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Requirement for NBS1 in the S phase checkpoint response to DNA methylation combined with PARP inhibition

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PARP inhibitor; methyl methanesulfonate; NBS1; SMC1; cell cycle; Chk1; Chk2

1. Introduction

Treatment of cells with a DNA methylating agent, such as methyl methanesulfonate (MMS), results in formation of methylated DNA base adducts. Repair of these lesions by the most simple base excision repair (BER) pathway [1] is initiated by a damage-specific monofunctional glycosylase, followed by strand cleavage by apurinic/apyrimidinic endonuclease, gap filling and 5'-sugar phosphate removal by DNA polymerase β , and ligation by DNA ligase I or III.

The damage surveillance protein poly(ADP-ribose) polymerase (PARP)-1 detects and binds to nicks and strand breaks in DNA, including those formed as intermediates of BER [2]. When bound to DNA, PARP-1 becomes catalytically activated and poly(ADP-ribosyl)ates numerous proteins including itself [3]. The formation of poly(ADP-ribose) (PAR) is thought to assist in the recruitment of other DNA repair proteins to damaged DNA, and plays a role in the attendant chromatin modification [4]. PARP autoribosylation also results in the loss of its affinity for DNA [5].

Inhibition of PARP activity by a non-toxic dose of 4-amino-1,8-naphthalimide (4-AN) results in extreme sensitization of human and mouse fibroblasts to MMS-induced cytotoxicity [6]. Activity inhibited PARP can still bind DNA, but in the absence of PAR formation, may remain bound to damaged DNA and prevent repair [7]. Treatment of mouse fibroblasts with a sub-lethal concentration of MMS plus 4-AN leads to S phase arrest in PARP-1-expressing cells [6]. In human fibroblasts, similar treatment resulted in an ATR- and Chk1-dependent accumulation of S phase cells [8]. Later, it was shown that ATM and its downstream effector kinase, Chk2, also were activated and involved in this S phase

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Conflict of interest

The authors declare that there are no conflicts of interest.

response [9]. Immobilized PARP at strand breaks may result in stalling of replication forks, replication fork collapse and production of lethal double-strand-breaks (DSBs). In a recent study, DSBs were found to accumulate in S phase cells after treatment with the combination of MMS and 4-AN [10].

In mammalian cells, NBS1 is a part of the Mre11/Rad50/NBS1 (MRN) complex that localizes in nuclear foci in response to DSB-inducing agents, and links DSB recognition and repair, recruitment of ATM, cell-cycle checkpoint signaling, and regulation of chromatin remodeling [11–13]. Mutations in the human NBS1 gene result in Nijmegen breakage syndrome (NBS), a rare disorder with abnormal responses to ionizing radiation (IR) that resemble those in patients with ataxia-telangiectasia (AT) [14].

Human Chk1- and Chk2-dependent S phase checkpoint responses have been well documented [15]. In an alternate pathway, structural maintenance of chromosomes (SMC)1 is phosphorylated on two sites (Ser957 and Ser966) in response to IR in an ATM- and NBS1-dependent manner and serves as an effector in an ATM/NBS1-dependent S phase checkpoint [16]. Phosphorylation of NBS1 (Ser343) by ATM is required for recruitment of ATM to sites of DNA damage and for the ATM-mediated phosphorylation of SMC1 that is required for activation of the S phase response [16]. Human cells over-expressing SMC1 mutant proteins that cannot be phosphorylated lack an IR-induced S phase checkpoint, and are also hypersensitive to the DNA damaging agents IR and MMS [17]. After exposure of cells to UV or hydroxyurea, SMC1 is phosphorylated in an ATM-independent manner [18]. NBS1 is phosphorylated by ATR when replication forks are stalled, or in response to UV exposure [19]. Phosphorylation of NBS1 facilitates the SMC1 activation required for the UV-induced intra-S phase checkpoint. Additionally, phosphorylation of SMC1 as a partner in the SMC1/SMC3 cohesin complex established during S phase is recruited to damage sites and facilitates DSB repair [20,21].

In view of the continued interest in development of PARP inhibitors for use as single and combination agents in cancer chemotherapy [22,23], it is important to understand cellular pathways or targets that could further sensitize cells beyond that afforded by PARP inhibition alone. As outlined above, combination of a sub-lethal dose of methylating agent (i.e., MMS) with a PARP inhibitor (4-AN) is considerably more cytotoxic than is treatment with either agent alone. Progress has been made toward understanding the mechanism of this enhancement that results in activation of ATR/Chk1 and ATM/Chk2 signaling pathways, production of DSBs and an S phase checkpoint.

Any other factors involved in this response are considered to be potential targets. NBS1, as a known modulator of ATR and ATM activities, and a component of the MRN complex that responds to DSBs, is a potential candidate. Here, we investigate involvement of an NBS1/SMC1-mediated S phase checkpoint in MMS + 4-AN-treated cells. This pathway has been observed following UV and IR, and also other types of DNA damage, e.g., nucleoside analog replication stalling [24], thymidine-induced replication fork stress [25] and in response to hexavalent chromium [26]. Little is known regarding the influence of PARP inhibition, or of the methylating agent/PARP inhibitor combination treatment, on NBS1 activation. To further recognize how to mediate the cellular response to MMS + 4-AN treatment, we now characterize NBS1 and SMC1 phosphorylation in human fibroblasts and also determine the influence of NBS1 on the well documented ATR/Chk1 and ATM/Chk2 pathways.

