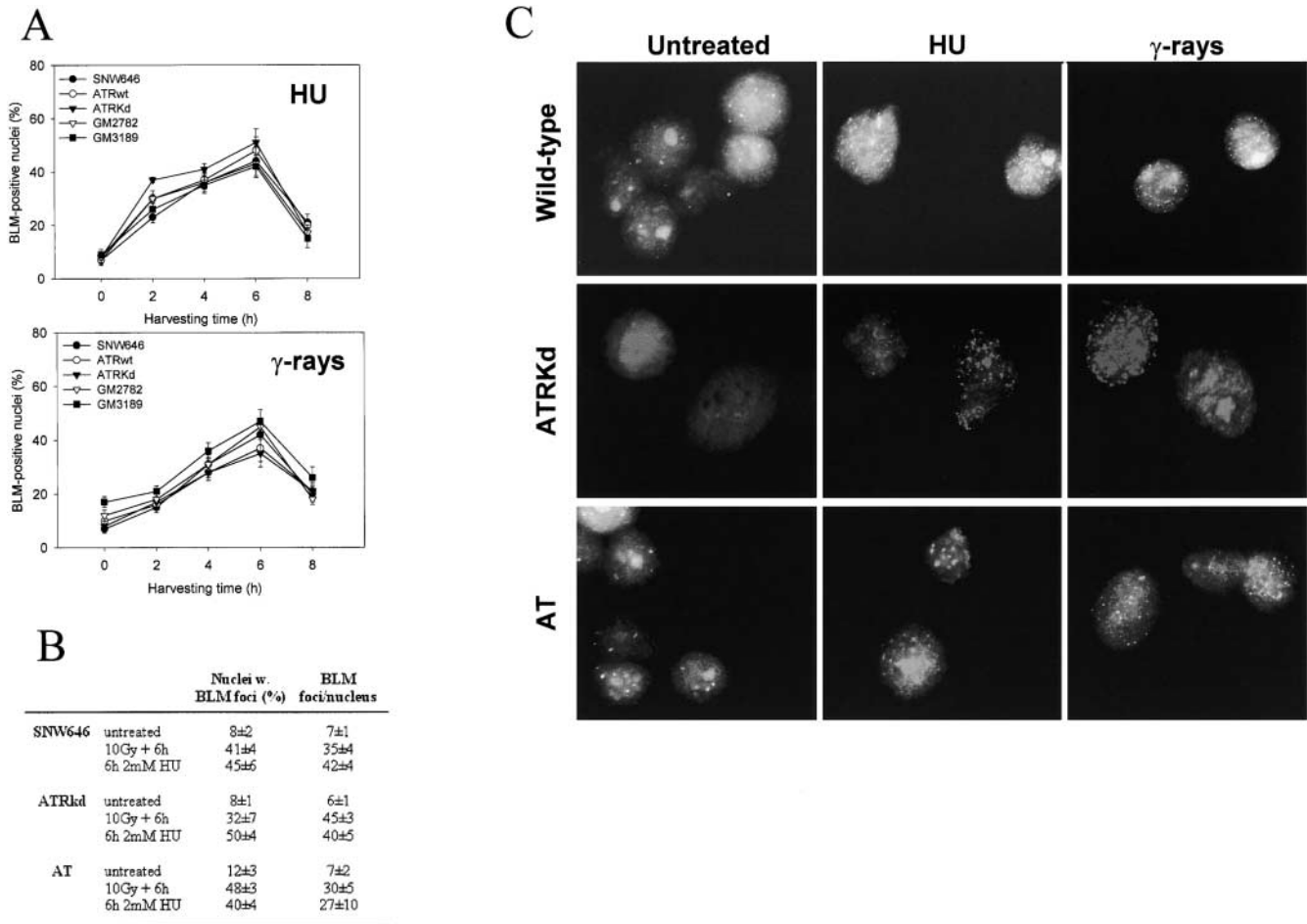


Figure 7. **BLM focal localization occurs also in the absence of phosphorylation.** (A) Normal (SNW646 and GM3657), ATRwt, ATRKd and AT cells (GM2782 and GM3189) were either treated with 2 mM HU or with 10 Gy of γ -rays and processed for immunofluorescent staining with BLM antibody at the indicated time points after treatment. The percentage of cells displaying BLM foci was calculated after scoring 200 nuclei for each time point. Data are the mean \pm the standard deviation of three independent experiments. (B) Table summarizing the number of BLM-positive nuclei and BLM foci/nucleus in wild-type (SNW646), ATRKd and AT (GM3189) cells. The values are the mean \pm the SD from three independent experiments. (C) Representative pattern of BLM relocalization after HU or 10 Gy of γ -rays in wild-type (GM3657), ATRKd and AT (GM3189) cells. Images were taken from cells harvested at 4 h after treatment but similar results were observed at the other harvesting times in which relocalization was detected.

