The Dispersal of Replication Proteins after Etoposide Treatment Requires the Cooperation of Nbs1 with the Ataxia Telangiectasia Rad3-Related/Chk1 Pathway

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Abstract

In mammalian cells, DNA replication takes place in functional subnuclear compartments, called replication factories, where replicative factors accumulate. The distribution pattern of replication factories is diagnostic of the different moments (early, mid, and late) of the S phase. This dynamic organization is affected by different agents that induce cell cycle checkpoint activation via DNA damage or stalling of replication forks. Here, we explore the cell response to etoposide, an anticancer drug belonging to the topoisomerase II poisons. Etoposide does not induce an immediate block of DNA synthesis and progressively affects the distribution of replication proteins in S phase. First, it triggers the formation of large nuclear foci that contain the single-strand DNA binding protein replication protein A (RPA), suggesting that lesions produced by the drug are processed into extended single-stranded regions. These RPA foci colocalize with DNA replicated at the beginning of the treatment. Etoposide also triggers the dispersal of replicative proteins, proliferating cell nuclear antigen and DNA ligase I, from replication factories. This event requires the activity of the ataxia telangiectasia Rad3-related (ATR) checkpoint kinase. By comparing the effect of the drug in cell lines defective in different DNA repair and checkpoint pathways, we show that, along with the activity of the ataxia telangiectasia Rad3-related (ATR; ref. 4). These kinases modulate the activity of a variety of targets involved in many cellular functions, including DNA synthesis, cell cycle, DNA damage repair, and chromatin remodeling. Prominent among the substrates of the apical ATM and ATR checkpoint kinases are the transducing kinases Chk1 and Chk2, which are responsible for spreading the alert signal (5, 6).

Different checkpoints, aimed at reducing the rate of DNA synthesis after DNA damage, operate during S phase. The replication checkpoint is initiated when replication forks stall, as in the case of inhibition of DNA polymerases, or as a consequence of the collision of replication forks with damaged DNA and/or aberrant DNA structures. This checkpoint inhibits initiation of DNA replication from hitherto unfired origins. Moreover, it preserves the integrity of the machinery operating at replication forks, thus allowing the recovery of DNA synthesis after DNA repair. An independent checkpoint, referred to as the intra-S-phase checkpoint, is activated by double-strand breaks. This checkpoint requires ATM and ATR activities (7). Nbs1 protein is the product of the gene mutated in Nijmegen breakage syndrome (NBS) patients and is a core component of the Mre11/Rad50/Nbs1 complex critical for detection, processing, and repair of double-strand breaks. It has been recently reported that Nbs1 protein participates in the cell cycle checkpoint response to double-strand breaks being involved in the activation of both ATM- and ATR-dependent pathways (8, 9).

In mammalian cells, the timing of origin firing can be examined by monitoring the dynamics of replication factories (i.e., the nuclear districts where DNA replication takes place; ref. 10). Replication factories are composed of groups of coordinately replicated chromosomal domains (11) and their subnuclear distribution reflects the replication of different portions of the genome identifying the different moments of S phase (from early to late; ref. 12). A large number of proteins involved in DNA replication, repair and recombination, in cell cycle regulation and checkpoint signaling are recruited to the replication factories, underscoring the relevance of these functional domains in the integration and coordination of DNA replication with DNA repair.
and cell cycle progression (13). The recruitment to replication factories of several enzymes, including DNA ligase I, is directed by a conserved proliferating cell nuclear antigen (PCNA)–binding motif (14) and is controlled by cell cycle–dependent phosphorylation (15, 16). The ordered assembly and disassembly of replication factories are monitored by the S-phase checkpoints. Indeed, the activation of replication checkpoint by stalled forks leads to the stabilization of replication factories (17), which are “frozen” until replication forks resume through an ATR-Chk1–dependent pathway (18). On the contrary, the anticancer drug etoposide strongly affects the functional organization of S-phase nuclei, leading to the disassembly of replication factories, redistribution of replicative factors DNA ligase I, PCNA, and replication protein A (RPA), and formation of DNA repair foci. We have previously suggested that these events are under the control of an S-phase checkpoint (19).