2. Materials and methods

2.1. Cell lines

NBS1-1LB1 NBS1-deficient fibroblasts (NBS1) are SV40-transformed and derived from primary NBS1-1LB cells obtained from an NBS1 patient [27]. The complemented NBS1-1LB1/#8-R2 cells (NBS1 comp) contain one extra human chromosome 8 [28]. Both cell lines were obtained and used with the permission of Dr. M.Z. Zdzienicka. ATRkd cells are SV40-transformed human fibroblasts expressing an ATR kinase dead (kd) mutant under the control of a tetracycline-inducible promoter [29], and were obtained from Fred Hutchinson Cancer Research Center. The AT cells are SV40-transformed human fibroblasts derived from an ataxia-telangiectasia (AT) patient. The AT-complemented (AT comp) cell line has an episomally maintained plasmid encoding a wild-type copy of the ATM gene [30]. As a control, the AT cell line contains the plasmid vector used in the complementation. Both AT cell variants were obtained from Coriell Cell Repository.

2.2. Primary antibodies

Anti p95/NBS1 mouse monoclonal antibody was obtained from BD Transduction Laboratories (611870), and anti p95/NBS1 rabbit antibody from Cell Signaling (#3002). Anti phospho-p95/NBS1 rabbit antibody (ser343) was from Abcam (ab 47272). Anti-SMC1 rabbit antibody was from Cell Signaling (#4802), and anti phospho-SMC1 (Ser957 and Ser966) were from Bethyl Laboratories (A300-045A and A300-050A, respectively). Anti phospho-Chk1 (Ser345) rabbit antibody was from Cell Signaling (#2348) and anti Chk1 rabbit and mouse antibodies were from Santa Cruz (sc-7898 and sc-8408, respectively). Anti Chk2 rabbit antibody was from Santa Cruz (sc-9064), and anti phospho-Chk2 (Thr68) rabbit antibody was from Cell Signaling (#2661). Anti phospho-ATM (Ser1981) monoclonal and anti-ATM polyclonal antibodies were from Santa Cruz (sc-47739 and sc-7230, respectively). Probing with anti α -tubulin, clone DM 1A mouse monoclonal antibody (Sigma T-9026) was used as a loading control.

2.3. Cytotoxicity studies

NBS1 or NBS1 comp cells were seeded at a density of 20,000 cells per well in six-well plates. The next day, cells were treated for 1 h with a range of MMS concentrations (Sigma-Aldrich), plus 4-AN (10 μ M) (Acros) as indicated. Cells were then washed with Hanks' balanced salt solution (HBSS) (HyClone) and incubated with control medium or medium containing 4-AN. Incubations were continued for a total of 48 h. The medium was then removed, cells were washed with HBSS, and further incubated in control medium for an additional 7–10 days until untreated control cells were 80% confluent. Cells (triplicate wells for each drug concentration) were counted by a cell lysis procedure [31], and results were expressed as the number of cells in drug-treated wells relative to control wells (% control growth).

2.4. Flow cytometric cell cycle analysis

NBS1 and NBS1 comp cells (3.3×10^6) were seeded in 100-mm dishes. The following day, cells were treated for 1 h with MMS (0.25 mM) in the absence or presence of 4-AN (10 μ M), as indicated. After exposure to MMS, cells were washed with HBSS and incubated with fresh medium alone or medium containing 4-AN for up to 24 h. Before the times specified (2–24 h) after the beginning of exposure to MMS, 10 μ M bromodeoxyuridine (BrdUrd) (Sigma-Aldrich) was added to the dishes for 2 h to pulse-label the cells. Cells were washed with phosphate-buffered saline (PBS), harvested by trypsinization, and then washed a second time with PBS. The cell pellet obtained after centrifugation was resuspended in 100 μ l of cold PBS, and the cells were dropped slowly into 70% ethanol and allowed to fix at

4°C overnight. The samples were washed, suspended in 2 N HCl containing 0.5% Triton X-100 and incubated for 30 min at room temperature to denature DNA. The cell samples were pelleted, resuspended in 0.1 M sodium borate (pH 8.5) to neutralize the acid, and then washed with PBS. Cells were then incubated at 4°C overnight with 20 µl of anti-BrdUrd fluorescein isothiocyanate (FITC)-conjugated antibody (BD Biosciences) in PBS containing 0.5% Tween 20 and 1% bovine serum albumin (BSA) and 5 µl of 10 mg/ml RNase (Sigma-Aldrich) stock solution. The following day, the cells were pelleted, washed with PBS, and resuspended in 1 ml of PBS containing 5 µg/ml of propidium iodide (PI) (Sigma-Aldrich). The samples were analyzed by flow cytometry using ModFit LT software (Verity Software House, Inc.). Data represent the mean of two experiments or the mean ± S.E.M. of at least three independent experiments.

