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NBS1 Mediates ATR-Dependent RPA Hyperphosphorylation Following Replication-Fork Stall and Collapse

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Summary

Post-translational phosphorylation of proteins provides a mechanism for cells to switch on or off many diverse processes, including responses to replication stress. Replication-stress-induced phosphorylation enables the rapid activation of numerous proteins involved in DNA replication, DNA repair and cell cycle checkpoints, including replication protein A (RPA). Here, we report that hydroxyurea (HU)-induced RPA phosphorylation requires both NBS1 (NBN) and NBS1 phosphorylation. Transfection of both phosphospecific and non-phosphospecific anti-NBS1 antibodies blocked hyperphosphorylation of RPA in HeLa cells. Nijmegen breakage syndrome (NBS) cells stably transfected with an empty vector or with S343A-NBS1 or S278A/S343A phospho-mutants were unable to hyperphosphorylate RPA in DNA-damage-associated foci following HU treatment. The stable transfection of fully functional NBS1 in NBS cells restored RPA hyperphosphorylation. Retention of ATR on chromatin in both NBS cells and in NBS cells expressing S278A/S343A NBS1 mutants decreased after DNA damage, suggesting that ATR is the kinase responsible for RPA phosphorylation. The importance of RPA hyperphosphorylation is demonstrated by the ability of cells expressing a phospho-mutant form of RPA32 (RPA2) to suppress and delay HU-induced apoptosis. Our findings suggest that RPA hyperphosphorylation requires NBS1 and is important for the cellular response to DNA damage.

Supplementary material available online at
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Key words: Phosphorylation, DNA-damage response, DNA repair, Replication-fork stall, Replication stress

Introduction

Cell, organism and species survival depend on the fidelity of DNA replication. Therefore, it is not surprising that highly complex surveillance mechanisms, consisting of DNA repair, DNA replication and checkpoint proteins, have evolved to maintain genomic integrity. Stalled replication forks pose a grave threat to cell survival and genomic integrity (Kolodner et al., 2002). Therefore, understanding how cells cope with aberrant replication forks is essential to understanding the mechanisms of genome maintenance. A key component of the cellular response to stalled replication includes the localization of an intricate set of DNA-repair and checkpoint proteins to sites surrounding DNA lesions in a precise and cohesive manner (van den Bosch et al., 2003). These large aggregates of proteins form dynamic multi-tiered structures known as foci (Bishop, 1994; Lisby and Rothstein, 2004). Using indirect immunofluorescence microscopy, these foci are observed as brightly colored spots within the nucleus. Examples of proteins in these foci include DNA-damage sensors such as H2AX (H2AFX), RAD9-HUS1-RAD9, RPA and MRE11-RAD50-NBS1 (MRN; NBS1 is also known as NBN); proximal kinases such as ATR and ATM; and adaptor proteins such as 53BP1 (TP53BP1), MDC1, BRCA1 and claspin; effector kinases such as CHK1 (CHEK1) and CHK2 (CHEK2); the homologous-recombination proteins RAD51 paralogs and RAD52; and the end-joining proteins ARTEMIS (DCLRE1C), KU70 (XRCC6), KU80 (XRCC5), DNA-PK and XRCC4 (Fernandez-Capetillo et al., 2003; Lisby et al., 2004; Riballo et al., 2004; Rouse and Jackson, 2002).

The assembly and disassembly of these aggregates appears to be directed by protein-protein interactions rather than by direct binding of each protein to the DNA lesion (Lisby et al., 2004). Depending on the type of lesion, specific proteins needed to sense and repair the lesion are recruited. The rapid initial recruitment of proteins is tightly regulated and phosphorylation is a crucial post-translational modification that can rapidly change the interaction of proteins. For example, DNA-damage-dependent phosphorylation of BACH1 is required for its interaction with BRCA1 (Yu et al., 2003).

The conserved proteins MRE11, RAD50 and NBS1 have central roles in both DNA replication and DNA repair, and are essential in maintaining genomic stability and function. Evidence that implicates the involvement of the MRN complex in normal DNA replication includes the requirement of the MRN complex to prevent DNA double-strand breaks during normal replication and its co-localization with PCNA throughout S-phase (Costanzo et al., 2001; Mirzoeva and Petrini, 2003). It has also been shown that NBS1 binds to E2F transcription factors and directs the MRN complex to origins of replication; this data suggests a role for NBS1 in the processing of secondary structures formed during the initiation of DNA synthesis and normal fork progression (Maser et al., 2001). Mutations within the human genes *MRE11* and *NBS1* cause the human disorders ataxia telangiectasia-like disorder and Nijmegen breakage syndrome, respectively (Carney et al., 1998; Stewart et al., 1999; Varon et al., 1998). Cells derived from individuals with these disorders display multiple DNA-damage-response defects, including chromosomal fragility, the inability to arrest DNA replication after DNA damage, hypersensitivity to DNA-damaging agents and defective checkpoint responses (D'Amours and Jackson, 2002).

Previous studies have shown that RPA, a heterotrimeric single-stranded-DNA-binding protein, is involved in the initiation of DNA replication and remains localized to replication forks during elongation in S-phase (Brill and Stillman, 1989; Dimitrova et al., 1999; Liu et al., 2003; Fairman and Stillman, 1988; Wold and Kelly, 1988). The middle subunit of RPA, RPA32, is phosphorylated primarily at two sites, serine 23 and serine 29, during the normal cell cycle in the G_1/S transition, G_2 and M-phase, followed by dephosphorylation at the end of M-phase (Din et al., 1990). DNA-damaging agents known to stall replication, including UV, inhibitors of topoisomerase I and II and hydroxyurea (HU), induce hyperphosphorylation of RPA at additional serines and threonines on the N-terminus of RPA32, including serines 4, 8 and 33 and threonine 21 (Block et al., 2004; Nuss et al., 2005; Zernik-Kobak et al., 1997). RPA phosphorylation is thought to modulate protein-protein interactions as well as its ability to bind dsDNA (Abramova et al., 1997; Binz et al., 2003; Liu et al., 2005; Patrick et al., 2005; Yoo et al., 2005). RPA hyperphosphorylation, either via its effect on protein-protein interactions or on DNA-binding ability, decreases RPA involvement in replication (Carty et al., 1994; Olson et al., 2006) and regulates RPA functions in mismatch repair (Guo et al., 2006).

Both RPA and the MRN complex co-localize to discrete foci and interact in response to DNA replication-fork collapse induced by HU and UV (Robison et al., 2004). Here, we demonstrate that HU-induced RPA hyperphosphorylation requires NBS1. In cells lacking NBS1 expression or expressing S343A NBS1 protein, RPA in HU-induced foci was not hyperphosphorylated. Our results suggest that ATR is the kinase responsible for HU-induced RPA hyperphosphorylation and support the model that NBS1 is crucial for ATR retention at the sites of HU-induced DNA damage. Cells that lacked RPA32 hyperphosphorylation exhibited a decreased and delayed apoptotic response following HU treatment, suggesting an important role for RPA hyperphosphorylation in the cellular response to DNA damage.

Results

Functional NBS1 is required for RPA hyperphosphorylation at repair foci

It has been shown previously that RPA interacts with the MRN complex at sites of DNA damage (Olson et al., 2007; Robison et al., 2004). To determine the DNA-damage-dependent interactions of NBS1 and phosphorylated NBS1 on RPA, we examined HU-induced hyperphosphorylation of RPA32 in HeLa cells after blocking endogenous NBS1 with the introduction of an anti-NBS1 antibody, which detects both unphosphorylated and phosphorylated NBS1 (supplementary material Fig. S1). Chariot transfection procedures did not affect normal cell cycle progression, as verified by flow cytometry (supplementary material Fig. S2). With the peptide-mediated transfection of control non-immune IgG protein, hyperphosphorylated RPA foci were visible in approximately 30% of the cells following HU treatment. The peptide-mediated transfection of anti-NBS1 antibodies resulted in a decrease of RPA32 hyperphosphorylation at DNA-damage foci to mock-treated levels, whereas the recruitment of unphosphorylated RPA32 to the sites of damage remained unchanged (Fig. 1J-L). In a similar manner, the introduction of phospho-specific antibodies against NBS1 phosphorylated at serine 343 [anti-NBS1-S(*P*)343] into HeLa cells decreased hyperphosphorylation of RPA32 at DNA-damage foci following HU treatment (Fig. 1A,P-R).