Etoposide is an effective agent in cancer therapy that specifically inhibits topoisomerase II (Topo II; ref. 20). It acts after cleavage of DNA by Topo II, leaving a lesion in which the enzyme is covalently linked to 5’-ends whereas 3’ termini are free. These lesions likely develop into double-strand breaks and single-strand DNA gaps. As a consequence, etoposide triggers checkpoint activation (21). It has been shown that in Xenopus egg extracts, etoposide inhibits origin firing through ATR (22).

Here, we explore the effect of etoposide on checkpoint activation and on the concomitant dispersal of replicative proteins PCNA and DNA ligase I from replication factories in human cells. We show that the etoposide-induced redistribution of replicative proteins requires the activity of the ATR kinase and involves both Nbs1 and Chk1 proteins.

Materials and Methods

**Drugs, cell lines, and cell treatments.** AT1 BR (European Collection of Cell Cultures no. BM0020) and HeLa cells were grown as monolayers in DMEM (Sigma, St. Louis, MO) supplemented with 10% FCS, 4 mmol/L glutamine, and 50 μg/ml gentamicin (all from Sigma). GM847 and GM847/ATRkd cell lines were kindly provided by Dr. Shlomo Handeli (Fred Hutchinson Cancer Research Center, Seattle, WA). GM847/ATRkd cells contain a kinase-inactive allele of ATR (ATRkd) under the positive control of a doxycycline-responsive promoter (23) and were cultured in complete DMEM medium with the addition of 400 μg/ml G418 (Sigma). Induction of ATRkd was accomplished by supplementing the growth medium with 2 μg/ml doxycycline (Sigma) for 48 to 72 hours (see Supplementary Fig. S1). M09J cell lines (ATCC CRL-2366) were grown in a 1:1 mixture of DMEM and Ham’s F12 (Sigma) media supplemented with 2.5 mmol/L L-glutamine and 10% FCS. GM15989 fibroblasts (Coriell Institute, Camden, NJ) mutated in Nbs1 gene were cultured according to the instructions of the supplier. ATLD2 fibroblasts defective in Mre11 protein (24) were kindly supplied by Prof. Yosef Shiloh (University of Tel Aviv, Tel Aviv, Israel). ATLD2 cells were grown on complete DMEM supplemented with 20% heat-inactivated FCS. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO₂. Cells were treated with etoposide (Sigma), ICRF-193 (NBS Biologicals, Huntingdon, United Kingdom), 7-hydroxyx斯塔uorosporine (UCN-01; kind gift from Prof. David M. Gilbert, Upstate Medical University, Syracuse, NY), caffeine (Sigma), and bleomycin (Sigma) for the time periods and the concentrations indicated in the text.

**Immunofluorescence.** Cells grown on coverslip were rinsed with cold PBS and fixed for 4 minutes in cold methanol as previously described (19). The following primary antibodies were used for detection of protein antigens: H18 monoclonal antibody (mAb) to RPA2 (NeoMarkers, Fremont, CA); PC10 mAb to PCNA (Santa Cruz Biotechnology, Santa Cruz, CA); mouse 5H5 mAb and rabbit polyclonal antibodies to DNA ligase I (developed in collaboration with Areta International, Gerenzano, Italy); Alexa Fluor 488–conjugated anti-Brdu mAb (clone PRB-1, Molecular Probes, Eugene, OR); anti-BrdUrd mAb (clone BMC9318, Chemicon, Temecula, CA); anti–phospho-histone H2A.X (Ser139) mAb (clone JBW301, Upstate, Charlottesville, VA); and anti-FEN1 polyclonal antibody (kindly supplied by Dr. Emma Warbrick, University of Dundee, Dundee, United Kingdom). The secondary antibodies used were TRITC-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were stained with 0.1 μg/ml 4,6–diamidino-2-phenylindole (DAPI; Sigma). To detect sites of replicative DNA synthesis, cells were grown in 100 μmol/L BrdUrd (Sigma) for 5 minutes before fixation and processed as previously described (25). Conventional epifluorescence microscopy was done with Optical Microscope Olympus IX71 equipped with a 63× objective. Photomicrographs were taken with digital camera Cool SNAP ES (Photometrics, Tucson, AZ). Data acquisition was done using the MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Confocal microscopy was done with a Leica TCS SP2 confocal laser microscopy apparatus equipped with a 63×/NA 1.32 oil immersion objective. We have used the 488-nm laser line.