2.5. Western blot analysis

For western blot analysis, 7×10^6 cells were plated on 145-mm dishes one day prior to treatment with MMS, 4-AN or a combination of the two agents. In some experiments, the ATR kinase dead mutant protein was induced in ATRkd cells by treatment with doxycycline (dox) (1 µg/ml) for 48 h prior to any other treatments. One day after plating, or following the induction of the ATRkd mutant, cells were treated for 1 h with MMS (0.25 mM for NBS1 cells, 0.5 mM for ATRkd cells, 0.1 mM for AT cells), and with 4-AN (10 µM) and dox (1 µg/ml), as indicated. Cells were then washed once with HBSS and incubated with medium alone or in medium containing 4-AN (also dox for the induced ATRkd cells) for up to 24 h. For IR exposure, cells were plated as described above one day prior to exposure. Before irradiation, cold medium was added to the cells and ice-cold cells were irradiated in a ^{137}Cs irradiator (5 or 10 Gy). Afterwards, cells were returned to warm medium and harvested at time points up to 2 h. For UV exposure, cells were plated as previously described. The following day, cell monolayers were washed twice with HBSS and the buffer aspirated before UV (254 nm) exposure in a Stratalinker. Cells were then returned to growth medium and harvested at 2 h.

For preparation of whole cell extracts, cells were washed with PBS, scraped, collected into PBS and centrifuged. Cell pellets were flash frozen in dry ice and thawed by resuspension in Buffer I (10 mM Tris-HCl, pH 7.8, 0.2 M KCl, 25 mM NaF and complete protease inhibitor cocktail (Roche)). An equal volume of Buffer II (10 mM Tris-HCl, pH 7.8, 0.2 M KCl, 25 mM NaF, 2 mM EDTA, 40% glycerol, 0.2% NP-40 and 2 mM dithiothreitol (DTT)) was then added as described previously [32]. The suspension was rotated for 1 h at 4°C and extracts were clarified by centrifugation in a microcentrifuge at full speed for 15 min at 4°C. Total protein concentration of extracts was determined by the Bio-Rad assay using BSA as protein standard.

Extract protein samples (60 µg) were loaded onto 4–12% Bis-Tris NuPAGE gels (Invitrogen) and electrophoresed in NuPAGE MOPS or MES running buffer at 4°C. Proteins were transferred to nitrocellulose filters at 25 V overnight, also in the cold. Following transfer, filters were blocked overnight at 4°C in 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBST). Filters were then incubated for 2 h at room temperature or overnight at 4°C with primary antibody. After washing with TBST, filters were further incubated with anti-rabbit or anti-mouse IgG-horseradish peroxidase (HRP) conjugated secondary antibody as appropriate (1:2,000–1:20,000 dilution, Bio-Rad) and visualized using Super Signal chemiluminescent detection (Thermo Scientific) according to the manufacturer's instructions. In most experiments where blots were probed with multiple primary antibodies, they were stripped for up to 30 min at 37°C or room temperature in Restore Western Blot Stripping Buffer (Thermo Scientific), washed three times in TBST, then blocked in 5% nonfat dry milk/TBST overnight.

2.6 Immunoprecipitation of ATM

For IR experiments, NBS1 and NBS1 comp cells (7.5×10^6) were plated on 150-mm dishes one day prior to irradiation. Cold medium was added to the cells and ice-cold cells were exposed to 5 Gy of radiation in a ^{137}Cs irradiator. Afterwards, cells were returned to warm medium and incubated for 15 min. For MMS and 4-AN experiments, cells were treated for 1 h with MMS (0.25 mM) and 4-AN (10 μM). Cells were then washed once with HBSS, and incubated with 4-AN for an additional hour before harvesting.

At the appropriate time, monolayers of cells were washed with PBS, scraped, collected into PBS and centrifuged. Cells were suspended in 100 μl of extraction buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 450 mM NaCl, 50 mM NaF, 0.2 mM EDTA, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, 0.25 mg/ml AEBSF, and 0.5 mM DTT), snap frozen in liquid nitrogen and thawed on ice. Freeze/thaw cycles were repeated two more times. Extracts were clarified by centrifugation in a microcentrifuge at full speed for 10 min at 4°C. Extracts were then supplemented with NP-40 to 0.1% and mixed overnight at 4°C with 1 μg of rabbit polyclonal antibody directed against ATM. Antibody was then collected with 10 μl of protein A sepharose (GE Healthcare) for 2 h at 4°C. The beads were then washed three times in 1 ml of wash buffer (20 mM HEPES-KOH, pH 7.9, 10% glycerol, 100 mM NaCl, 50 mM NaF, 0.2 mM EDTA, 0.1% NP-40, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, 0.25 mg/ml AEBSF, and 0.5 mM DTT).

Proteins were eluted from the beads with SDS-sample buffer and loaded onto 4–12% Bis-Tris NuPAGE gels and electrophoresed in NuPAGE MOPS running buffer. Proteins were transferred to nitrocellulose filters at 25 V overnight. Following transfer, filters were blocked in 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBST) at room temperature for 1 h. Filters were then incubated overnight at 4°C with mouse monoclonal antibody directed against ATM phosphorylated at Ser1981 (1:1,000 dilution). After blotting with anti-mouse IgG-HRP secondary antibody (1:10,000 dilution), chemiluminescent detection was performed. Afterwards, filters were stripped for 10 min at 37°C and 5 min at room temperature in Restore Western Blot Stripping Buffer and washed twice in PBST. Filters were then incubated overnight at 4°C with rabbit polyclonal antibody directed against total ATM (1:1,000 dilution). Following blotting with anti-rabbit IgG-HRP secondary antibody (1:10,000 dilution), protein was again detected by chemiluminescence.