Similar to HeLa cells blocked with NBS1 antibodies, cells lacking fully functional NBS1 exhibited decreased hyperphosphorylation of RPA at DNA-damage foci upon HU treatment, whereas the overall recruitment of RPA32 to foci was not altered (Fig. 2J-L). Expression of fully functional NBS1 activated hyperphosphorylation of RPA32 at DNA-damage foci to a similar level observed in HeLa cells (Fig. 2D-F), whereas cells expressing phospho-mutant NBS1 protein (S343A) demonstrated impaired phosphorylation, similar to cells lacking fully functional NBS1.

Western blot shows that HU-induced RPA hyperphosphorylation is impaired in NBS cell lines and in NBS1 phospho-mutant cell lines

A decrease of RPA32 hyperphosphorylation in NBS cells expressing phospho-mutant NBS1 (S343A) (Fig. 2) and in HeLa cells transfected with anti-NBS1-S(*P*)343 antibodies (Fig. 1) suggests a requirement for NBS1 phosphorylation as a prerequisite for hyperphosphorylation of RPA32. To further characterize the relationship between NBS1 phosphorylation and RPA32 hyperphosphorylation, we compared NBS cells restored with full-length NBS1 to NBS cells containing an empty vector or expressing phospho-mutant forms of NBS1 (S343A and S278A/S343A) in their abilities to hyperphosphorylate RPA32 using western blot analysis. Because HU-induced replication stress is a cell-cycle-specific effect, we synchronized the cells with aphidicolin before HU treatment. Aphidicolin at low doses decreases the rate of fork progression without activating checkpoints (Shimura et al., 2007) and has been shown to be effective in cell synchronization (Pedrali-Noy et al., 1980); this allowed for the induction of RPA hyperphosphorylation at a lower dose of HU. Consistent with foci data, RPA32 hyperphosphorylation was absent in NBS cells expressing phospho-mutant forms of NBS1 and cells lacking NBS1 (Fig. 3A), whereas expression of wild-

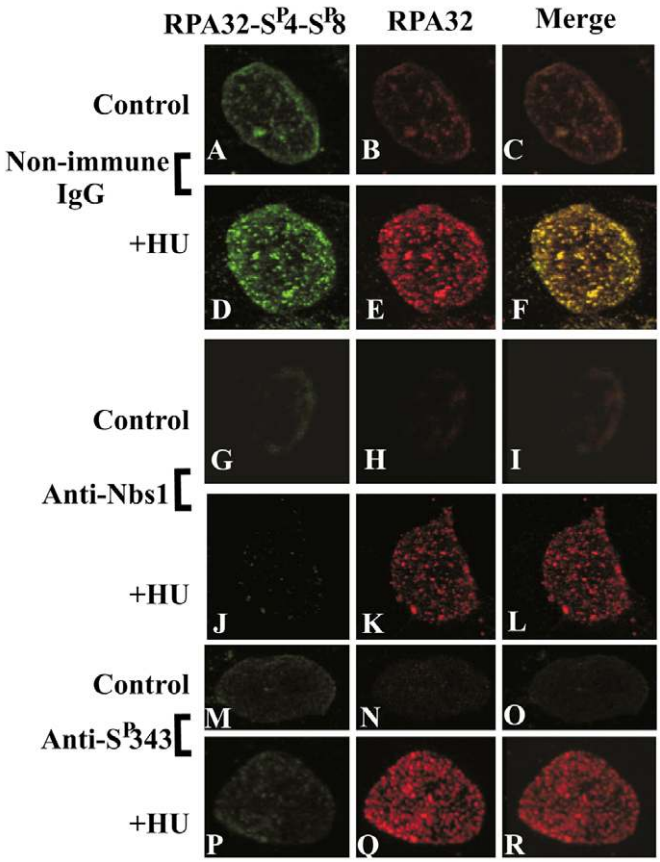

Fig. 1. Anti-NBS1 and anti-NBS1-S(*P*)343 block the formation of RPA32-S(*P*)4-S(*P*)8 and RPA foci. (A-R) Following the introduction of the negative-control IgG (A-F), anti-NBS1 (G-L) or anti-NBS1-S(*P*)343 (M-R), HeLa cells were treated with 5 mM HU, or were mock-treated (control), for 3 hours. Cells were detergent-extracted and fixed, incubated with primary and secondary antibodies, and visualized by confocal microscopy. (D-F) HU treatment led to RPA32-S(*P*)4-S(*P*)8 (green) and RPA32 (red) focus formation in negative-control IgG-transfected cells. (J-L,P-R) anti-NBS1- and anti-NBS1-S(*P*)343-transfected cells contained RPA32 foci but RPA32-S(*P*)4-S(*P*)8 foci were not observed. HU-induced damage was verified by H2AX phosphorylation (supplementary material Fig. S4).

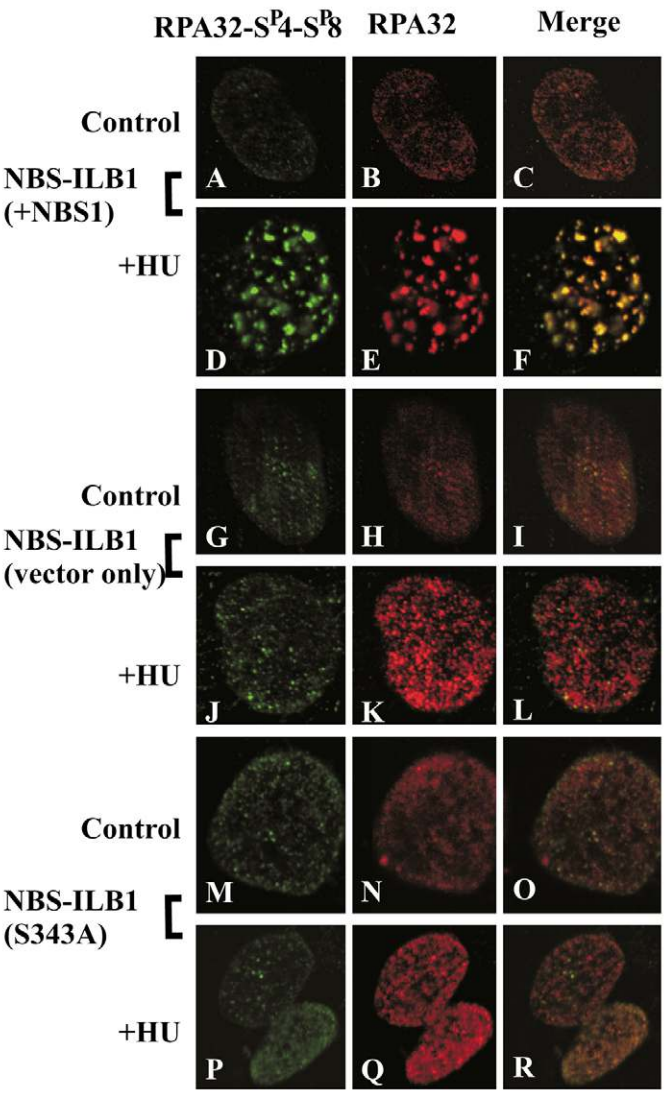

Fig. 2. Impaired formation of RPA32-S(*P*)4-S(*P*)8 foci in NBS-ILB1 (vector only) and NBS-ILB1 (S343A) cells. (A-R) The indicated isogenic cell lines were treated with 5 mM HU for 3 hours prior to detergent-extraction, fixation and immunofluorescence using RPA32-S(*P*)4-S(*P*)8 (green) or RPA32 (red) antibodies. (D-F) HU treatment induced RPA recruitment to sites of DNA damage and normal RPA hyperphosphorylation occurred in NBS-ILB1 (NBS1) cells. (J-L,P-R) NBS-ILB1 (vector only) and NBS-ILB1 (S343A) cells recruited RPA to sites of DNA damage; however, RPA32 was not phosphorylated at these sites. HU toxicities were comparable for all cell lines tested (supplementary material Fig. S3).

type NBS1 restored hyperphosphorylation of RPA32 to NBS cells.