**Figure 1.** The dispersal of replication factories in response to different drugs. Exponentially growing HeLa cells were treated with the indicated drugs and stained with anti–DNA ligase I (LigI) polyclonal antibody and with TRITC-conjugated goat anti-rabbit IgG secondary antibody (left). Nuclei were counterstained with DAPI (right). Untreated control cells (A) and cells grown for 3 hours in 100 μmol/L etoposide (B), 100 μmol/L ICRF-193 (C), and 100 μg/ml bleomycin (D).

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for excitation of FITC (detected at 500 nm < λ<sub>FITC</sub> < 540 nm) and the 543-nm laser line for the TRITC fluorescence (detected at λ > 570 nm). Images were exported to Adobe Photoshop (Adobe, San Jose, CA).

**Cell lysate, chromatin isolation, and Western blotting.** To prepare cell lysate, cells were harvested by centrifugation (5 minutes, 1,300 × g, 4°C), resuspended in Laemmli buffer, and boiled for 10 minutes. Enriched chromatin fraction was prepared as previously described (26) and analyzed by Western blotting. When required, the membrane (Hybond-ECL, Amersham Biosciences) was stripped according to the instructions of the manufacturer. The following primary mouse mAbs were used for detection of protein antigens: anti-RPA2 (9H8); anti-PCNA (PC10) and anti-Chk1 (clone G-4, Santa Cruz Biotechnology); anti-Flag (M2) and anti-a-tubulin (Sigma); anti–phospho-histone H2A.X (Ser139; Upstate); anti-Chk2 (clone 7, Upstate); and anti–phospho-Chk2 (clone MS110, Oncogene, La Jolla, CA). The following rabbit polyclonal antibodies were used: anti–DNA ligase I (Areta International); anti-Orc2 (kindly supplied by Dr. Bruce Stillman, CSH Laboratory, Cold Spring Harbor, NY); anti–FEN1 (kindly supplied by Dr. Emma Warbrick); anti-SMC1 and anti-SMC1pSer966 (Bethyl Laboratories, Montgomery, TX); anti–Rad9 (Santa Cruz Biotechnology); anti–Mre11 (Abcam, Cambridge, United Kingdom); and anti–Chk2pThr68 and anti–Chk1pSer345 (Cell Signaling Technology, Danvers, MA). ATR protein was detected with goat polyclonal anti-ATR antibody (Santa Cruz Biotechnology). Primary antibodies were revealed with horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit antibodies or donkey anti-goat antibodies and enhanced chemiluminescence (ECL) systems [Super Signal West Dura Extended, Super Signal West Pico (Pierce, Rockford, IL) and ECL (Amersham Biosciences)].

**Results**

**Etoposide affects the distribution of replication proteins in S phase.** We have previously shown that treatment of HeLa cells with the anticancer drug etoposide results in the progressive reduction of the fraction of cells in which replicative proteins (PCNA and DNA ligase I) are associated with mid and late S-phase replication factories (ref. 19; Fig. 1A and B). This redistribution seems to be specifically induced by etoposide because it does not occur after treatment with other stress agents, such as UV irradiation, or inhibitors of DNA synthesis, such as aphidicolin and hydroxyurea (ref. 19 and data not shown).

To understand whether or not redistribution of replicative proteins triggered by etoposide was due to topological constraints or to the formation of double-strand breaks, we tested the effect of ICRF-193 and bleomycin. ICRF-193 is a specific inhibitor of Topo II activity, which prevents the formation of the cleavable complex. This drug affects the topology of DNA without inducing double-strand breaks and leads to metaphase arrest (27). Although a 3-hour incubation with ICRF-193 induced checkpoint activation (Supplementary Fig. S1A), it did not affect the distribution of DNA ligase I (Fig. 1C) or PCNA (not shown). Bleomycin is a radiomimetic agent that induces double-strand breaks detectable by phosphorylation of the histone variant H2AX (Supplementary Fig. S1A). As shown in Fig. 1D, this drug also did not prevent the association of DNA ligase I (Fig. 1D) and PCNA (not shown) with mid/late replication factories.