cells. Interestingly, BLM phosphorylation was absent in AT cells after ionizing radiation but was not abolished after HU treatment (Fig. 6 C), suggesting that kinases other than ATM could phosphorylate BLM in response to replication fork arrest. The ATR kinase has been reported to be crucial in the modification of several proteins after replication fork stall (Shiloh, 2001); therefore, we sought to determine whether ATR could be involved in BLM modification after HU. In order to test this possibility, we exposed cells expressing an ATR dominant negative mutant form (ATRKd) (Cliby et al., 1998) to HU or γ -rays. BLM phosphorylation was not observed after HU, whereas it was apparently normal after γ -rays exposure (Fig. 6 D). Interestingly, treatment of cells with dose levels of Wortmannin resulting only in the inhibition of DNA-PK activity (Sarkaria et al., 1998, 1999) did not affect the phosphorylation state of BLM either after γ -rays or HU (unpublished data).

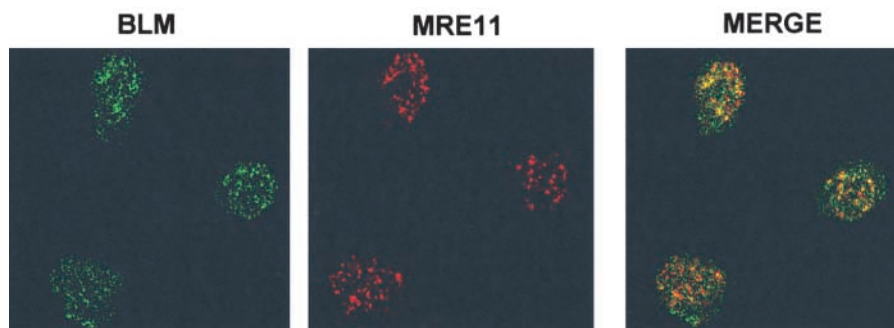
These results suggest that BLM is phosphorylated after replication fork arrest through an ATR-dependent mechanism.

BLM foci after HU are formed independently by phosphorylation

Phosphorylation of BLM by ATM does not seem to be important for its subnuclear assembly, as BLM foci are found in AT cells (Bischof et al., 2001), so we investigated whether phosphorylation was essential for BLM focus-forming activity after HU treatment.

Ionizing radiation and HU-induced BLM relocalization was analysed in normal and AT cells, and in cells expressing a dominant negative inactive form of the ATR kinase. We found that the percentage of nuclei with BLM foci and the number of foci per nucleus increased in ATRKd and AT cells after HU treatment (Fig. 7, A and B). Consistent with previous reports (Bischof et al., 2001), a higher number of BLM-positive nuclei was found in AT cells, also after HU treatment (Fig. 7, A and B). Interestingly, almost all the BLM foci were found in MRE11-positive cells after 4 h HU of treatment with $>50\%$ of foci colocalizing (Fig. 8 A and Table I). These results show that BLM phosphorylation by

Figure 8. BLM foci colocalizes early with the RMN complex after HU-induced replication arrest. Normal, wild-type cells (GM3657) were mock treated or exposed to 2 mM HU for the indicated time, and then processed for double immunofluorescent staining with either MRE11 antibody and BLM polyclonal antiserum. The percentage of cells positive for the indicated double staining was calculated after scoring 50 nuclei for each time point. Images were handled as described in Materials and methods. Nuclei were considered positive for the colocalization if at least one third of the foci showed colocalization as judged by a yellow-orange appearance in the merged image.



ATR after replication fork arrest is not important for its relocalization.

BLM phosphorylation is important for formation of the RMN complex after HU

In order to investigate whether BLM phosphorylation after HU was correlated to the relocalization of the RMN complex at sites of replication fork stall, we examined MRE11 focus-forming activity in ATRKd cells.

