Phosphorylation of SDT repeats in the MDC1 N terminus triggers retention of NBS1 at the DNA damage–modified chromatin

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DNA double-strand breaks (DSBs) trigger accumulation of the MRE11–RAD50–Nijmegen breakage syndrome 1 (NBS1 [MRN]) complex, whose retention on the DSB-flanking chromatin facilitates survival. Chromatin retention of MRN requires the MDC1 adaptor protein, but the mechanism behind the MRN–MDC1 interaction is unknown. We show that the NBS1 subunit of MRN interacts with the MDC1 N terminus enriched in Ser-Asp-Thr (SDT) repeats. This interaction was constitutive and mediated by binding between the phosphorylated SDT repeats of MDC1 and the phosphate-binding forkhead-associated domain of NBS1. Phosphorylation of the SDT repeats by casein kinase 2 (CK2) was sufficient to trigger MDC1–NBS1 interaction in vitro, and MDC1 associated with CK2 activity in cells. Inhibition of CK2 reduced SDT phosphorylation in vivo, and disruption of the SDT-associated phosphoacceptor sites prevented the retention of NBS1 at DSBs. Together, these data suggest that phosphorylation of the SDT repeats in the MDC1 N terminus functions to recruit NBS1 and, thereby, increases the local concentration of MRN at the sites of chromosomal breakage.

Introduction

Double-strand breaks (DSBs) pose a major threat to genomic integrity by promoting mutations, gene deletions, and/or chromosomal translocations (Shiloh, 2003; Kastan and Bartek, 2004). These adverse effects are counteracted by genome surveillance machinery that is designed to rapidly detect DSBs, delay cell cycle progression, initiate DNA repair, or, in case of excessive damage, activate the cell death program (Zhou and Elledge, 2000; Nyberg et al., 2002; Lukas et al., 2004b). Because the maintenance of genome integrity requires extreme accuracy and coordination of elaborate, often intertwined molecular pathways, cells evolved numerous means to increase the efficiency of this process. Among others, virtually every eukaryotic cell responds to DSBs by concentrating signaling, repair, and various adaptor proteins in the vicinity of DNA lesions (Lukas et al., 2005). Although the purpose of this phenomenon (cytologically manifested by the formation of nuclear foci enriched in various DSB regulators) is not yet fully understood, several intriguing scenarios have been considered: for instance, DNA damage–induced protein accumulation may facilitate the formation of active repair holocomplexes, increase the availability of these enzymes at the sites of genetic lesions, generate a local chromatin microenvironment supportive for repair-associated DNA transactions, and/or amplify the DSB-associated signaling (Fernandez-Capetillo et al., 2004; Essers et al., 2006; Stucki and Jackson, 2006; Bartek and Lukas, 2007). Thus, the local concentration of DSB regulators emerges as a vital tool to increase the fidelity of the genome surveillance machinery.

Prominent among the cellular factors that accumulate at DSBs is the MRE11–RAD50–Nijmegen breakage syndrome 1 (NBS1 [MRN]) complex, an essential genome caretaker that regulates crucial steps of the DSB response such as DSB detection, activity of the ataxia telangiectasia mutated (ATM) kinase (the key upstream component of DSB signaling), cell cycle checkpoints (D'Amours and Jackson, 2002; Petrini and Stracker, 2003; Kobayashi et al., 2004; Stracker et al., 2004), and, as the most recent advancements suggest, induction of apoptosis (Difilippantonio et al., 2007; Stracker et al., 2007). In addition, the MRN complex participates in the resection of

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; BRCT, BRCA1 C terminal; CK2, casein kinase 2; DSB, double-strand break; FHA, forkhead-associated; IR, ionizing radiation; MRN, MRE11–RAD50–NBS1; NBS, Nijmegen breakage syndrome; SDT, Ser-Asp-Thr; shRNA, short hairpin RNA; WT, wild type.