3. Results

For each of the experiments described involving wild-type, ATR-deficient, ATM-deficient, or NBS1-deficient cells, we include data obtained following exposure to UV and IR. Firstly, we wished to confirm that our western techniques and cell lines give results in agreement with previously published data, and secondly, to obtain reference information for comparison with the MMS and 4-AN data. Figure panels presenting reference or introductory data (for UV, IR or MMS + 4-AN) are smaller in size than those illustrating novel information.

3.1. Role of ATR in the phosphorylation of NBS1 in response to MMS + 4-AN

Treatment of human fibroblasts with the combination of MMS + 4-AN results in phosphorylation of the checkpoint protein Chk1, but this is not observed in control untreated cells, or in cells treated with MMS or 4-AN as single agents [8]. Shown in Fig. 1A are immunoblotting results obtained 24 h after dosing ATR kd cells. Phosphorylation of Chk1 at Ser345 observed following the combination of MMS + 4-AN (lane 4) was abrogated under conditions where the transdominant inhibitor, kinase dead ATR, is expressed (+ dox) and ATR is inactivated (lane 8). These results indicated an extreme ATR-dependence in the

activation of Chk1, and are consistent with the hypothesis of an ATR/Chk1-dependent MMS + 4-AN-induced S phase delay [8].

NBS1 is phosphorylated by ATR under conditions where replication forks are stalled [19]. Similar to our Chk1 data, we find that NBS1 is phosphorylated 24 h after treatment of cells with MMS + 4-AN (Fig. 1B, lane 4), but not when cells were treated with either agent alone (Fig. 1B, lanes 2 and 3). Phospho-NBS1 (Ser345) was present, but significantly reduced in ATR-inactivated cells 24 h after treatment with MMS + 4-AN (Fig. 1B, lane 8), indicating a partial dependence on ATR in this NBS1 phosphorylation event.

The MRN complex, including NBS1, is involved in recruitment of ATR to damaged DNA, and is required for the ATR-mediated phosphorylation of NBS1 after both replication fork stalling and UV exposure [19,33,34]. As reported previously [19], ATR-dependent phosphorylation of NBS1 was apparent in cells after low (5 J/m²) dose UV exposure (data not shown). In addition, the UV-dependent phosphorylation of NBS1 observed 2 h after exposure of ATRkd cells to higher dose UV (30 and 50 J/m²) was also strongly ATR-dependent (Fig. 1C). NBS1 is phosphorylated following exposure of cells to IR, but in contrast to UV, phosphorylation of NBS1 observed 2 h after IR was completely ATR-independent (see Fig. 1D).

Next, we determined the requirement for ATR in the phosphorylation of NBS1 during the 24 h period following treatment of cells with MMS + 4-AN. Throughout the entire time course studied (2–24 h), the level of NBS1 phosphorylation was considerably elevated in cells under conditions (– dox) where ATR could be activated. Thus, the level of phospho-NBS1 determined over the entire 24 h time course showed a consistent pattern of significant ATR-dependence (Fig. 1E, top panel). These results indicate that ATR activity is important, though not essential, for NBS1 phosphorylation in MMS + 4-AN-treated cells.

3.2. Role of NBS1 in survival and S phase checkpoint response after MMS + 4-AN

Since previous data showed that inhibition of ATR activation resulted in sensitization of human fibroblasts to MMS + 4-AN and abrogation of the S phase checkpoint [8], we next examined MMS + 4-AN-induced phenotypes in NBS1-deficient and NBS1 comp cells. The PARP inhibitor, 4-AN, significantly sensitized both NBS1 cell types to MMS (Fig. 2A). NBS1 has been shown to influence BER of MMS-induced damage and to be important for cell survival [35]. Here, NBS1 complementation resulted in enhanced resistance to MMS and also to MMS + 4-AN (Fig. 2A). The results with these cells are very similar to those observed in AT and AT complemented cells [9].

Phosphorylation of NBS1 is important for down-regulation of DNA replication and an S phase checkpoint in response to UV- and IR-induced DNA damage [19,36]. Treatment of NBS1-deficient cells with MMS + 4-AN failed to achieve an accumulation of S phase cells or any delay in S phase of the cell cycle compared with control untreated cells (Fig. 2B). In contrast, an increased proportion of S phase cells was observed following exposure of NBS1 comp cells to MMS + 4-AN (Fig. 2C). The accumulation of S phase cells compared with control untreated cells became apparent at 4 h after treatment and peaked at 8 h (Fig. 2C). These results suggest that NBS1 plays a critical role in signaling during the MMS + 4-AN-initiated S phase checkpoint. As the proportion of S phase cells decreased between 8 and 12 h (Fig. 2C), there was a parallel accumulation of MMS + 4-AN-treated NBS1 comp cells in G2/M (Fig. 2D). This phenotype has been commonly observed at later times in MMS + 4-AN-exposed wild-type equivalent cells [8,9].