We found that overexpression of an inactive dominant negative form of ATR prevented, almost completely, formation of MRE11 nuclear foci after HU treatment (Fig. 9, A and B). MRE11 foci were found at a rather normal level in AT cells (Fig. 9, A and B; unpublished data), in agreement with previously reported data (Maser et al., 1997; Mirzoeva and Petrini, 2001), and in cells overexpressing a wild-type form of ATR (ATRwt). Because ATR function has been reported to mediate multiple checkpoint response (Shiloh, 2001), we test the possibility that the observed impaired relocalization of the RMN complex in ATRKd cells should be a secondary effect of S-phase checkpoint release. Hence, we measured the number of cells in S-phase after treatment with HU in ATRwt and ATRKd cells. We found that overexpression of the ATR inactive form did not largely affect the S-phase arrest imposed by HU (Fig. 9 C), suggesting that this checkpoint response is mainly mediated by ATR/ATM-independent mechanisms as previously reported (Guo et al., 2000). Thus, the observed effect on the assembly of the RMN complex of ATR inhibition is unlikely associated to S-phase checkpoint override.

Our results indicate a positive correlation between that ATR-dependent phosphorylation of BLM and accurate subnuclear relocalization of the RMN complex in response to HU treatment.

Discussion

BLM protein seems to be important in maintaining genomic stability; in fact, BS cells show increased rates of homologous recombination and possibly an aberrant resolution of such events. However, the precise functions of BLM helicase remain to be fully elucidated. BLM protein is found associated with several proteins involved in genome stability, such as RAD51, PML, MLH1, and also BRCA1 and the RMN complex, to form the so-called BASC complex (Wang et al., 2000; Hu et al., 2001; Langland et al., 2001; Wu et al., 2001). The association of BLM with BASC is thought to be important as a surveillance system, in order to recognize anomalous structures in DNA (Wang et al., 2000).

In this study, we have shown for the first time that BLM is required for correct relocalization of the RMN complex at sites of stalled replication fork after HU treatment. In addition, we have provided evidence that cellular localization of the RMN complex is correlated with BLM subnuclear redistribution and its phosphorylation by the ATR kinase. On the contrary, we demonstrated that BLM is not important in the focal localization of the RMN complex after γ -rays, and that BRCA1 relocalization does not necessitate BLM activity. Our findings of an essential role of BLM in loading other factors, such as the RMN complex, at sites of replication fork stall should be consistent with a role of BLM in recognizing abnormal structures at the stalled replication machinery. A similar role has been proposed for the yeast orthologue of BLM, SGS1 (Frei and Gasser, 2000). Accordingly, it has been reported that BLM helicase can bind Holliday junctions (Karow et al., 2000; van Brabant et al., 2000) and other structures that could arise from replication fork blockage (Mohaghegh et al., 2001). In this context, the recruitment of the RMN complex could assist BLM helicase in the resolution of abnormal structures at the stalled repli-

Table I. Colocalization of BLM with RMN or BRCA1 foci after replication arrest

2 mM HU (h)	BLM/RMN-positive nuclei (%of total BLM-positive nuclei)	RMN/BRCA1-positive nuclei (%of total RMN-positive nuclei)	BLM/BRCA1-positive nuclei (%of total RMN-positive nuclei)
2	20	0	0
4	85	13	12
6	24	75	23