Thus, the signal that triggers the dispersal of replication proteins from replication factories is not the mere inhibition of Topo II activity or the induction of double-strand breaks but is somehow related to the formation of a covalent Topo II-DNA complex induced by etoposide.
Etoposide-induced RPA2 foci colocalize with replicated chromatin. We have previously reported that 1-hour incubation of HeLa cells in 100 μmol/L etoposide induces the formation of foci of p34 subunit (RPA2) of RPA, which do not colocalize with replication factories revealed as sites of BrdUrd incorporation or stained by antibodies against replicative proteins (PCNA and DNA ligase I). Notably, RPA2 foci are usually adjacent to sites of residual DNA synthesis detected with a BrdUrd pulse (19). We wondered whether this spatial organization reflected preferential formation of RPA2 foci on postreplicative chromatin. To test this hypothesis, HeLa cells were pulse labeled with BrdUrd immediately before incubation with etoposide (T0). After 1 hour, cells were fixed (T1) and co-stained with an anti-BrdUrd antibody, to detect replicated DNA, and with an antibody specific for the RPA2 protein. As shown in Fig. 2A, RPA2 foci colocalized with DNA synthesized at T0 (i.e., with the portions of the genome that were replicating when etoposide was added to the medium). This is compatible with the idea that RPA2 foci are involved in the repair of DNA damage on replicated DNA. Remarkably, RPA foci did not form on treatment with ICRF-193 and bleomycin (Fig. 2B), suggesting that these drugs do not induce the formation of extended single-strand DNA regions.

ATR kinase is required for the etoposide-induced redistribution of replication proteins. Staurosporin prevents the redistribution of replicative enzymes in response to etoposide, suggesting the involvement of checkpoint kinases (19). To identify the kinase involved, we studied the effect of etoposide in three cell lines with defects in the DNA-damage checkpoint response: (a) AT1 cells lacking ATM kinase activity; (b) human glioma cells MO59J in which DNA-dependent protein kinase is down-regulated (28); and (c) GM847/ATRkd cells that overexpress a dominant-negative form of ATR under the control of a promoter inducible with doxycycline (23). We determined the percentage of nuclei displaying DNA ligase I (and PCNA) in mid/late S-phase patterns in untreated and stressed cells. Consistent with our previous results (19), after 3 hours of growth in etoposide-containing medium, the fraction of nuclei with DNA ligase I (and PCNA) in mid/late S-phase patterns was reduced to <10% of the control values in HeLa cells, GM847 control fibroblasts, and GM847/ATRkd cells grown without doxycycline. The same reduction occurred in AT1 and MO59J cells, indicating that ATM and DNA-dependent protein kinase are dispensable. On the contrary, dispersal of replicative enzymes was abrogated by caffeine (5 mmol/L), a checkpoint inhibitor, and drastically affected in GM847/ATRkd cells grown for 48 hours in doxycycline-containing medium. Under these conditions, the fraction of nuclei with mid/late patterns was >90% of that observed in the untreated cells (Fig. 3A and B). Notably, expression of ATRkd did not inhibit the formation of RPA2 foci (not shown).

To further investigate the effect of etoposide, we checked whether the drug could affect binding of replicative proteins to chromatin. As shown in Fig. 3C and D, both in HeLa cells and in GM847 control fibroblasts, PCNA and DNA ligase I dissociated from chromatin after etoposide treatment. This dissociation paralleled the redistribution observed in immunofluorescence. On the contrary, RPA2 was still bound to chromatin but in a hyperphosphorylated form. Unexpectedly, FEN1, another PCNA-interacting protein that relocates after etoposide treatment (Supplementary Fig. S2), was still found in the chromatin fraction. This could reflect a switch from replication to DNA repair complexes as recently reported (29). Finally, etoposide did
not perturb the behavior of Orc2. In agreement with the immunofluorescence analysis (Fig. 3B), all these effects required the ATR function and were abrogated in ATRkd-expressing cells. Interestingly, the dissociation of replication factors was accompanied by an increased interaction of ATR with chromatin (Fig. 4B). Collectively, these results indicate a relationship between the subnuclear distribution of replication factors during S phase and their association with chromatin.