3.3. Phosphorylation of Chk1 in response to DNA damage as a function of NBS1 expression

Since phosphorylation of Chk1 after MMS + 4-AN exposure is dependent on ATR activity (Fig. 1A) [8], we considered the possibility that NBS1 may facilitate the ATR-dependent phosphorylation of Chk1. NBS1 has been shown to be required for efficient recruitment or retention of ATR after replication fork stalling [34], but was not required for Chk1 phosphorylation after UV [37]. In line with this latter observation, strong phosphorylation of Chk1 at Ser345 was seen at 2 h after UV irradiation (10–40 J/m²) of both NBS1-deficient and NBS1-proficient cells (Fig. 3A). However, an additional band representing NBS1-dependent hyperphosphorylation of Chk1 was also observed (top panel, Fig. 3A). The results suggest that NBS1 expression does not have a strong influence on Chk1 phosphorylation after UV. Interestingly, phosphorylation of Chk1 was observed after 5 Gy IR (Fig. 3B), and there was some elevation in the phosphorylation level of Chk1 in NBS1-expressing cells, particularly at early times (0.25 and 0.5 h; see lanes 4 and 6).

In contrast to the significant ATR-dependent phosphorylation of Chk1 following treatment of cells with MMS and 4-AN (Fig. 1A) [8], little NBS1-dependence was observed for Chk1 phosphorylation, possibly only at 2 h following treatment (Fig. 3C, compare lanes 3 and 4 of top panel). At all later times (4–16 h), similar levels of phospho-Chk1 were found in both NBS1-expressing and non-expressing cell lines (Fig 3C). The results indicate that, similar to UV data outlined above, there is minimal requirement for NBS1 for the documented MMS + 4-AN-induced phosphorylation of Chk1.

3.4. Role of ATM in the phosphorylation of NBS1 in response to MMS + 4-AN

We recently reported that treatment of human fibroblasts with MMS + 4-AN resulted in phosphorylation of Chk2, but this was not observed in control untreated cells, or cells treated with MMS or 4-AN as single agents [9]. Representative data obtained 20 h after treatment are shown in Fig. 4A. While the MMS + 4-AN-induced level of phospho-Chk2 (Thr68) was greatest in AT comp cells (lane 8), there was still a significant level in AT-deficient cells (lane 4) indicating a partial ATM independence. Interestingly, there was robust phosphorylation of NBS1 (Ser343) in both AT and AT comp cells 24 h after MMS + 4-AN treatment (Fig. 4B, compare lanes 4 and 8), suggesting that ATM was not required for NBS1 phosphorylation at this time point. Similar to Chk2 (Fig. 4A), phosphorylation of NBS1 at 24 h was specific to the combined MMS + 4-AN treatment, and phospho-NBS1 was not detected in control untreated cells or following exposure to either agent alone (Fig. 4B, lanes 1–3 and 5–7).

NBS1 is phosphorylated in a mostly ATM-independent manner following exposure of cells to UV (see Fig. 4C) [18], whereas ATM is required for NBS1 phosphorylation after IR (see Fig. 4D)[16]. Here, we confirm strong phosphorylation of NBS1 in ATM-expressing cells 2 h after exposure to 10 Gy IR (+ ATM; Fig. 4D, lane 4), but not in cells deficient in ATM (– ATM, lane 2). Comparing the time course of NBS1 phosphorylation following MMS + 4-AN treatment of AT-proficient and -deficient cells, only at early times (i.e., 2 and 8 h) was there some suggestion of ATM involvement (Fig. 4E, compare lanes 3 and 4), whereas similar levels of phospho-NBS1 were observed at later times (16–24 h, Fig. 4B and E). Detection of phosphorylated H2AX (γ H2AX) is widely used as an early indicator of DSB formation in cells [38]. Flow cytometric analysis detected γ H2AX, thus predicted formation of DSBs, in these cells as early as 2 h after MMS + 4-AN treatment (data not shown), a likely explanation for early activation of ATM.

Taken together, the results presented in Figs. 1E and 4E indicate overlapping functions of ATR and ATM in NBS1 phosphorylation after MMS + 4-AN, perhaps due to the complexity

of DNA damage lesions formed as a result of this combination. Whereas ATM is most important at 2 h for the NBS1 phosphorylation resulting from MMS + 4-AN treatment, ATR is more critical throughout long-term (24 h) experiments (compare Figs. 4E and 1E).

3.5. Phosphorylation of Chk2 in response to DNA damage as a function of NBS1 expression

Phosphorylation of Chk2 (Thr68) showed some NBS1-dependence at 15 and 30 min after exposure to 5 Gy IR (see Fig. 5A). This is consistent with previous results indicating that Chk2 phosphorylation after IR is modestly impaired in cells lacking NBS1 [39]. Levels of phospho-Chk2 in NBS1-proficient and NBS1-deficient cells after MMS + 4-AN treatment were similar, with only a slight enhancement at early times (2–8 h) in NBS1-expressing cells (+ NBS1, Fig. 5B). Therefore, NBS1 has only a minimal role in the known activation of both Chk1 (Fig. 3C) and Chk2 (Fig. 5B) following exposure of cells to MMS + 4-AN.

Autophosphorylation (activation) of ATM in response to DNA damage, in addition to its recruitment to DNA breaks, is modulated by the MRN complex [40]. Next, we examined the role of NBS1 in ATM autophosphorylation after MMS + 4-AN treatment, utilizing IR as a positive control DNA damaging agent. In NBS1 comp cells (+ NBS1), significant ATM autophosphorylation was seen 15 min after exposure to IR (Fig. 5B, top panel, lane 4), whereas there was negligible ATM phosphorylation in NBS1-deficient cells (– NBS1, Fig. 5B, top panel, lane 3) or in non-irradiated control cells (lanes 1 and 2). After MMS + 4-AN treatment, significant autophosphorylation of ATM was observed at 2 h in NBS1 comp cells (+ NBS1, Fig. 5B, 3rd panel, lane 4), whereas a significantly lower level of ATM phosphorylation was seen in NBS1-deficient cells (– NBS1, Fig. 5B, 3rd panel, lane 3).