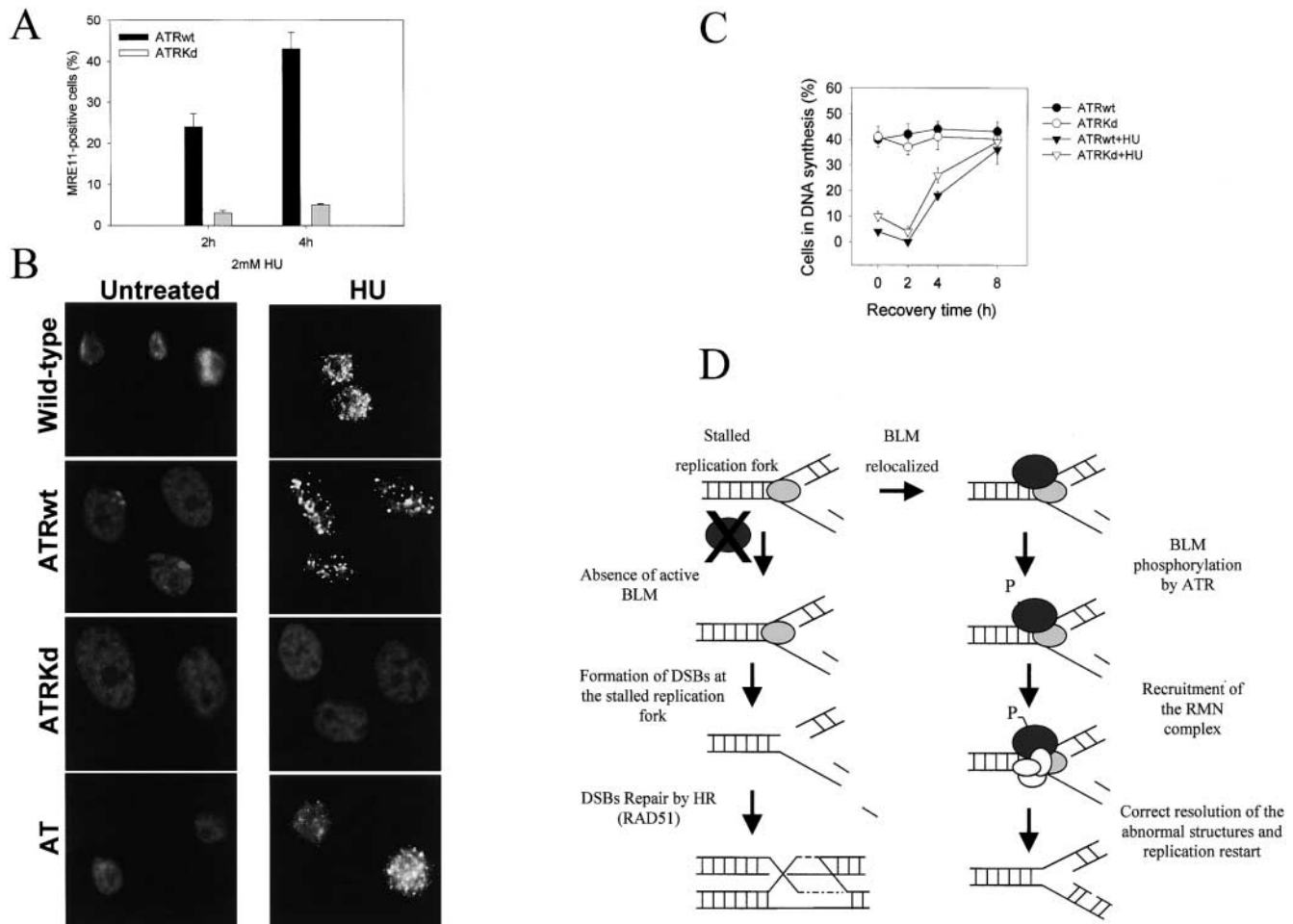


Figure 9. BLM phosphorylation is required to relocalize the RMN complex after replication fork stall. (A) Cells overexpressing the wild-type (ATRwt) or the inactive form (ATRKd) of the ATR kinase were treated with 2 mM HU for the indicated times and then processed for immunofluorescent staining with MRE11 antibody. The percentage of cells displaying MRE11 foci was calculated after scoring 200 nuclei for each time point. Data are the mean \pm the standard deviation of three independent experiments. (B) Representative pattern of MRE11 relocalization after HU in the absence or presence of ATR-mediated BLM phosphorylation. The cells used were the GM3657 (wild-type), the ATRwt, the ATRKd and the GM3189 (AT). MRE11 foci were not detected in cells overexpressing the inactive form of ATR after HU treatment, whereas were observed in AT cells (panel B, third and fourth rows). Images were taken from cells harvested at 4 h after treatment, but similar results were also observed at 2 h. (C) Overexpression of the kinase-inactive form of ATR did not result in abrogation of the HU-imposed S-phase replication checkpoint. Cells overexpressing wild-type (ATRwt) and kinase inactive (ATRKd) form of ATR were mock treated or exposed to 2 mM HU and harvested at the indicated time. Percentage of S-phase cells was determined labeling DNA synthesis by adding BrdUrd in the last hour before harvesting. BrdUrd incorporation was assessed as described in Materials and methods. Similar results as shown for the ATRwt cells were obtained with other cell lines expressing the ATR kinase at a normal level (GM3657 and SNW646). Points represent mean \pm SE from at least three experiments. (D) Proposed model of action of BLM, RMN complex, ATR, and BRCA1 in the resolution of stalled replication forks.

