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; BrdU, 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, TOPOIIIα, 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 arrest or ionizing radiation-induced DNA damage (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).

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 re-distributes 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 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 ± 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 ± 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 ± 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 ± SE from at least three experiments.
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
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 replication 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 8 h (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
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.

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 point. Data are the mean ± 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 ± 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.

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
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 ATRK\textsubscript{d} 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 (ATR\textsubscript{wt}). 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 ATRK\textsubscript{d} 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 ATR\textsubscript{wt} and ATRK\textsubscript{d} 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 \( \gamma \)-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-

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**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          | 0                                                        | 0                                                        | 0                                                        |
| 4          | 85                                                       | 13                                                       | 12                                                       |
| 6          | 24                                                       | 75                                                       | 23                                                       |

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.
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 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 a specific functional interaction between these two proteins. Consistently, we find that BLM focalization already occurs at 2 h 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).

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boiled in electrophoresis sample buffer prior to Western blotting analysis. Resuspended in phosphatase buffer and incubated for 30 min with 300 noprecipitates were boiled in electrophoresis sample buffer and analyzed lysate were precleared with Sepharose protein A/G beads, and then 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 (10^7) 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 orthovana- date, 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 the appropriate normal serum or only with the secondary antibody, confirmed that the observed fluorescence pattern was not attributable to artefacts.

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