Taken together, the results suggest formation of DSBs and demonstrate ATM activation 2 h following exposure to MMS + 4-AN (Fig. 5C). ATM is important for the phosphorylation of NBS1 at the 2 h time point (Fig. 4E, compare lanes 3 and 4 of top panel). Similarly, NBS1 has most influence on Chk2 phosphorylation at 2 h (Fig. 5B, compare lanes 3 and 4 of top panel). However, it is apparent that ATM-independent phosphorylation of NBS1 (Fig. 4E) and ATM-and NBS1-independent Chk2-phosphorylation (Fig. 4A and 5B) are increasingly apparent at later times.

3.6 Role of NBS1 in the DNA damage-dependent phosphorylation of SMC1

Since SMC1 acts as a downstream effector in the NBS1-dependent S phase checkpoint, we examined a role for NBS1 in SMC1 phosphorylation. As expected, after both UV (see Fig. 5D) and IR exposure (see Fig. 5E), phosphorylation of SMC1 was severely compromised in the NBS1-deficient cells (– NBS1). Following exposure of cells to MMS + 4-AN, the level of phospho-SMC1 observed in NBS1-proficient cells was elevated compared to NBS1-deficient cells, but the difference between the cell lines became less at later time points (Fig. 5F). Thus, in contrast to the strong requirement for NBS1 for SMC1 phosphorylation after UV and IR exposure, a lesser NBS1-dependence was seen following MMS + 4-AN exposure.

3.7. Requirement of ATR and ATM for MMS + 4-AN-induced phosphorylation of SMC1

We next investigated the roles of the upstream kinases ATR and ATM in the NBS1/SMC1 signaling observed as a result of MMS + 4-AN-induced DNA damage. As expected [33], phosphorylation of SMC1 after UV exposure was strongly ATR-dependent (see Fig. 6A), whereas there was little evidence for involvement of ATR in SMC1 phosphorylation following IR exposure (see Fig. 6B). Following MMS + 4-AN treatment, there was a partial requirement for ATR in phosphorylation of SMC1 throughout the 24 h time course studied. This partial ATR-dependence (Fig. 6C) paralleled the dependence on ATR for

phosphorylation of NBS1 (Fig. 1E). The results are consistent with involvement of an ATR/NBS1/SMC1 signaling pathway in the S phase checkpoint observed in NBS1-expressing cells after MMS + 4-AN.

As described [18], phosphorylation of SMC1 after UV exposure was ATM-independent (see Fig. 6D), whereas SMC1 phosphorylation after exposure to 10 Gy IR was entirely ATM-dependent (see Fig. 6E). Similar to the ATM dependence pattern seen for NBS1 phosphorylation (Fig. 4E), ATM-dependent SMC1 phosphorylation was observed at early times (2–8 h) in cells treated with MMS + 4-AN (Fig. 6F), but not at later times. Thus in MMS + 4-AN-treated cells, phosphorylation of SMC1 showed dependence on ATR- and ATM-mediated phosphorylation of NBS1, suggesting important roles for SMC1 and NBS1 in this cellular stress response.

4. Discussion

A sub-lethal dose of the DNA methylating agent MMS, combined with a non-toxic concentration of the PARP inhibitor 4-AN, results in an ATR/Chk1-dependent accumulation of cells in S phase [6,8] with ATM and Chk2 critical for sustaining the S phase checkpoint [9]. Here, we investigate whether treatment of cells with MMS + 4-AN also involves phosphorylation of NBS1 and SMC1, and activation of a Chk1/Chk2-independent branch of the S phase checkpoint. Since DNA DSBs accumulated in MMS + 4-AN-treated cells [10], we suspected involvement of NBS1, a component of the MRN complex, in some component of the signaling or repair response.

The first indication for NBS1 involvement was the finding that, similar to phosphorylation of Chk1 (Fig. 1A), ATR-dependent phosphorylation of NBS1 could be observed 24 h after treatment of cells with the combination of MMS + 4-AN, but not in control cells or those treated with a single agent (Fig. 1B). A significant degree of ATR-dependence for the appearance of phospho-NBS1 was observed throughout the entire 24 h period following treatment with MMS + 4-AN (Fig. 1E). Additionally, NBS1 was required for accumulation of S phase cells following exposure to the MMS + 4-AN combination (compare Fig. 2B and C). This NBS1-dependent S phase checkpoint was likely not Chk1-dependent, since MMS + 4-AN resulted in phosphorylation of Chk1 in a relatively NBS1-independent manner (Fig. 3C). These findings suggested a major role for ATR in phosphorylation of NBS1 after cellular exposure to MMS + 4-AN (Fig. 1E), in addition to the ATR-dependent, but mostly NBS1-independent, phosphorylation of Chk1 (Fig. 3C).