Dissecting the etoposide response. Several players acting in the checkpoint-mediated DNA-damage response have been described in the last few years (for a review, see refs. 7, 30). They include phosphorylation of histone variant H2AX (γH2AX) and assembly of γH2AX foci on double-strand breaks; phosphorylation of sensor proteins, such as members of the PCNA/RFC like complex (Rad9); phosphorylation of the Mre11 subunit of Mre11/Rad50/Nbs1 complex; phosphorylation of mediators/adaptors like BRCA1 and SMC1; and finally, activation of the effectors Chk1 and Chk2 kinases. We investigated by Western blotting the phosphorylation pattern of all these proteins after etoposide treatment in normal fibroblasts and in ATRkd-expressing cells. As shown in Fig. 4, all the proteins listed above are phosphorylated after etoposide treatment in normal fibroblasts and in ATRkd-expressing cells. As shown in Fig. 4, all the proteins listed above are phosphorylated after etoposide treatment of GM847 control fibroblasts. Phosphorylation of H2AX, Rad9, BRCA1, and Chk2 proteins (Fig. 4A and B), as well as formation of γH2AX foci (not shown) and binding of Rad9 and BRCA1 to chromatin (Fig. 4B), occurred also in ATRkd-expressing cells. On the contrary, ATRkd severely reduced phosphorylation of Chk1 (Fig. 4A), in agreement with the notion that ATR is the main activator of Chk1 kinase (21). Finally, expression of ATRkd strongly reduced phosphorylation of Mre11 and SMC1 (Fig. 4A).

This analysis suggests the involvement of the Chk1 and/or Mre11/Rad50/Nbs1-SMC1 pathways (7) in the ATR-dependent dispersal of replicative factors triggered by etoposide.

Involvement of Nbs1 and Chk1 in the etoposide-induced redistribution of PCNA. To assess the relevance of the Mre11/Rad50/Nbs1 complex in the redistribution of PCNA, we compared the effect of etoposide in GM847 control fibroblasts, GM5989 fibroblasts mutated in the NBS gene (Nbs1), and ATLD2 cells defective for the expression of Mre11. As shown in Fig. 5A, comparable redistribution of PCNA after etoposide was observed in GM847 control fibroblasts and ATLD2 cells, arguing against an involvement of Mre11 in this phenomenon. On the contrary, dispersal of PCNA was reduced but not completely abrogated in NBS cells where the frequency of mid/late S-phase patterns was still ~60% of the value observed in untreated cells. The relevance of Nbs1 in the etoposide response is indicated also by the Western blot analysis in Fig. 5B. Similarly to ATRkd-expressing cells, no phosphorylation of Mre11 protein was detectable in NBS cells after etoposide treatment. Moreover, the level of RPA2 and Chk1 phosphorylation was drastically reduced whereas phosphorylation of Chk2 and H2AX was not perturbed. Collectively, these results indicate that Nbs1 is required for the full activity of the ATR-mediated response to etoposide. Notably, a difference exists between the two cell lines on SMC1 cohesin protein, which is phosphorylated in NBS cells but not in ATRkd-expressing cells.
To understand whether or not Chk1 was also involved in the dispersal of PCNA, we investigated the effect of the Chk1 inhibitor UCN-01 (31). As shown in Fig. 5A, UCN-01 prevented the effect of etoposide in GM847 control fibroblasts and ATLD2 cells. This effect was even more dramatic in NBS fibroblasts where the percentage of cells with mid/late S-phase patterns was 140% of the control value (untreated cells). This analysis indicates that Chk1 and Nbs1 contribute to the redistribution of replicative proteins triggered by etoposide acting through the ATR pathway. However, as indicated by the behavior of NBS cells, they probably cover complementary aspects because their contemporary inhibition increases the fraction of cells with replication proteins in mid/late S-phase patterns.