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DNA ends, an essential step required for an error-free DSB repair by homologous recombination (Jazayeri et al., 2006). Such diverse involvement in the DSB response is reflected by the nuclear dynamics of MRN. Most notably, recent studies revealed that the distribution of NBS1 (and other MRN components) at the DSB sites is not uniform but splits into distinct subcompartments (Lukas et al., 2004a; Bekker-Jensen et al., 2006). Although a fraction of MRN interacts with single-stranded DNA formed after enzymatic DSB resection (consistent with the essential role of MRN in DSB resection), the bulk of the DSB-associated MRN accumulates within the vast regions of chromatin marked by ATM-phosphorylated histone H2AX (γ-H2AX). It is indeed this chromatin-associated fraction of MRN that cytologically manifests as the so-called ionizing radiation (IR)–induced foci.

The function of the chromatin-bound MRN has been subjected to intensive investigation and yielded important insights. Thus, it was found that the forkhead-associated (FHA) domain of NBS1 is necessary for its retention at the DSB-flanking chromatin and that its disruption impairs IR-induced foci formation of the entire MRN complex (Zhao et al., 2002; Cerosaletti and Concannon, 2003). Reconstitution experiments in human cells derived from the NBS patients suggested that the NBS1 N terminus, where the FHA domain resides, is important for survival, optimal ATM activity, and the intra–S-phase checkpoint after IR (Tauchi et al., 2001; Zhao et al., 2002; Cerosaletti and Concannon, 2003, 2004; Lee et al., 2003; Horejsi et al., 2004; Cerosaletti et al., 2006). However, because these assays were performed on the background of low levels of hypomorphic NBS1 alleles, the exact role of the chromatin-bound MRN remained a matter of debate. An important breakthrough in this discussion has been recently provided by Difilippantonio et al. (2005, 2007), who generated a mouse strain in which the endogenous NBS1 gene was replaced by a mutant with the disrupted FHA domain. Remarkably, although these mice were viable, they displayed defects in the DNA damage–induced G2/M- and S-phase checkpoints, an increased incidence of chromosomal aberrations, attenuated ATM activity after low doses of IR, and a decreased radiation survival. Although it is possible that the NBS1 N terminus supports other functions than the chromatin tethering of MRN, these results provide the most compelling evidence so far that the chromatin-bound MRN contributes to reach the threshold of the DNA damage signaling (especially after a low dose of IR), thereby guarding against chromosomal instability.

Although the requirement of the NBS1-FHA domain for the MRN focus formation has been established, the way this domain communicates with the DSB-flanking chromatin and/or the associated proteins is less clear. An earlier study suggested that the NBS1-FHA domain directly binds to γ-H2AX (Kobayashi et al., 2002). However, more recent results showed that the interaction of NBS1 with the DSBs requires MDC1, a large adaptor protein and an important upstream coordinator of the DSB-induced chromatin response (Goldberg et al., 2003; Stewart et al., 2003; Lukas et al., 2004a; Lou et al., 2006). MDC1 contains tandem BRCA1 C-terminal (BRCT) domains, which bind with high specificity to phosphorylated Ser139 of γ-H2AX (Stucki et al., 2005). As a result, MDC1 is among the first proteins to accumulate at the DSB sites, and its productive assembly in this compartment is a prerequisite for retention of most of the known chromatin-binding DSB regulators, including the MRN complex (Stucki and Jackson, 2006). The key evidence for the causative role of MDC1 for MRN focus formation was provided by several independent studies, which all showed that the siRNA-mediated knockdown of MDC1 by RNA interference in human cells (Goldberg et al., 2003; Stewart et al., 2003; Lukas et al., 2004a) or a complete MDC1 knockout in mouse cells (Lou et al., 2006) prevented MRN retention at the DSB-flanking chromatin. Importantly, kinetic measurements in living cells extended these results by showing that the MDC1 knockdown abolished MRN retention at DSBs from the earliest stages after DNA damage and that in the absence of MDC1, NBS1 could not concentrate at the DSB-flanking chromatin despite the fact that H2AX was efficiently phosphorylated in these compartments (Lukas et al., 2004a).