RPA redistributes to chromatin, but is not hyperphosphorylated following HU treatment in cells expressing catalytically impaired ATR

To understand the mechanism of the recruitment of RPA to sites of damage and to identify the kinase responsible for phosphorylation of RPA after replicative stress, we looked at RPA recruitment and phosphorylation in ATR-kinase dead (ATR-kd) and ATM deficient (A-T) cells. Consistent with previous reports, HU treatment caused considerable redistribution of RPA to the chromatin-bound fraction in A-T cells, A-T cells expressing ATM, ATR wild-type cells and ATR-kd cells (Fig. 3B), indicating that RPA recruitment to stalled replication forks is independent of ATM and ATR (Balajee and Geard, 2004; Liu et al., 2003; Stiff et al., 2005). The absence of ATM did not affect RPA hyperphosphorylation; however, overexpression of ATR-kd decreased hyperphosphorylation of RPA bound to chromatin (Fig. 3B). This suggests that the majority of hyperphosphorylation of chromatin-bound RPA following HU-induced replicative stress is ATR dependent.

Decreased ATR chromatin retention in NBS1-deficient and NBS1-phospho-mutant cells

It is probable that the kinase responsible for RPA hyperphosphorylation following HU-induced DNA damage is ATR, the kinase activity of which might also require fully functional NBS1. This would suggest that the lack of RPA32

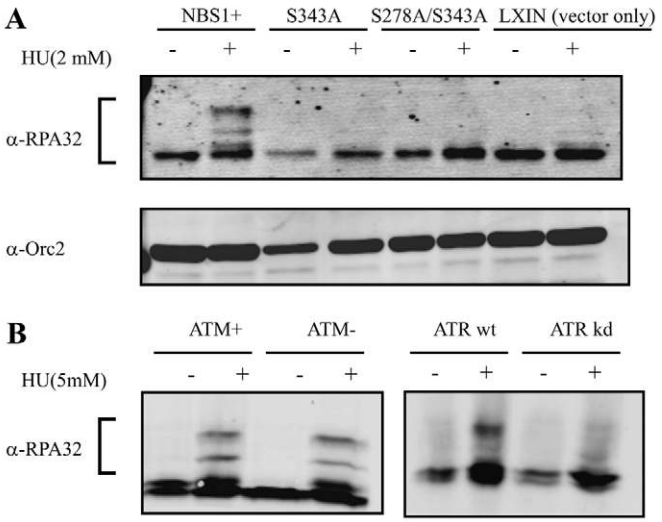

Fig. 3. NBS1 and ATR are required for RPA phosphorylation following HU-induced replicative stress. (A) NBS-ILB1 (NBS1), NBS-ILB1 (S343A), NBS-ILB1 (S278A/S343A) and NBS-ILB1 (LXIN, vector only) cells were synchronized with 6 μM aphidicolin for 16 hours. The medium was replaced with fresh medium for 3 hours before HU treatment. Following 3 hours of treatment with 2 mM HU, cells were harvested and chromatin fractions were prepared. Proteins were detected by immunoblotting with the corresponding antibodies. (B) Chromatin isolated from the A-T cell line AT22IJE-T (ATM+) and its isogenic derivative cell line expressing recombinant ATM (ATM–) showed little difference in RPA-chromatin binding or RPA phosphorylation. Similar to the A-T cell lines, U2OS cells expressing either wild-type ATR or kinase-dead ATR (ATR-kd) displayed similar RPA-chromatin binding following DNA damage. Distinct from A-T cells, ATR-kd expression in U2OS cells significantly decreased RPA hyperphosphorylation.

hyperphosphorylation might be due to impaired recruitment and retention of ATR at the site of DNA damage. To test this hypothesis, we compared NBS1-deficient and phospho-mutant-NBS1 cells to NBS1-corrected cells for detergent-extraction-resistant ATR binding. Cells were synchronized with aphidicolin and released into fresh medium for 3 hours prior to HU treatment. ATR foci in NBS1-corrected cells were intense in approximately 30-35% of the treated cells, whereas ATR foci observed in NBS1-deficient and NBS1-phospho-mutant cells were detectable at a much lower level, comparable to control levels (Fig. 4A-R). The requirement of NBS1 for ATR chromatin retention has been shown previously (Stiff et al., 2005). To confirm the importance of NBS1 phosphorylation for ATR chromatin retention, we carried out cell fractionation experiments, choosing the NBS1-corrected and the NBS1 double-phospho-mutant (S278A/S343A) cells. Compared with NBS1-corrected cells, weak ATR chromatin retention could be observed in the double-phospho-mutant cells in both untreated and HU-treated conditions, whereas ATR chromatin retention in NBS1-corrected cells increased slightly with HU treatment (Fig. 4S).

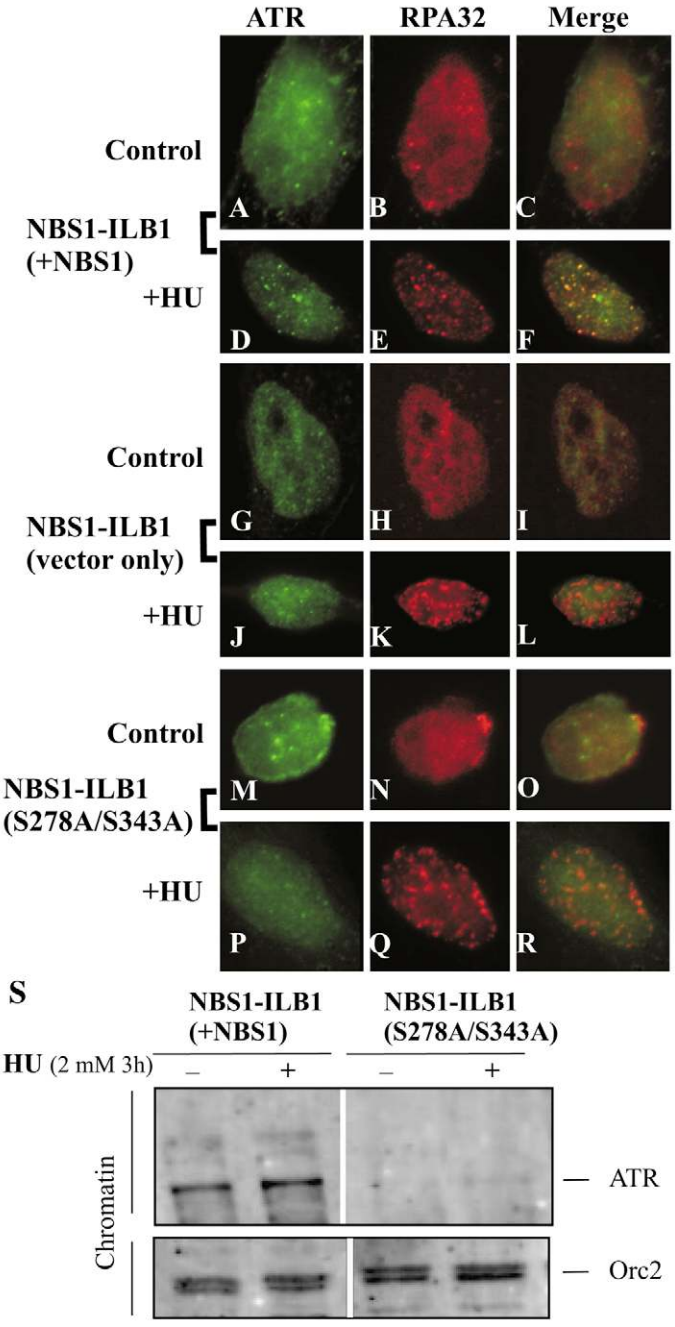

Fig. 4. HU-induced ATR foci and chromatin retention is decreased both in NBS1-deficient and NBS1 phospho-mutant cells. (A-R) NBS-ILB1 (NBS1), NBS-ILB1 (vector only) and NBS-ILB1 (S278A/S343A) cells were synchronized and treated with 2 mM HU for 3 hours. To visualize detergent-resistant ATR foci, cells were extracted with detergent before fixation. (D-F) In NBS cells restored with wild-type NBS1, ATR foci (green) were still visible after detergent extraction (D) and ATR foci colocalized with HU-induced RPA32 foci (red) (F). (G-R) Decreased retention of ATR (J,P) and a loss of colocalization with RPA (L,R) was observed in NBS1-deficient and in NBS1 phospho-mutant cells. (S) ATR retention in chromatin fractions of synchronized NBS-ILB1 (NBS1) and NBS-ILB1 (S278A/S343A) cells was examined by immunoblotting with anti-ATR antibodies. NBS cells expressing NBS1 retained ATR in the chromatin fractions, whereas NBS cells expressing a phospho-mutant form of NBS1 (S278A/S343A) did not retain ATR in the chromatin. Orc2-chromatin binding was not altered following DNA damage and was used as a loading control.