In contrast to the substantial role of ATM in Chk2 activation following the combination of MMS + 4-AN [9], ATM-dependent NBS1 phosphorylation occurred only at early time points (2 and 8 h) after MMS + 4-AN (Fig. 4E). Thus, analysis at 24 h revealed equivalent MMS + 4-AN-dependent phosphorylation of NBS1 in both AT and AT comp cells (Figs. 4B and 4E). Again, this result is more in line with data obtained following UV exposure (see Fig. 4C), and in contrast to the ATM-dependence observed following IR (see Fig. 4D).

The appearance of phospho-Chk2 is highly NBS1-dependent after IR exposure (see Fig. 5A). The enhanced phosphorylation of Chk2 (a downstream substrate of ATM) 15 min after IR exposure of NBS1 comp (+ NBS1) cells (Fig. 5A, top panel, lane 4) is consistent with the significantly enhanced IR-induced NBS1-dependent activation of ATM at 15 min (Fig. 5C, top panel). In contrast, there was little evidence for an NBS1 requirement for Chk2 phosphorylation after MMS + 4-AN, other than at 2 h (Fig. 5B, compare lanes 3 and 4). A corresponding enhanced activation of ATM was observed in NBS1 comp cells 2 h after MMS + 4-AN (Fig. 5C, bottom panels), plus phosphorylation of NBS1 after MMS + 4-AN was enhanced in AT-proficient cells primarily at this early time point (Fig. 4E).

Following UV exposure, phosphorylation of NBS1 and SMC1 were strongly ATR-dependent (see Fig. 1C and Fig. 6A). UV enhanced phosphorylation of SMC1 by ATR activation (– dox, Fig. 6A) parallels the observed ATR-enhanced phosphorylation of NBS1 (Fig. 1C). These results confirm activation of the ATR/NBS1/SMC1 pathway in these cells following UV-induced DNA damage. In cells treated with MMS + 4-AN, higher levels of both phospho-NBS1 (Fig. 1E) and phospho-SMC1 (Fig. 6C) were observed throughout the 24 h time point in cells in which ATR was active, suggesting an enhancement of phosphorylation of both NBS1 and SMC1 by ATR. Activation of the ATR/NBS1/SMC1 signaling pathway could explain why an MMS + 4-AN-induced S phase checkpoint was seen in NBS1 comp, but not NBS1-deficient cells (Fig. 2).

Phosphorylation of SMC1 was almost entirely NBS1- and ATM-dependent following IR (see Fig. 5F and Fig. 6E). These results are consistent with activation of the ATM/NBS1/SMC1 pathway following IR-induced DNA damage. In contrast, there was some enhancement of NBS1 and SMC1 phosphorylation by ATM at early times (<8 h) after treatment of cells with MMS + 4-AN (Fig. 4E and 6F), but not to the level of ATM dependence described above after IR. Also, some dependence on NBS1 expression was seen when comparing phospho-SMC1 levels in NBS1-deficient and NBS1-proficient cells (Fig. 5F). These data indicate early activation of the ATM/NBS1/SMC1 S phase signaling pathway in response to MMS + 4-AN, since the timing would correspond to the peak times of S phase accumulation (6–8 h) in MMS + 4-AN-treated cells (Fig. 2C).

In summary, SMC1 phosphorylation after MMS + 4-AN exhibited varying degrees of dependence on NBS1 (Fig. 5F), ATR (Fig. 6C) and ATM expression (Fig. 6F). SMC1 phosphorylation as a result of DNA damage is proposed to be responsible for the NBS1-mediated S phase checkpoint. Thus, the MMS + 4-AN-induced S phase checkpoint defect in NBS1-deficient cells (compare Fig. 2B and 2C) may be due to a reduced cellular level of the critical downstream effector, phospho-SMC1.

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Fig. 1.

Role of ATR activation in the DNA damage-induced phosphorylation of Chk1 and NBS1. Analysis of (A) Chk1 and phospho-Chk1 (Ser345) and (B) NBS1 and phospho-NBS1 (Ser343) in cell extracts prepared at 24 h from ATRkd cells, either dox induced (+ dox) or not (- dox). Shown are control untreated cells and cells treated with MMS (0.5 mM for 1 h), with 4-AN (10 μ M continuously) or with MMS + 4-AN combined. ATRkd cells $\pm$ dox induction were (C) exposed to UV (0, 30 and 50 J/m²) and harvested after 2 h, (D) dosed with IR (0 or 10 Gy) and harvested at 2 h, or (E) were mock treated (control), or treated with MMS (0.5 mM for 1 h) combined with 10 μ M 4-AN and present for the entire 24 h. MMS + 4-AN treated cells were harvested for extract preparation between 2–24 h. Cell extracts were probed for phospho-NBS1 (Ser343), total NBS1 and tubulin as a loading control. Cell treatments and immunoblotting methods are described in detail in Section 2.

Fig. 2.

Hypersensitivity and cell cycle phenotypes of NBS1-deficient compared with NBS1 comp cells. (A) Hypersensitivity of NBS1-deficient cells (NBS1, open symbols) to MMS alone (circles) and the combination of MMS (1 h) + 4-AN (10 μ M for a total of 48 h, squares). Analysis was by growth inhibition as described in Section 2. Results represent the mean $\pm$ S.E.M. of three independent experiments. (B–D) Cell cycle analysis following staining with PI and BrdUrd, and flow cytometry as described in Section 2. (B) Absence of S phase accumulation of NBS1-deficient cells following treatment with MMS (0.25 mM) combined with 4-AN (10 μ M continuously). (C) Accumulation of S phase NBS1 comp cells treated with MMS + 4-AN (as in B) compared with control untreated cells. (D) Later accumulation of MMS + 4-AN-treated NBS1 comp cells in G2/M. Results with error bars represent the mean $\pm$ S.E.M. of at least three independent experiments, other data points represent the mean of two experiments.