At lest three additional pieces of evidence suggested that the retention of MRN at the DSB-flanking (and γ-H2AX decorated) chromatin is not direct but is mediated by interaction with MDC1. First, peptides derived from the phosphorylated γ-H2AX C terminus interacted with MRN only in the presence of MDC1 (Lukas et al., 2004a). Second, MDC1 can be efficiently copurified with MRN from cell extracts (Goldberg et al., 2003; Stewart et al., 2003; Lukas et al., 2004a). Importantly, this interaction was dependent on the phosphate-binding FHA domain of NBS1, suggesting that the formation of a productive MDC1–MRN complex is phosphorylation dependent (Lukas et al., 2004a). Third, real-time imaging of protein assembly in live cells revealed that NBS1 accumulates at the DSB-flanking chromatin with a dynamics that is indistinguishable from that of MDC1 (but significantly faster than other chromatin-binding proteins such as 53BP1 or BRCA1), an observation that further supported the emerging model of the intimate relationship between MDC1 and the MRN complex (Lukas et al., 2004a; Bekker-Jensen et al., 2005; Mailand et al., 2007).

Although intriguing, these results left several mechanistic questions unanswered: most notably, it remains unclear whether the phosphosubstrate of the NBS1-FHA domain is indeed MDC1 itself or whether the MDC1–NBS1 interaction requires yet another, hitherto unrecognized (phospho)intermediate. Furthermore, the identity of the protein kinases and the phosphorylation sites that generate the recognition signal for the NBS1-FHA domain (and thus promote retention of MRN on the DSB-flanking chromatin) have not been determined. In an attempt to resolve these issues, we performed a detailed analysis of the MDC1–NBS1 interaction in vitro and in cells. We show that the NBS1-FHA domain binds to the N-terminal part of MDC1 enriched in acidic Ser-Asp-Thr (SDT) repeats. Furthermore, we provide evidence that the SDT repeats are constitutively phosphorylated, at least in part, by casein kinase 2 (CK2). These SDT phosphorylations are functionally significant because they trigger productive interaction between MDC1 and NBS1 and determine the retention of MRN at the DSB-modified chromatin.