HU-induced apoptosis is suppressed and delayed in RPA32 S4A-S8A phospho-mutant cells

Among the identified phosphorylation sites on the N-terminus of RPA32, S4 and S8 are unique in that their phosphorylation is only observed following DNA damage (Patrick et al., 2005). To examine the functional significance of RPA phosphorylation in response to DNA damage, we retrovirally infected UM-SCC38 cells with HA-tagged copies of wild-type or S4A-S8A-mutant alleles of RPA32 (A4A8 RPA32). Endogenous RPA32 was downregulated with siRNA directed towards a 3′ UTR region of *RPA32* mRNA, with recombinant RPA32 protein expressed at similar levels to endogenous protein (Fig. 5A). HA-RPA32, bound to anti-HA antibodies, efficiently immunoprecipitated both endogenous RPA70 and RPA14 from lysates prepared from cells transfected with wild-type RPA32 and A4A8-RPA32, verifying that HA-RPA32 is capable of forming a complex with the other RPA subunits (Fig. 5B). Anti-HA antibodies did not immunoprecipitate endogenous RPA70 and RPA14 from lysates derived from cells transfected with an empty vector.

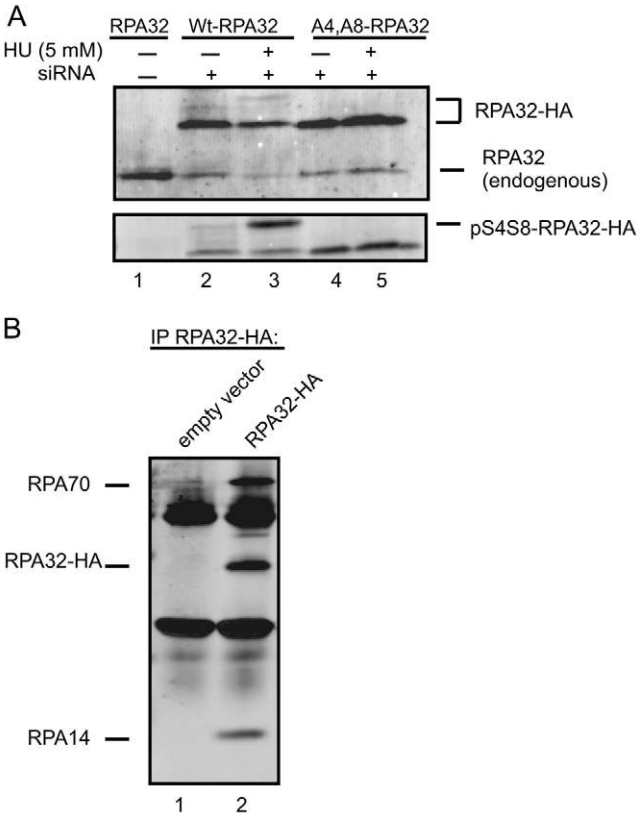

Fig. 5. Wild-type HA-RPA32-containing heterotrimers are hyperphosphorylated following siRNA downregulation of endogenous RPA32 and HU treatment. (A) SCC38 cells were transfected with HA-wt-RPA32 (lanes 2,3) or HA-A4A8-RPA32 (lanes 3,4). Cells were treated with siRNA directed towards the 3′ non-coding sequence of RPA32 for 48 hours. Following siRNA downregulation of endogenous RPA32, cells were subjected to HU (5 mM) treatment for 3 hours. Lysates from either control or HU-treated cells were analyzed for the presence of RPA32 by western blot analysis using RPA32 antibodies that recognize both endogenous and transfected RPA32. The membrane was reblotted without stripping with antibodies that recognize phosphorylated S4S8 forms of RPA32 (bottom panel). (B) HA-tagged RPA32 formed a complex with endogenous RPA70 and RPA14, as demonstrated by co-immunoprecipitating HA-tagged RPA32. Lysates prepared from UM-SCC38 cells transfected with an empty vector or HA-wt-RPA32 were subjected to immunoprecipitation using HA antibodies. Immunoprecipitates were then analyzed for RPA70, RPA32 and RPA14 by western blot analysis (lanes 1-2).

Another cellular response to the presence of overwhelming DNA damage is the process of programmed cell death, apoptosis. Staining with an Annexin-V–APC conjugate was used for the detection of apoptotic cells by flow cytometry. Cells were transfected with siRNA directed towards endogenous *RPA32* 48 hours prior to HU treatment. Cells were then treated for 24 hours with 2 mM HU then returned to fresh medium. At 24 hours after HU treatment, over 25% of the wild-type-RPA32-expressing cells and uninfected cells transfected with scrambled siRNA stained positive for Annexin V. A4A8-RPA32-expressing cells did not exhibit Annexin V staining at 24 hours in response to HU treatment (Fig. 6A, middle row, right panel). By 48 hours, approximately 50% of the wild-type and uninfected cells were positive for both propidium iodide (PI) and Annexin V staining, whereas A4A8-RPA32 cells had only a modest increase in PI/Annexing V staining (8.7%, Fig. 6A, bottom row). The apoptotic fraction exhibited at 72 hours in A4A8-RPA32 cells continued to appear suppressed and delayed, suggesting a role for S4S8 phosphorylation in apoptotic signaling (Fig. 6B).

Discussion

In this study, we have enhanced our understanding of the interaction between RPA, ATR and the MRN complex, and their respective roles in the replicative-stress-induced DNA-damage response. ‘Replicative stress’ generally refers to the physical impairment and uncoupling of DNA helicases and polymerases by DNA lesions, which leads to stalled replication forks and long stretches of ssDNA. We observed foci that were positive for hyperphosphorylated RPA in HU-induced DNA damage, suggesting that hyperphosphorylation of RPA is involved in the stabilization and repair of stalled forks.

Evidence has been provided that phosphorylation of NBS1 is required for an efficient response to DNA damage; however, the functional significance of NBS1 phosphorylation is not fully understood. In our study, we show that phosphorylation of NBS1 is crucial for hyperphosphorylation of RPA in response to HU-induced DNA damage. Cells with impaired NBS1 phosphorylation had decreased hyperphosphorylation of RPA following HU treatment, without affecting overall RPA chromatin retention. These findings suggest a requirement of NBS1 phosphorylation for the hyperphosphorylation of RPA.

One possibility is that the lack of RPA phosphorylation observed in NBS1 phospho-mutant cells is unique to RPA. However, it has previously been demonstrated that NBS1 phosphorylation is required for SMC1 phosphorylation and CHK2 phosphorylation, following low-dose ionizing radiation (IR) treatment, suggesting other DNA-damage response proteins are affected by a lack of NBS1 phosphorylation (Buscemi et al., 2001; Yazdi et al., 2002). However, not all DNA-damage response proteins are impaired by the lack of NBS1 phosphorylation. In NBS1 phospho-mutant cells, ATM activation is not affected following IR and NBS1 phosphorylation is not required for UV-induced CHK1

phosphorylation (Jazayeri et al., 2006). This suggests that some proteins are impaired, whereas others are not affected by NBS1 phosphorylation following various types of DNA damage.

Based on our finding that expression of kinase-dead ATR suppressed RPA hyperphosphorylation and that ATR retention on chromatin is dependent on the presence of fully functional NBS1, we propose that RPA hyperphosphorylation requires an NBS1-dependent retention of ATR to the damaged site. A similar model in which the kinase has a non-catalytic docking site that helps to retain it in close proximity to its target proteins has been observed previously in other signaling pathways. For example, mitotic phosphorylation of NIR2 (PITPNM1) provides a docking site for the PLK1 kinase involved in cytokinetic events of cell division (Litvak et al., 2004).