cation forks after their recognition. In fact, the RMN complex itself presents characteristics, such as the exonuclease and endonuclease activities (Haber, 1998), that could be important in resolution of aberrant replication forks. Therefore, MRE11 complex has been reported to be important in dealing with UV-induced replication arrest in some circumstances (Limoli et al., 2000) and, very recently, Maser and colleagues (2001) demonstrated a possible role for the RMN complex during replication. From this point of view, the possible association of a helicase, BLM, with the RMN complex after replication arrest could actually represent, functionally, the mammalian analogue of the bacterial RecBCD complex (Connelly et al., 1998). Bacteria replication restart, after fork stall, requires the RecBCD complex and RecA.

However, in the absence of an active RecBCD complex, replication fork stall is resolved through the action of resolvases, resulting in the formation of double-strand breaks, which trigger homologous recombination (Michel et al., 2001). In fission yeasts, the RecQ helicase, Rqh1, was proposed to remove abnormal structures formed at stalled replication forks in a nonrecombinogenic way, by reversed forks reaction (Doe et al., 2000). In the absence of Rqh1, the reversed fork would be processed only through recombination. Accordingly, the absence of an active BLM could result in an uncorrected resolution of the abnormal structures at the stalled replication fork, which possibly forces cells to use a RAD51-dependent mechanism of replication fork repair, giving rise to genetic instability, rearrangements, and possibly cell

death. Consistent with such possibility, the RAD51 response in BS cells is found to be unusually elevated (Wu et al., 2001), and a higher yield of apoptotic and micronucleated cells are also observed after replication arrest (Fig. 4). These events would result from deregulated recombination, as observed in the *Sgs1* yeast mutants or in cells from Werner's syndrome (WS), which lack the WRN protein (Gangloff et al., 2000; Pichierri et al., 2001). However, in WS cells, apoptotic cell death was specifically observed in cells engaged in recombination, thus resulting in depletion of RAD51-positive nuclei, whereas in BS, such a depletion of RAD51-positive cells is not observed (Wu et al., 2001; unpublished data). Such an observation suggests a role for BLM soon after replication arrest, rather than a role in controlling the correct resolution of RAD51-dependent recombination events. Moreover, WS cells are able to assemble the MRE11 complex in a manner that is indistinguishable from the wild-type, suggesting further that WRN and BLM helicases perform different and nonoverlapping roles in the cells. Furthermore, BLM foci are detected starting at 2 h after replication arrest, whereas RAD51 foci are observed from 6 to 10 h (Pichierri et al., 2001; unpublished data).

Interestingly, the recruitment of the RMN complex, at sites of replication arrest, requires not only BLM focus-forming activity, but also the ATR kinase activity, as RMN complex does not focalize when the ATR kinase is catalytically inactive but BLM is functional. Interestingly, we found BS cells highly sensitive to HU like that expressing inactive form of ATR (Cliby et al., 1998), supporting positively a probable functional interaction between these two proteins. It is noteworthy that an active BLM seems to be unnecessary to the activation of BRCA1, either after γ -rays or HU, even though BRCA1 and BLM helicase are activated by ATR in response to stalled replication (Tibbetts et al., 2000, and this study), and despite the fact that they colocalize after replication arrest (Wang et al., 2000). This actually suggests that BLM and BRCA1 act during different steps of replication fork repair. Further studies are needed to clarify the different roles for these two proteins in the response to replication arrest. However, it has been reported that a functional BRCA1 is not important for the activity of the RMN complex (Wu et al., 2000a), and it seems that BRCA1 inhibits the activity of the RMN complex (Paull et al., 2001).

Taken together, these findings could suggest a model (Fig. 9 D) in which BLM relocates (probably at sites of stalled replication fork, soon after DNA synthesis blockage) and is phosphorylated by ATR. BLM and the RMN complex would promote resolution of the stalled replication fork and rescue of the DNA synthesis in a nonrecombinogenic way. It is possible that BRCA1 can function later to properly resolve replication fork arrest or to repair double-strand breaks formed as a consequence of the absence of either BLM or RMN relocation, possibly cooperating with RAD51. Consistently, we find that BLM focalization already occurs at 2 h after HU, when we observe almost complete inhibition of DNA synthesis, whereas BRCA1 relocation takes place later. Also, the RMN complex is recruited at sites of replication arrest shortly, showing the maximum of colocalization with BLM at 4 h after replication arrest, whereas colocalization with BRCA1 is highest at 6 h. Interestingly,

the peak of BRCA1 and RAD51 colocalization has been reported only at 8–12 h after treatment (Zhong et al., 1999; unpublished data), actually suggesting a later role for this complex in recombination and arguing for our model.