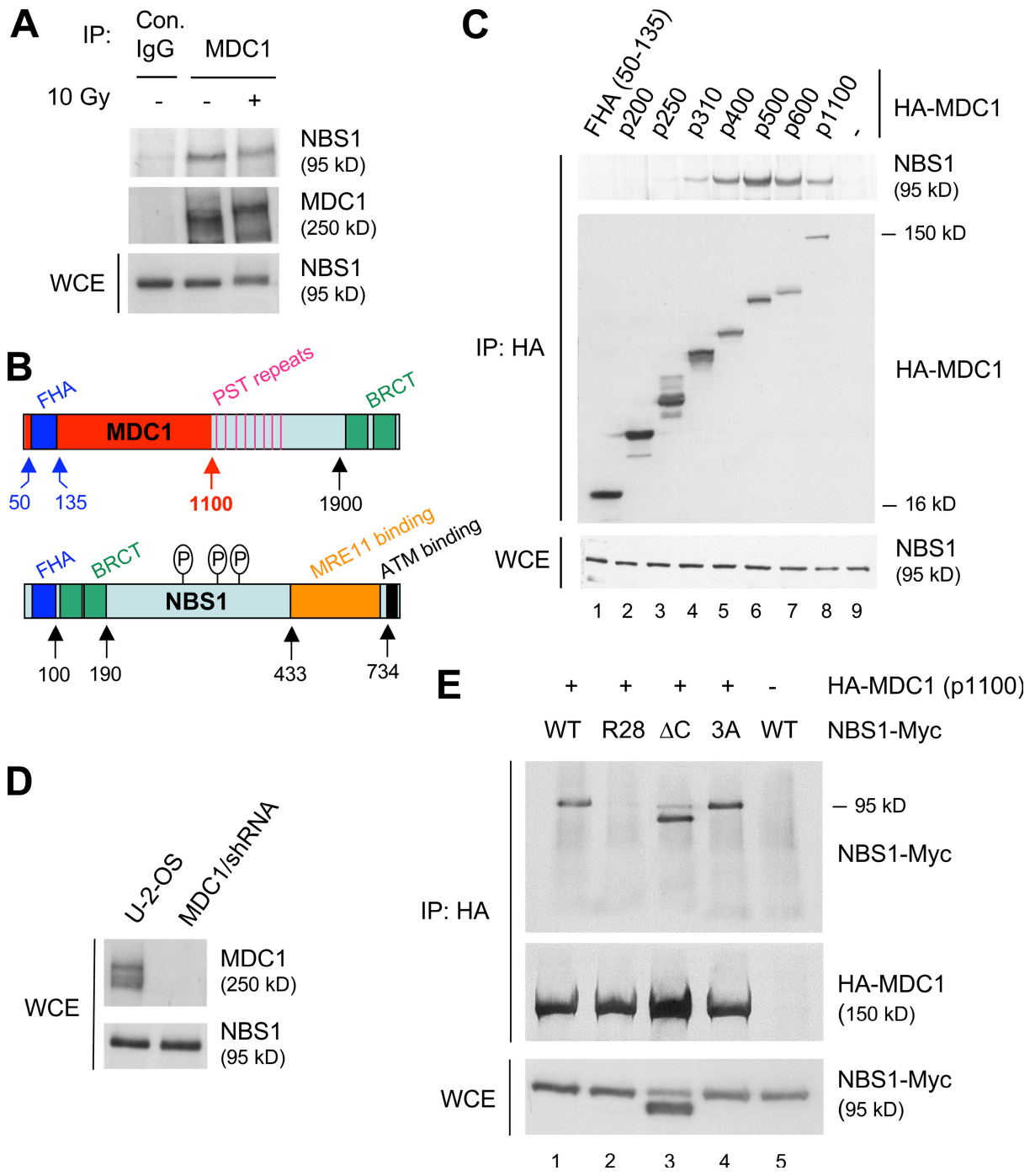

Figure 1. **Interaction between NBS1 and MDC1 is mediated by the FHA domain of NBS1 and the first 500 amino acids of MDC1.** (A) Endogenous MDC1 and NBS1 interact both before and after DNA damage. Lysates from U2OS cells either mock or γ irradiated (10 Gy for 1 h) were subjected to immunoprecipitation with an anti-MDC1 antibody and analyzed for the presence of NBS1 by immunoblotting. A nonimmune species-matched antibody was used as a control. Expression levels of NBS1 in whole cell extracts (bottom) indicate an equal input in each lane. (B) A schematic structure of MDC1 and NBS1. The red segment indicates the N-terminal region of MDC1 (amino acids 1–1,100) subjected to progressive deletion and interaction analysis with NBS1. (C) The N-terminal region of MDC1 spanning the first 500 amino acids efficiently interacts with cellular NBS1. The indicated HA-tagged fragments of the MDC1 N terminus were transfected into MDC1/shRNA cells (the numbers indicate the C-terminal amino acid of each fragment). After 24 h, lysates were prepared, subjected to immunoprecipitation with an anti-HA antibody, and analyzed by immunoblotting with antibodies to NBS1 (top) and HA (middle). The input into each reaction was controlled by NBS1 immunoblotting of the whole cell extracts as in A (bottom). (D) Efficient knockdown of endogenous MDC1. Lysates from U2OS cells and its derivative in which MDC1 is knocked down by stably integrated shRNA (MDC1/shRNA) were analyzed by immunoblotting with an MDC1-specific antibody (top). The NBS1 immunoblot serves as a loading control and indicates that the overall levels of NBS1 are not affected by the MDC1 knockdown (bottom). (E) The FHA domain of NBS1 interacts with the MDC1 N terminus. MDC1/shRNA cells were cotransfected with the HA-tagged MDC1 N terminus (p1100) and the following Myc-tagged variants of NBS1: WT (wild type), R28 (nonfunctional FHA domain), ΔC (C-terminal deletion without the MRE11 and ATM interaction domains), and 3A (alanine substitutions of serines 278, 343, and 397, the ATM phosphorylation sites). After 24 h, lysates were immunoprecipitated with an anti-HA antibody and analyzed by immunoblotting with anti-Myc (top) and anti-HA (middle) antibodies. The bottom panel is an immunoblot of whole cell extracts and shows the input of each of the Myc-tagged NBS1 proteins. WCE, whole cell extract; PST, Pro-Ser-Thr rich.