ATR retention in NBS1 phospho-mutant cells appeared to be decreased in the absence of HU, suggesting that NBS1 might be equally as important for ATR chromatin retention during normal replication. It has been shown that ATR is crucial for replication through 'slow zones' and prevents double-strand-break accumulation during normal replication (Cha and Kleckner, 2002; Trenz et al., 2006). This requirement of NBS1 phosphorylation might help to explain the necessity of both ATR and the MRN complex in preventing replication stress during normal replication.

Our data, and previous studies, implicate ATR as the primary kinase responsible for replicative-stress-induced phosphorylation of RPA (Block et al., 2004; Peng and Chen, 2005). Members of the phosphoinositide 3-kinase related kinases (PIKKs) – ATM, DNA-PK and ATR – have been reported to hyperphosphorylate RPA. Each of these kinases has been shown to phosphorylate RPA in vitro and in vivo (Barr et al., 2003; Brush et al., 1994; Gately et al., 1998; Liu et al., 2006; Oakley et al., 2001; Unsal-Kacmaz and Sancar, 2004; Wang et al., 2001). Recent reports have demonstrated ATM-dependent activation of ATR in response to IR, and ATR-dependent activation of ATM in response to HU and UV (Jazayeri et al., 2006; Stiff et al., 2006). Why does HU-induced RPA hyperphosphorylation require NBS1 and ATR but does not appear to require ATM even though ATM is activated by replication stress? One explanation is that it might be a matter of timing. With the initial stress in the form of extended regions of ssDNA, increased binding of RPA occurs and attracts ATR and ATRIP as the initial response. NBS1, possibly through binding to ATRIP, appears to be

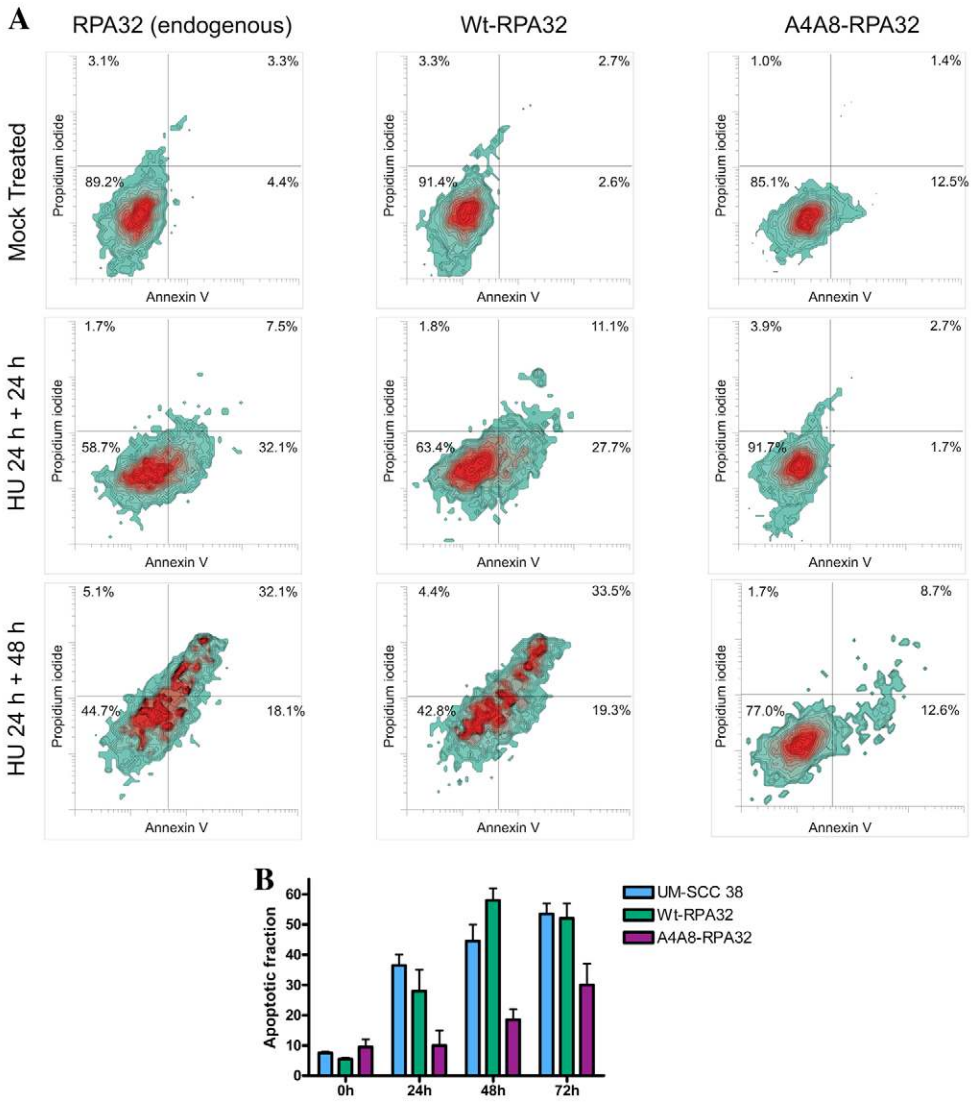

Fig. 6. Loss of S4S8 phosphorylation suppresses HU-induced apoptosis. (A) Time of onset of apoptosis after 24 hours of HU treatment (2 mM). HU-induced apoptosis was assessed by Annexin V staining and flow cytometry. Right-hand quadrants indicate Annexin-V-positive cells, indicative of apoptosis. Cells in the upper quadrants have begun to lose membrane integrity and stain positive for propidium iodide and Annexin V. The results from one of three representative experiments are shown. (B) The percentage of apoptotic cells was calculated from at least three separate experiments. The percentage is presented as the mean ± s.d. of the total number of gated cells in the upper- and lower-right quadrants. Following siRNA downregulation of endogenous RPA32, cells were treated with 2 mM HU for 24 hours. At the indicated time points, cells were harvested, fixed and stained with Annexin V to detect apoptotic cells.

necessary to retain the signal activation by RPA and ATR. This does not rule out the possibility of a direct role for ATM-dependent phosphorylation of RPA at later time points, as well as an indirect role for ATM in the activation of downstream kinases such as CHK2, which might contribute to the direct phosphorylation of RPA in vivo.

Cells expressing alanine at S4 and S8 on the N-terminus of RPA32 exhibited a loss of apoptosis. This suppression of apoptosis might be attributable to a defect in the ability of a cell to sense DNA damage and to activate the appropriate downstream response, including apoptotic signaling. The inability to induce apoptosis following DNA damage has the potential to be advantageous to cancer cells. Indeed, studies have shown that the loss of apoptosis contributes to tumorigenesis and to the resistance of cancer cells to a variety of therapeutic agents (Igney and Krammer, 2002; Lowe et al., 1993).

In conclusion, our findings provide novel evidence that fully functional NBS1 is required for the hyperphosphorylation of RPA present at DNA-repair-associated foci. Although the function of NBS1 phosphorylation and RPA hyperphosphorylation in protein interaction and protein recruitment are only beginning to be understood, we have shown that RPA hyperphosphorylation is involved in the cellular response to DNA damage. Additional studies will be needed to elucidate the relationship between ATR, RPA and NBS1 in sensing DNA damage.

Materials and Methods

Cell lines and treatments

HeLa cells were obtained from the American Type Culture Collection (ATCC) and maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) plus 100 U/ml penicillin and 100 μg/ml streptomycin. The simian-virus-40-transformed NBS fibroblast cell line, NBS-ILB1, which had been transfected with retroviral constructs encoding wild-type and phospho-mutant NBS1 forms to generate the NBS-ILB1 (Vector), NBS-ILB1 (NBS1), NBS-ILB1 (S343A) and NBS-ILB1 (S278/S343A) cell lines were generously provided by Patrick Concannon (Benaroya Research Institute, WA). Cells were grown in DMEM supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 500 μg/ml G418 (Cerosaletti et al., 2000). ATR-kd mutant and ATR-wt U2OS cells, kindly provided by Paul Nghiem (University of Washington Medical Center, DC), were grown and maintained as previously described (Nghiem et al., 2002). UM-SCC 38 cells were established at the University of Michigan and generously provided by Thomas Carey (University of Michigan, MI). UM-SCC 38 cells expressing wild-type RPA 32 and S4A/S8A-substituted RPA32 were maintained in DMEM containing 150 μg/ml hygromycin (Sigma, St Louis, MO) for selection. For retroviral packaging, Phoenix amphotrophic cells (Orbigen, San Diego, CA) were grown in Dulbecco DMEM supplemented with 10% fetal bovine serum with penicillin-streptomycin. For HU treatment, cells were incubated in growth medium containing HU (1-5 mM) for the times indicated before harvesting. To obtain cells primarily in S-phase, asynchronous cells were treated with 6 μM aphidicolin for 16-17 hours. The medium containing aphidicolin was removed, and cells were washed twice in serum-free medium and then incubated in serum containing medium for an additional 3 hours before treatment with HU. Where indicated, cells were transfected with 50 nM of siRNA against *RPA32* (5′-CCU-AGUUUCACAAUCUGUUUU-3′, Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen) according to the manufacturer's instructions.