In conclusion, this study establishes a regulatory relationship between BLM, ATR, and the RMN complex in the response to replication fork arrest and collapse raising the possibility that they perform a coordinate action ensuring genomic stability in human cells.

Materials and methods

Cell lines

We used three normal (SNW646, GM3657, and AHH1), two BS (GM09960 and GM03403), and two WS (KO375 and AG14426) EBV-transformed lymphoblasts. SNW646 and KO375 lymphoblasts were a gift of Dr. V.A. Bohr (National Institutes of Health, Baltimore, MD), whereas BS, AG14426, and GM3657 normal lymphoblasts were from Coriell Cell Repositories. EBV-transformed lymphoblasts from two ataxia telangiectasia donors (GM02782 and GM03189) were provided by Dr. F. Rosselli (CNRS, Villejuif, France). Cell lines overexpressing the kinase-dead or wild-type form of ATR kinase (ATRkd and ATRwt, respectively) have been described elsewhere (Cliby et al., 1998). The GM03403 cell line was phenotypically reverted (GM03403BLM) through transfection with a linearized pREP4 plasmid (Invitrogen) containing the wild-type *BLM* cDNA. GM03403 cells contain a frameshift mutation leading to the expression of a truncated BLM protein (Ellis et al., 1995). According to Dutertre et al. (2000), we have not been able to detect this truncated protein by Western blotting. However, we were able to detect wild-type BLM protein in phenotypically reverted cells (Fig. 1 C). Functional complementation in GM03403BLM was verified by analyzing the SCEs levels (Fig. 1, A and B).

All lymphoblastoid cell lines were routinely maintained in exponential growth in RPMI 1640 medium (Life Technologies) supplemented with 10–15% heat-inactivated fetal calf serum (Boehringer Mannheim) by a daily dilution to 3.5×10^5 cells/ml; GM03403BLM cells were grown in the presence of 200 μ g/ml of G418 (Sigma-Aldrich). The ATRwt and ATRkd cells were grown in DME medium (Life Technologies) supplemented with 15% fetal calf serum (Hyclone) in the presence of 400 μ g/ml of G418 (Sigma-Aldrich). Expression of the transgene was achieved by the addition of 1 μ M deoxycycline (Sigma-Aldrich) for 48 h.

Chemicals and treatments

HU (Sigma-Aldrich) was added from a stock solution (200 mM in PBS) and left in the cultures until harvesting. Alternatively, cells were exposed to γ -rays from a ^{60}Co source at a dose rate of 2 Gy/min. Because we did not observe a significantly higher sensitivity of BS cells towards ionizing radiation-induced DNA damage, either in terms of apoptotic cell death or cell survival (unpublished data), we have chosen to use a dose level of 10 Gy, corresponding to \sim 10% of cell survival either in normal or BS cells. This dose level is widely used to investigate relocalization of proteins involved in DNA repair and/or DNA damage signalling. After γ -ray exposure, cells were cultured in complete medium at 37°C until being processed.

Sister chromatid exchanges analysis

GM3657, GM03403, and GM03403BLM cells were grown in the presence of 3 μ g/ml BrdUrd and metaphase cells collected after 48 h by adding colcemid (0.2 μ g/ml) in the last 4 h. Slides prepared according to standard techniques were stained by 10 μ g/ml Hoechst for 20 min to visualize SCEs. 50 metaphase cells for each experimental point were scored.

Evaluation of the apoptotic response

Apoptotic and abnormal nuclear morphology was determined as previously described (Pichierri et al., 2001). Apoptosis was also visualized using the TdT-mediated end-labelling assay using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) according to the procedure indicated by the manufacturer for analysis on slides. Results obtained with the two different methods were similar.