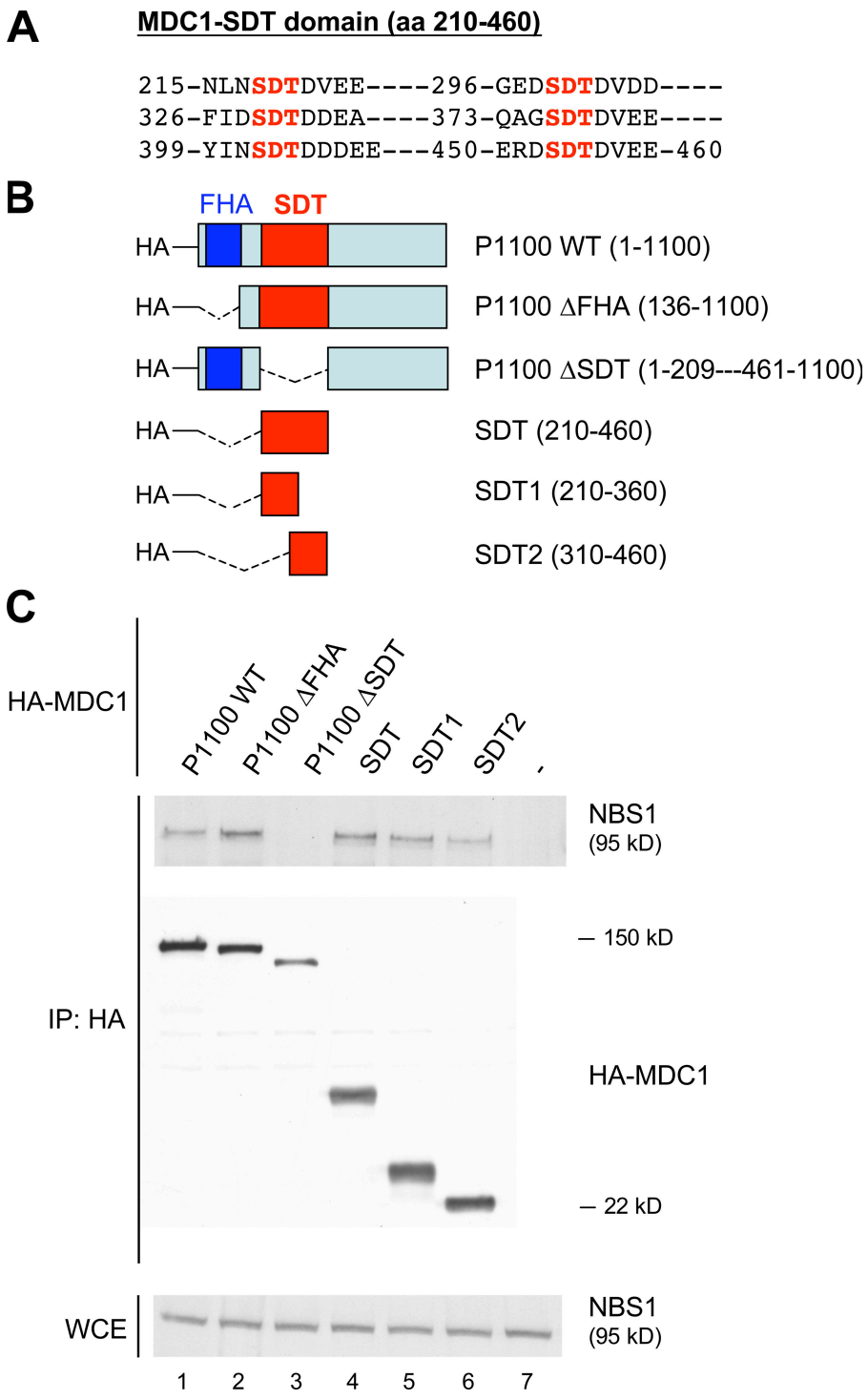

Figure 2. **The SDT-rich region within the MDC1 N terminus promotes MDC1–NBS1 interaction.** (A) The N-terminal part of MDC1 delineated by amino acids 218 and 455 contains six acidophilic Ser-Thr-Asp (SDT) repeats. (B) A schematic depiction of the MDC1 fragments used in C for the NBS1 binding assay. (C) The SDT region is necessary for and sufficient to promote interaction of the MDC1 N terminus with NBS1. MDC1/shRNA cells were transfected with the HA-tagged MDC1 fragments described in B. After 24 h, lysates were prepared, immunoprecipitated with an anti-HA antibody, and analyzed by immunoblotting with NBS1 (top) and HA (middle) antibodies. The bottom panel is an immunoblot of whole cell extracts and shows equal input of each NBS1 in each lane. WCE, whole cell extract.

Results

The FHA domain of NBS1 interacts with the N-terminal part of MDC1

Endogenous MDC1 and NBS1 proteins efficiently interact both before and after DNA damage (Fig. 1 A) and contain numerous structural motifs and potential targets for posttranslational modifications (Fig. 1 B) that might be involved in this interaction. To elucidate the requirements for the MDC1–MRN interaction, we first constructed a series of HA-tagged fragments progressively spanning the N-terminal part of MDC1 (Fig. 1 C). We transiently expressed each of these fragments in a U2OS cell line in which the endogenous