Retroviral gene expression

Wild-type and A4A8-substituted *RPA32* cDNAs were inserted into the retroviral vector pQCXIH (Clontech, Mountain View, CA). Amino acids 18-271 were amplified from a pET-11d vector containing the RPA heterodimer (generously supplied by Marc Wold, University of Iowa, IA) using primers 5′-GCGCAC-CGGTGATATACATATGTGGAAC-3′ and 5′-CGCGGGATCCGTAAGCTCAGT-AATCTGGAACATCGTATGGGTATTCTGCATCTGTGGA-3′, and were digested with *Bam*HI and *Nae*I. Amino acids 1-18 were created by the ligation of two phosphorylated, double-stranded oligonucleotides with *Age*I and *Nae*I overhangs. The N-terminal double-stranded oligonucleotides and the amplified C-terminus containing a HA-tag sequence were ligated into *Age*I/*Bam*HI-digested pQCXIH.

Phoenix A cells were plated in 60-mm culture dishes at 5×10^6 cells/ml and transfected with 24 μg of plasmid using Lipofectamine 2000 (Invitrogen). After 48 hours at 37°C, cells were incubated for an additional 24 hours at 32°C. Supernatants were collected, centrifuged at 2000 ***g*** for 10 minutes and added to 25% confluent SCC-38 cells in the presence of 10 μg/ml polybrene (Sigma). After 48 hours, UM-SCC-38 cells were selected with 150 μg/ml hygromycin. Surviving cells were grown to near confluence and tested for expression of recombinant RPA32 by western blot analysis.

Western immunoblots

Cell lysates and immunoprecipitates were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were probed using primary antibodies against NBS1 (Novus Biological; 1:10,000), NBS1-S(*P*)343 (phospho-specific) (Novus Biologicals; 1:7500), RPA32 (Neomarkers; 1:5000), RPA32-S(*P*)4-S(*P*)8 (phospho-specific for hyperphosphorylated RPA32) (Bethyl Laboratories; 1:10,000), ATR (Genetex; 1:3000) and Orc2 (Genetex Inc.; 1:250). Secondary antibodies were Alexa-Fluor-680-linked anti-rabbit and anti-mouse (Invitrogen; 1:5000), and bound antibodies were visualized using infrared fluorescence (LI-COR, Lincoln, NE).

Peptide-mediated delivery of antibodies into HeLa cells

Chariot-antibody complexes were formed by incubating 2-3 μg of anti-RPA32-S(*P*)4-S(*P*)8, anti-NBS1, anti-NBS1-S(*P*)343 or negative control IgG with 4 μg of presonicated Chariot (Active Motif, Carlsbad, CA) in PBS (pH 7.4) for 30 minutes at 25°C. HeLa cells were overlaid with the pre-formed complexes according to the manufacturer's recommendations. Briefly, the delivery of Chariot-antibody complexes was performed by incubating complexes directly in cell medium with cells for 3 hours at 37°C before mock or HU treatment.

Immunofluorescence microscopy

Cells were grown on 12-mm coverslips (BD Biosciences, Franklin Lakes, NJ) for 36 hours before treatment. Cells were treated with 4-5 mM HU for 3 hours. To visualize extraction-resistant proteins, cells were washed with PBS containing 0.5% Triton X-100 at RT for 10 minutes and fixed for 3 minutes with PBS containing 4% paraformaldehyde. Cells were then blocked for 1 hour in PBS containing 15% fetal bovine serum. Primary-antibody dilutions used are as follows: anti-RPA32-S(*P*)4-S(*P*)8, 1:2000; anti-RPA70, 1:1000; anti-RPA32, 1:1000; and anti-NBS1-S(*P*)343, 1:1500. Secondary-antibody dilutions are as follows: anti-rabbit Alexa-Fluor-488, 1:300; and anti-mouse Alexa-Fluor-568, 1:300. Images were obtained with a Leica confocal microscope (Leica Microsystems, Milton Keynes, UK) equipped with a 488/514 nm dual band argon ion laser. An oil-immersion objective was used. Images were collected using Leica confocal software analysis package (TCS-NT) software. For each experiment, at least 200 cells were analyzed, and experiments were repeated three times. Quantification of fluorescence intensity was performed on blind captured images using standardized capture settings, image processing and analysis performed on Leica confocal software.

Subcellular fractionation

The cellular-protein fractionation protocol was performed as previously described with slight modifications (Liu et al., 2001). Briefly, cells were treated with HU or were mock-treated. The free cytoplasmic/nucleoplasmic fraction was prepared by allowing cells to lyse for 10 minutes on ice in 0.5% Triton X-100 in cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 5 μl/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride). The insoluble fraction was washed with PBS and treated with 100 μg/ml DNase I in cell lysis buffer containing 4 mM $MgCl_2$ at 37°C for 15 minutes. The suspension was centrifuged, and the supernatant was collected and designated the chromatin fraction.

Annexin V staining

Cells were stained using Annexin V staining kit (BD Biosciences), according to the manufacturers instructions. Briefly, 5×10^5 cells were seeded on 60 mm plates and treated with scrambled siRNA or siRNA directed towards endogenous *RPA32* for 48 hours. Cells were washed and fresh medium was added for 3 hours. Cells were then treated with HU (1 mM) for 24 hours. HU was removed from the medium and replaced with fresh medium, and cells were collected at the indicated times. Floating and adherent cells were washed twice in BD Perm/Wash buffer and resuspended in Annexin V Binding Buffer (BD Biosciences) for 30 minutes. Annexin-V-positive cells were detected with an Annexin-V–APC conjugate. PI was used to detect necrotic cells as per the manufacturer's instructions. Cells were analyzed for apoptosis by flow cytometry with the BD FACSArray (BD Biosciences). Data were analyzed using WinList (Verity Software) and Graph Pad Prism (Graph Pad Software).

We are thankful to Karen Cerosaletti and Patrick Concannon (Benaroya Research Institute) for the NBS-ILB1 cell lines; and to Paul Nghiem (University of Washington Medical School) for the

ATR-wt and ATR-kd U2OS cell lines. We thank Steve Patrick for critical reading of the manuscript.