**Involvement of Nbs1 and Chk1 in the effect of etoposide on BrdUrd incorporation.** We asked whether the redistribution of PCNA occurring after etoposide was accompanied by a reduction of the fraction of cells displaying replication foci. Therefore, cells either untreated or grown in the presence of etoposide, with or without UCN-01, were labeled with a short BrdUrd pulse, which could not detect sites of DNA repair. Cells were then scored for the presence of replication foci. Consistent with the analysis of replication factories in Figs. 3 and 5, etoposide drastically reduced incorporation of BrdUrd in control GM847 fibroblasts and ATLD2 cells, and ATRkd cells grown in the absence of doxycycline. In contrast, no effect on replication foci was detectable in cells expressing the ATRkd mutant and only a moderate reduction was observed in NBS fibroblasts (Fig. 6A). This effect of etoposide was reverted on inhibition of Chk1. A difference was instead observed in NBS cells both on etoposide sensitivity and the recovery of replication foci with UCN-01. Indeed, mid/late foci were more sensitive to etoposide than early foci (41% versus 78% of the control value, respectively). We interpreted this result as an indication that Nbs1 had a central role in the inhibition of early-S-phase foci after etoposide treatment. On the contrary, inhibition of DNA synthesis during mid/late S phase required also some other pathway. In accordance with this hypothesis, we found that Chk1 inhibition in NBS fibroblasts drastically increased the fraction of cells with mid/late foci without affecting early patterns. Remarkably, the fraction of cells with mid/late foci in the presence of etoposide and UCN-01 was identical to the fraction of mid/late replication factories measured independently (i.e., 141% and 144% of the control value, respectively; Figs. 5A and 6B). It is worth noticing that both in GM847 and in NBS fibroblasts, UCN-01 by itself does not increase the fraction of cells with mid/late S-phase patterns revealed by BrdUrd labeling (Fig. 6A).

**Discussion**

In this article, we have investigated the role of the intra-S-phase checkpoint pathway in the dispersal of replicative proteins, PCNA and DNA ligase I, from mid/late replication patterns after etoposide treatment. We propose that this redistribution is directly triggered by the processing of the Topo II-DNA covalent complex (ultimately inducing single-strand DNA) rather than by topological constraints or double-strand breaks originating thereafter. This...
interpretation is supported by several observations. Indeed, replication patterns are not perturbed by ICRF-193, a Topo II inhibitor that affects DNA topology without introducing DNA breaks, or by bleomycin, a radiomimetic drug that produces double-strand breaks and activates the intra-S-phase checkpoint. Contrary to etoposide, bleomycin and ICRF-193 do not induce the massive production of single-strand DNA as shown by the absence of RPA2 foci (Fig. 2) and RPA2 phosphorylation (Supplementary Fig. S1A). Interestingly, dispersal of PCNA from replication patterns is also induced by camptothecin (10 μmol/L ref. 32),1 a poison that stabilizes the covalent Topo I-DNA complex. Whether the processing of covalent protein-DNA complexes could elicit a signal transduction cascade aimed at inhibiting progression through S phase is an open and intriguing possibility.

Our data indicate that etoposide does not lead to a complete and immediate block of DNA synthesis as replication foci can still be labeled with BrdUrd, although to a reduced extent, in the presence of the drug (Fig. 6; ref. 19). This could be explained if Topo II-DNA complexes frozen by etoposide mainly form behind the replication fork as previously reported by others (33). Consistent with this interpretation, we have found that before inducing the dispersal of PCNA, etoposide triggers the formation of RPA2 foci colocalizing with DNA replicated at the beginning of the treatment (see Fig. 2A and ref. 19). Thus, etoposide would drastically differ from aphidicolin and UV irradiation that cause the stalling of the replication forks and block the elongation phase of DNA synthesis.

ATR, Chk1, and Nbs1 are required for the etoposide-induced dispersal of replication proteins. Our analysis shows that the etoposide-induced redistribution of replicative proteins during the S phase requires the ATR function. Other checkpoint pathways, identified by apical ATM and DNA-dependent protein kinases, do not appreciably contribute to this phenomenon although ATM is probably activated as indicated by phosphorylation of Chk2 kinase (see Fig. 4A). This is not the first evidence that inhibition of DNA replication by etoposide relies on the ATR pathway. Indeed, in in vitro assays with Xenopus egg extracts, etoposide was shown to prevent firing of DNA replication origins through ATR activation (22). However, this is the first time that etoposide-induced activation of ATR kinase has been shown to affect the dynamics of the nuclear districts involved in the replication process. Etoposide-induced redistribution of PCNA is intriguing because the activation of ATR-dependent checkpoint by agents that stall the replication forks is known to stabilize the replication factories (17). We think that the type of damage, its distribution relative to the moving fork, and the mechanism involved in the DNA damage recognition could explain this apparent paradox and determine the choice between stabilization and dispersal of replication proteins in S phase.