Fig. 3.

Role of NBS1 in the DNA damage-induced phosphorylation of Chk1. NBS1 and NBS1 comp cells were treated with UV, IR or the combination of MMS + 4-AN. Extracts were made at the appropriate time and probed for phospho-Chk1 (Ser345), total Chk1 and NBS1, or with tubulin as a loading control. NBS1 (–) and NBS1 comp cells (+) were (A) exposed to UV (0, 10, 20 and 40 J/m²) and harvested at 2 h, (B) were mock treated, or exposed to IR (5 Gy) and harvested at 0.25, 0.5, 1 and 2 h, or (C) were mock treated (control), or treated with MMS (0.25 mM for 1 h) combined with 10 μM 4-AN present continuously and harvested for extract preparation from 2–16 h. Treatment and immunoblotting methods are described in detail in Section 2.

Fig. 4. Role of ATM in the DNA damage-induced phosphorylation of Chk2 and NBS1. Analysis of (A) Chk2 and phospho-Chk2 (Thr68) in extracts prepared at 20 h and (B) NBS1 and phospho-NBS1 in cell extracts prepared at 24 h from AT and AT comp cells. Shown are control untreated cells and cells treated with MMS (0.1 mM for 1 h), with 4-AN (10 μ M continuously) or with MMS + 4-AN combined. (C–E) AT (– ATM) and AT comp cells (+ ATM) were treated with UV, IR or MMS + 4-AN. Extracts were made at the appropriate time and probed for phospho-NBS1 (Ser343), total NBS1 and tubulin as a loading control. Cells were (C) exposed to UV (0, 5, 10 and 30 J/m²) then harvested after 2 h, (D) dosed with IR (0 or 10 Gy) and harvested at 2 h or (E) were mock treated (control), or treated with MMS (0.1 mM for 1 h) combined with 10 μ M 4-AN present continuously for up to 24 h and harvested for extract preparation at times between 2 and 24 h. Cell treatments and immunoblotting methods are described in detail in Section 2.

Fig. 5.

Role of NBS1 in the DNA damage-induced phosphorylation of Chk2, ATM and SMC1.

NBS1 (- NBS1) and NBS1 comp (+ NBS1) cells were treated with (A) IR or (B) MMS + 4-AN. Extracts were made at the appropriate time and probed for phospho-Chk2 (Thr68), total Chk2 and NBS1, or with tubulin as a loading control. Cells were (A) mock treated (control), or dosed with IR (5 Gy) and harvested at 15 and 30 min, or (B) were mock treated (control), or treated with MMS (0.1 mM for 1 h) combined with 10 μ M 4-AN present continuously and harvested for extract preparation at 2–16 h. (C) NBS1 (-) and NBS1 comp (+) cells were dosed with IR (5 Gy) and harvested at 15 min (top panels), or treated with MMS (0.25 mM for 1 h) combined with 4-AN (10 μ M for 2 h, bottom panels), and prepared extracts were immunoprecipitated with an anti-ATM antibody. In each experiment, immunoprecipitated samples were probed for autophosphorylated ATM (Ser1981) and total ATM. Similarly, NBS1 (-) and NBS1 comp (+) cells were treated with (D) UV, (E) IR or (F) MMS + 4-AN and extracts were probed for phospho-SMC1 (Ser957) and total SMC1, with tubulin as a loading control. Cells were (D) exposed to UV (0, 10, 20 and 40 J/m²) and harvested at 2 h, (E) dosed with IR (5 Gy) and harvested at 0.25, 0.5, 1 and 2 h, or (F) mock treated (control), or treated with MMS (0.25 mM for 1 h) combined with 10 μ M 4-AN present continuously and harvested for extract preparation between 2–16 h. Treatment and immunoblotting methods are described in detail in Section 2.

Fig. 6.

Role of ATR and ATM in the DNA damage-induced phosphorylation of SMC1. ATRkd, AT and AT-comp cells were treated with UV, IR or MMS + 4-AN. Extracts were made at the appropriate time and probed for phospho-SMC1 (Ser957 or 966), total SMC1 and tubulin as a loading control. ATRkd cells ± dox induction were (A) exposed to UV (0, 30 and 50 J/m²) and harvested after 2 h, (B) dosed with IR (0 or 10 Gy) and harvested at 2 h, or (C) were mock treated (control), or treated with MMS (0.5 mM for 1 h) combined with 10 μM 4-AN present continuously and harvested for extract preparation between 2–24 h. Similarly, AT and AT comp cells were treated and extracts probed as described above. AT (–) and AT comp (+) cells were (A) exposed to UV (0, 5, 10 and 30 J/m²) and harvested after 2 h, (B) dosed with IR (0 or 10 Gy) and harvested at 2 h, or (C) were mock treated (control), or treated with MMS (0.1 mM for 1 h) combined with 10 μM 4-AN present continuously and harvested for extract preparation between 2–24 h. Cell treatments and immunoblotting methods are described in detail in Section 2.