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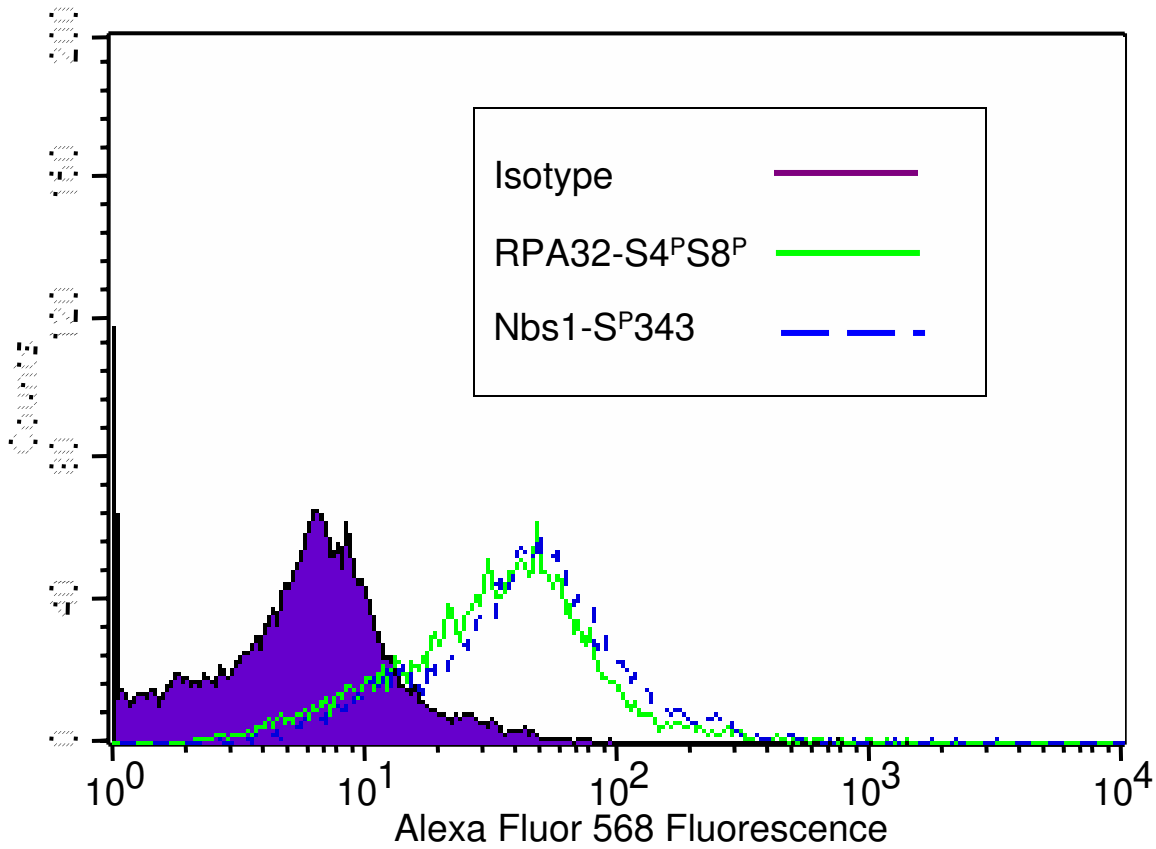
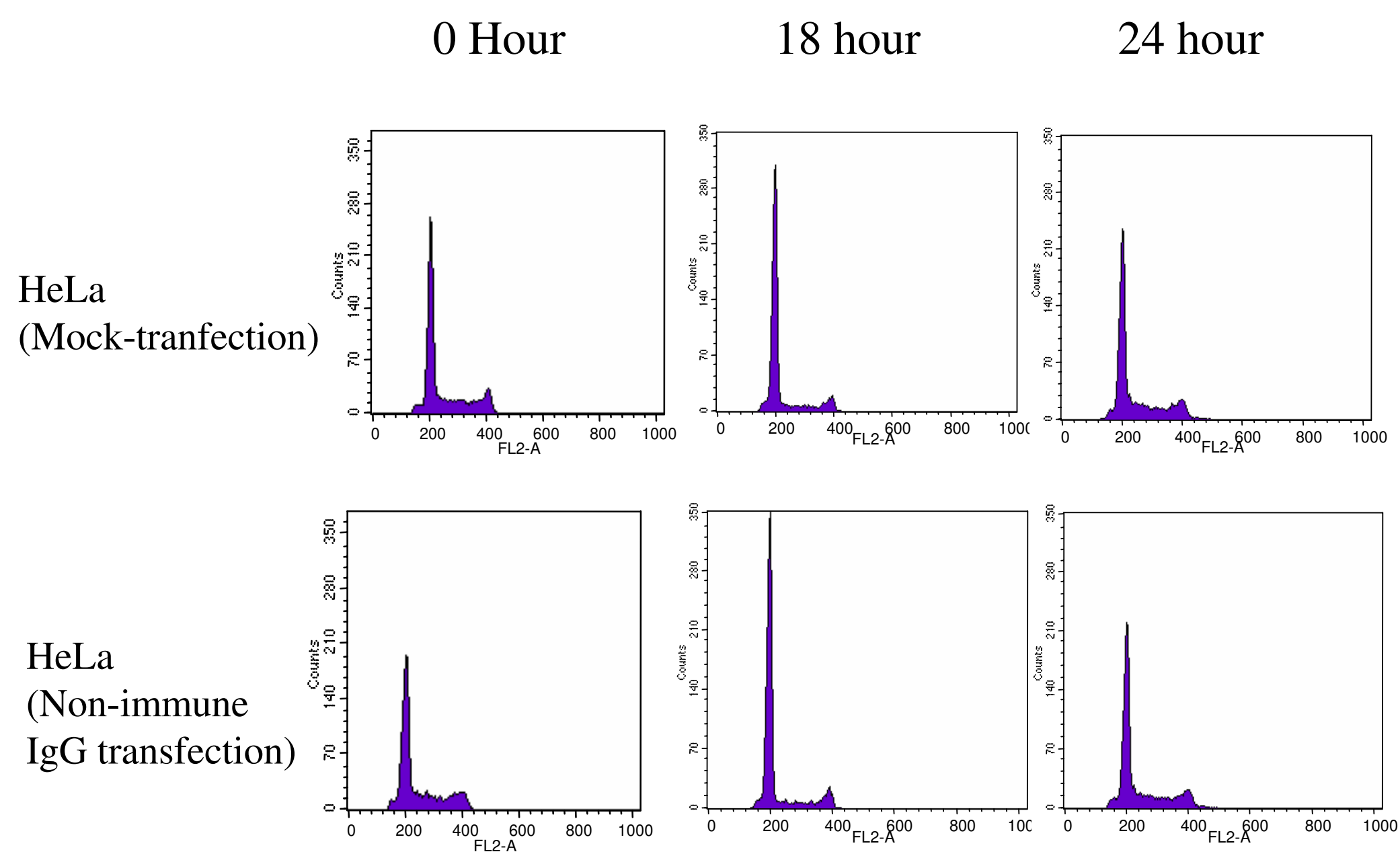
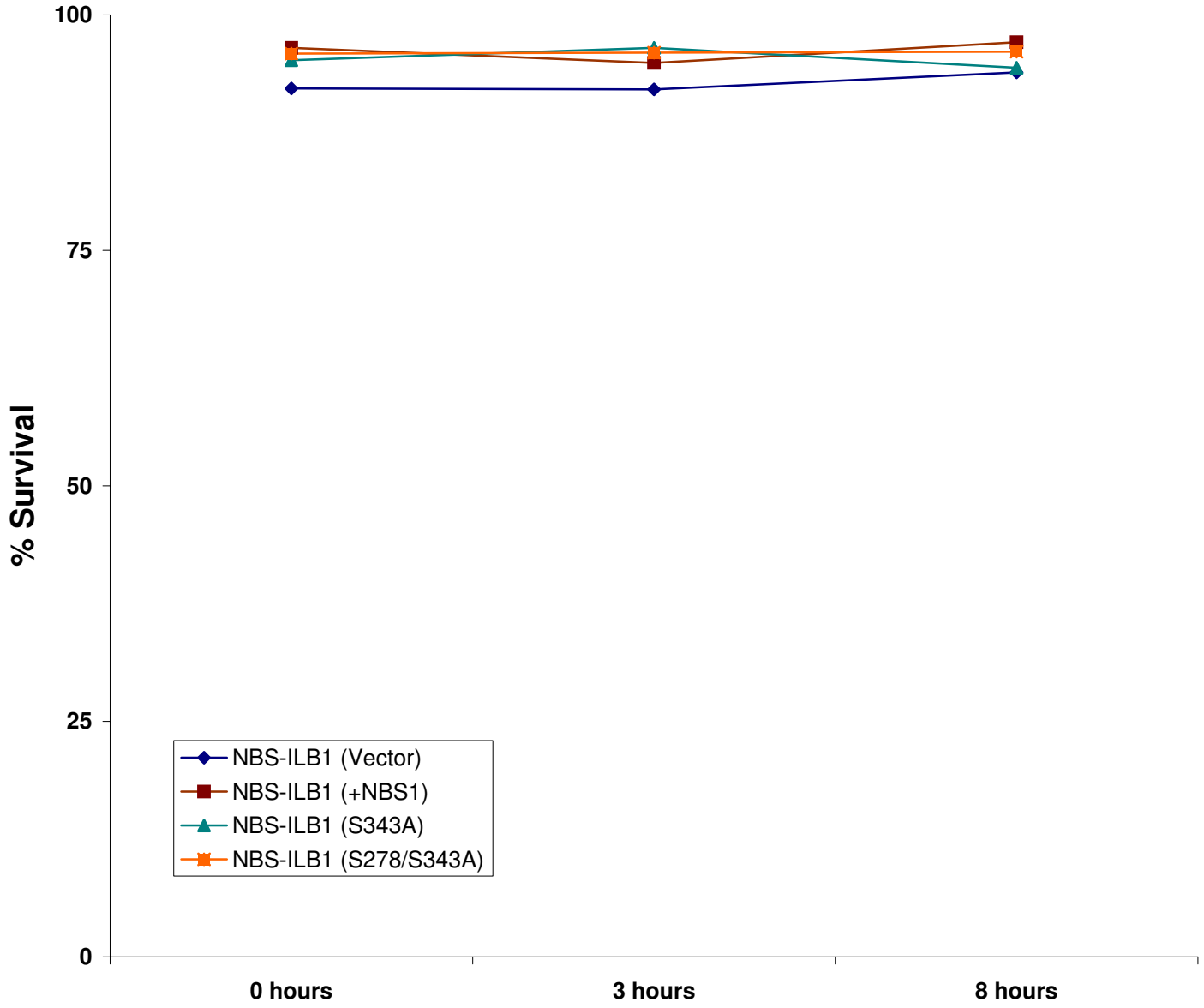