Two proteins, Nbs1 and Chk1, cooperate with ATR for the dispersal of PCNA and DNA ligase I. Nbs1, a subunit of the Mre11/Rad50/Nbs1 complex, probably plays a major role in the initial steps of the response because it is critical for the ATR-mediated phosphorylation of Mre11, RPA2, and Chk1. It is unclear whether the residual phosphorylation of Chk1 and RPA2 detectable in NBS fibroblasts is due to the leakiness of the Nbs1 mutation (34) or to the existence of a compensatory factor. It has been recently proposed that Nbs1 is required for ATR-dependent phosphorylation events on stalling of the replication fork (9). Our data extend the involvement of Nbs1 to the response to etoposide. Interestingly, mutation of Mre11, another subunit of the Mre11/Rad50/Nbs1 complex, does not affect the induction of the ATR pathway by etoposide. This could suggest a selective involvement of the Mre11/Rad50/Nbs1 complex in the ATR-dependent signaling. Alternatively, the difference between NBS and ATLD2 cells could reflect different levels of partially functional Mre11/Rad50/Nbs1 complex and/or the fact that the Nbs1 mutation perturbs the subcellular distribution of Mre11 protein (35).

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1 R. Rossi and A. Montecucco, unpublished observations.
Different sensitivity of early and mid/late replicons to etoposide. Our analysis suggests a different involvement of Nbs1 and Chk1 activities in the etoposide-induced inhibition of early and mid/late replication foci. In control GM847 fibroblasts, the almost complete inhibition of both early and mid/late BrdUrd foci by etoposide requires Chk1. In NBS cells, the effect of the drug on DNA synthesis is strongly reduced and the frequency of cells with early and mid/late BrdUrd foci after etoposide treatment is still 80% and 40% of the control value, respectively. Interestingly, Chk1 inhibition with UCN-01 does not affect the frequency of early foci in etoposide-treated NBS cells whereas the fraction of mid/late foci increases to 140% of the control value. This could indicate that Nbs1 and Chk1 control early BrdUrd foci acting in the same pathway whereas they exert largely independent and probably parallel control mechanisms in the case of mid/late foci. The molecular mechanisms underlying this difference are still to be clarified. However, it is plausible that the contemporary lack of Chk1 and Nbs1 activities underlying this difference are still to be clarified. However, it is plausible that the contemporary lack of Chk1 and Nbs1 activities

On the basis of the available data, we propose a model for the involvement of Nbs1 and Chk1 in the response to etoposide. According to this model, processing of Topo II-DNA complexes formed behind the replication fork would promote the assembly of RPA2 foci on replicated chromatid. Nbs1, probably associated with RPA2 foci (9), would assist phosphorylation of Mre11, RPA2, and Chk1 by ATR. Consistent with this, Nbs1 and RPA2 foci colocalize in a fraction of cells after etoposide treatment (36). Chk1 would act in trans to prevent firing of mid/late origins (18) and recruitment of replicative factors to late replication factories leading to the redistribution of PCNA and DNA ligase I throughout the nuclear volume and to their dissociation from chromatin. This, however, does not account for the effect of etoposide on early replication foci, of which the inhibition cannot be triggered by DNA damages occurring in previously activated replicons. Interestingly, the etoposide-induced inhibition of these foci is severely compromised in NBS cells. We speculate that the signal relevant for inhibition of early foci could be a Topo II-DNA complex formed in proximity of DNA replication origins activated at the onset of the S phase. A Nbs1-dependent checkpoint pathway could be involved in preventing origin firing by a cis-acting mechanism (7). Although several points remain to be proved, a partial support to this model is provided by the observation that a Topo II-DNA complex maps adjacent to the Lamin B2 origin, which is activated in the first minute of the S phase (37).

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