Immunofluorescence

Cells harvested at the indicated time were spread onto poly-L-lysine-coated slides and fixed as previously described (Pichierri et al., 2001). Staining with rabbit polyclonal anti-BLM (Novus BioLabs), rabbit poly-

clonal anti-MRE11 (Novus BioLabs), mouse monoclonal anti-MRE11 (GenTex), mouse monoclonal anti-RAD50 (GenTex), rabbit polyclonal anti-NBS1 (Novus BioLabs), or mouse monoclonal anti-BRCA1 (Oncogene Research) was performed overnight at 4°C in PBS/1% BSA, whereas specie-specific fluorescein- or Texas red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were applied for 1 h at room temperature, followed by counterstaining for 5 min at room temperature with 0.5 µg/ml DAPI. All the primary antibodies were used at a 1:300 dilution, whereas the secondary antibodies were employed at a 1:500 dilution. Slides were mounted in Vectashield (Vector Labs) and analyzed by a Zeiss fluorescence confocal microscopy or through a Leica epifluorescence microscope equipped with a CCD camera. Images were acquired as greyscale files using the Metaview software, and then pseudocolored and merged using Adobe Photoshop. For each time point, at least 200 nuclei were examined, and BLM, BRCA1, or MRE11-NBS1 foci were scored at a 100× magnification using the epifluorescence microscope. Only nuclei showing >5 foci were considered positive. Parallel samples, incubated either with the appropriate normal serum or only with the secondary antibody, confirmed that the observed fluorescence pattern was not attributable to artefacts.

Recovery of DNA synthesis after HU treatment

In order to verify whether BS cells properly arrest DNA synthesis after HU treatment, cells were exposed to 2 mM HU and harvested in drug-free medium for different time points. BrdUrd (30 µg/ml) was added 1 h before harvesting, and BrdUrd incorporation was evaluated as previously described (Franchitto et al., 1998). At least 500 interphase cells were scored to evaluate the percentage of labeled nuclei. Only nuclei displaying a more or less uniform BrdUrd labeling in the entire volume were considered as actively replicating.

The percentage of cells undergoing DNA synthesis at each time point was calculated as fraction of the treated cells versus untreated controls.

Immunoprecipitation and Western blot analysis

Cells (10⁷) were collected by low-speed centrifugation, washed in PBS, and lysed in standard RIPA buffer (PBS, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, 10 µg/ml Aprotinin, 10 µg/ml PMSF, 1 mM Na orthovanadate, and 1 mM NaF). Cell lysates (20 µg) were resolved by SDS-PAGE and transferred to nitrocellulose (PROTRAN; Schleicher & Schuell). Equal loading and transfer was monitored by Ponceau red staining of the membrane. Blots were separately incubated overnight at 4°C with primary antibodies against NBS1 (1:2,000), MRE11 (1:2,000), BLM (1:1,000), or BRCA1 (1:100). Horseradish peroxidase-conjugated goat specie-specific secondary antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:1,000, and visualization of the signal was accomplished using ECL plus (Amersham Pharmacia Biotech).

In immunoprecipitation experiments, cells (15 × 10⁷) were lysed in a mild lysis buffer (50 mM Tris/HCl, pH 8, 300 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 25 mM β-glycerophosphate, and 0.5% Igepal-40), including protease and phosphatase inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml PMSF, 1 mM Na orthovanadate, and 1 mM NaF). 300 µg of lysate were precleared with Sepharose protein A/G beads, and then incubated overnight at 4°C with anti-BLM antibodies and Sepharose protein A/G beads. After extensive washing in mild lysis buffer, BLM-containing immunoprecipitates were boiled in electrophoresis sample buffer and analyzed by immunoblotting. For phosphatase treatments, immunoprecipitates were resuspended in phosphatase buffer and incubated for 30 min with 300 units of lambda phosphatase (New England BioLabs) at 30°C, and then boiled in electrophoresis sample buffer prior to Western blotting analysis.

³²P in vivo labeling

In vivo labeling experiments used exponentially growing wild-type cells (SNW646). The cells were washed with phosphate-free RPMI1640 medium, supplemented with 10% heat-inactivated fetal calf serum, and incubated for 30 min at 37°C in the same media. [³²P]-orthophosphate (Amersham Pharmacia Biotech) was added directly to the medium (0.6 mCi/ml). After a 30-min incubation at 37°C, cells were either mock treated or exposed to 2 mM HU for different time periods. The cells were lysed as described above, and BLM was immunoprecipitated using 3 µl rabbit anti-BLM antiserum (Novus Biologicals). The immunoprecipitated proteins were resolved by SDS-PAGE, and analyzed by autoradiography and Western blotting.

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