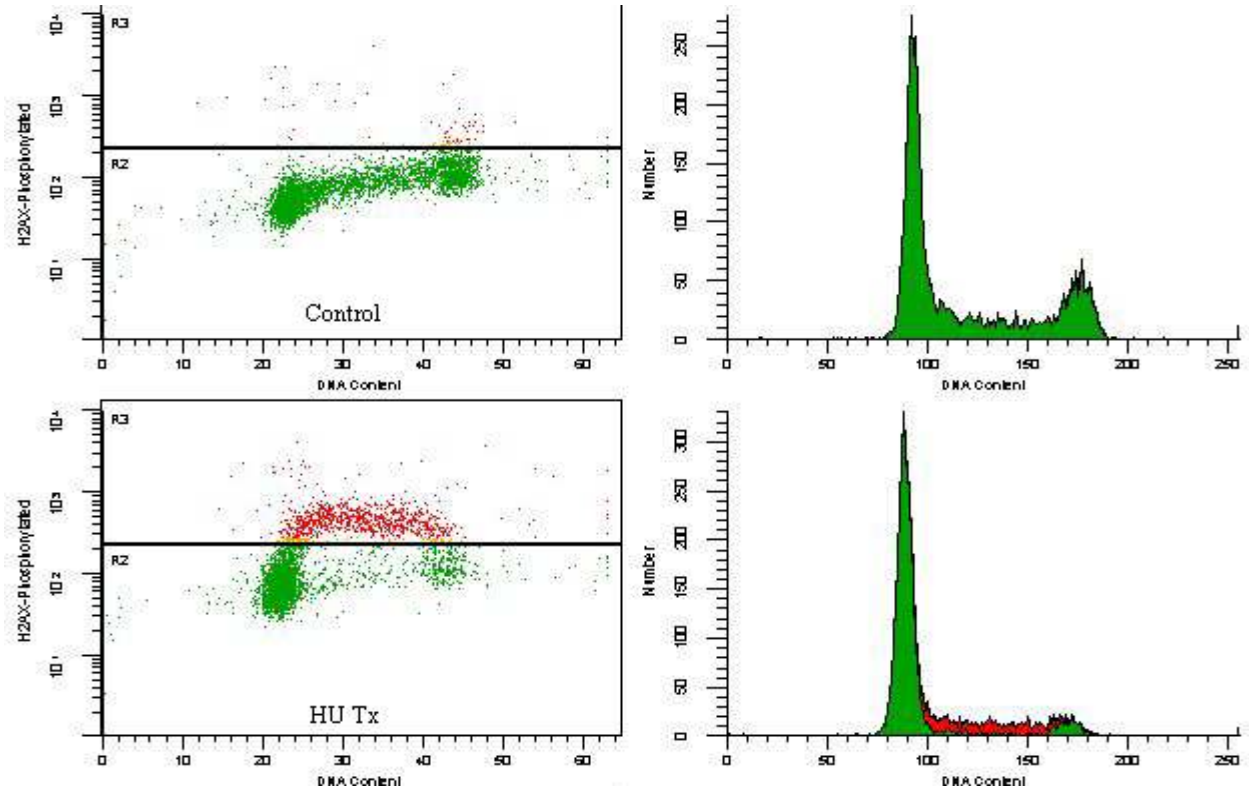
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Figure 3



Supplementary figure 4

Supplementary Data

Nbs1 is Required for RPA Phosphorylation Following Replication Fork Stall and Collapse

Manthey, K., Elliott, J. and Oakley, G.G.

Supplemental Experimental Procedures

Peptide-mediated delivery of antibodies into HeLa cells.

Chariot®–antibody complexes were formed by incubating 2-3 µg of non-immune IgG, with 4 µg of presonicated Chariot® (ChariotTM; Active Motif, Carlsbad, CA) in PBS (pH 7.4) for 30 min at 25 ◦C. HeLa cells were overlaid with the pre-formed complexes according to the manufacturer's recommendations. Briefly, the delivery of Chariot®–antibody complexes was performed by incubating Chariot®–antibody complexes directly in cell medium with cells for 3 h at 37 ◦C.

Chariot-antibody Transformation Efficiency. To determine antibody transformation efficiency, cell-associated fluorescence of a total of 10,000 cells was determined using flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ). Chariot®–antibody treated cells were fixed and permeabilized using the cytofix/ cytoperm kit (BD Biosciences) and stained with Alexa Fluor 568 secondary antibody (Molecular Probes, Carlsbad, CA) at a 1:250 dilution. Percentage of cells with Alexa Fluor 568 fluorescence was determined using CellQuest pro software (BD Biosciences).

Flow cytometric analysis. HeLa cells were harvested at 0 hr, 18 hr, and 24 hr time points and fixed with 70% ethanol. Approximately 10^6 cells/ml were incubated with propidium iodide and RNase A, then analyzed using a FACSCalibur (BD Biosciences). Data was plotted using CellQuest software; approximately 10,000 events were analyzed for each sample. To detect H2AX phosphorylation mock- and HU-treated cells were fixed in 1% methanol-free formaldehyde solution and permeabilized in 70% ethanol at -20°C over night. Cells were washed in PBS, resuspended in 1% BSA in 0.1% Tween PBS (BSA-T-PBS) for 5 min and incubated with 2μg/ml monoclonal γH2AX antibody (Upstate, Temecula, CA) over night at 4°C. Cells were washed in BSA-T-PBS and suspended in BSA-T-PBS containing 10μg/mL goat anti-mouse secondary antibody (Alexa Fluor 647, Invitrogen). After 1h incubation wash with BSA-T-PBS, cells were suspended in propidium iodide (PI) staining solution containing 5 μg/mL PI (Sigma) and RNase A in PBS (100 mg/mL, Sigma) and incubated for 30 min in the dark. The intensity of fluorescence was measured with the BD FACSArray (BD Biosciences) and data were analyzed using WinList (Verity) software.

Figure legends

Fig. S1. Transfection efficiency determined by flow cytometry. HeLa cells were transfected with non-immune IgG isotype control, anti-RPA32S4^{P}S8^P or Nbs1 SP343 antibody-Chariot® complexes. Transfection efficiencies averaged approximately 80%. The transfection efficiencies shown are 85% and 89% for anti-RPA32S4^{P}S8^P and Nbs1 S^{P}343, respectively.

Fig. S2. Cell cycle analysis following protein transfection with Chariot® reagent. HeLa cells were mock-transfected or transfected with non-immune IgG-Chariot® complexes, fixed at the indicated times and cell cycle analysis was performed.

Fig. S3. HU toxicity in NBS-ILB1 cells and transgenes following HU treatment. Cells were treated with 5 mmol HU for 3 and 8 hours; for the 8 hour time point HU-containing medium was replaced by regular medium after 3 hours and cells were incubated for additional 5 hours. Cell survival was analyzed using the Live/Dead assay.

Fig. S4. H2AX and cell cycle analysis of cells either mock-treated or treated with HU for 3 h. Bivariate analysis was performed with a BD-FACSArray demonstrating H2AX phosphorylation predominantly in S-phase of HU-treated cells.