MDC1 was down-regulated by stably integrated short hairpin RNA (shRNA; Fig. 1 D; Bekker-Jensen et al., 2006) and tested their ability to interact with endogenous NBS1. The MDC1 fragments were constructed so that they were not affected by the shRNA to endogenous MDC1 (see Materials and methods), and the robust down-regulation of MDC1 in this cell line (designated U2OS/shMDC1) allowed us to assess the binding efficiency of each fragment unbiased by higher-order complexes containing the

Table I. **In vivo phosphorylated SDT sites**

Site (amino acids)	Identified by phosphotryptic analysis (this study)	Identified by mass spectrometry (references)
218/220	–	–
299/301	+	Olsen et al., 2006
329/331	+	Beausoleil et al., 2004; Olsen et al., 2006
376/378	–	–
402/404	+	Olsen et al., 2006
453/455	+	Beausoleil et al., 2004; Olsen et al., 2006

endogenous protein. Under these conditions, a fragment spanning the first 500 amino acids of MDC1 appeared to be necessary and sufficient to bind cellular NBS1 (Fig. 1 C, lane 6), and this binding did not further increase after including the more C-terminal sequences (Fig. 1 C, lanes 7 and 8). Interestingly, although MDC1 also contains an FHA domain at the very N terminus, this domain (either isolated or in the context of a larger fragment spanning up to the first 200 amino acids of MDC1) appeared to be entirely dispensable for NBS1 interaction (Fig. 1 C, lanes 1 and 2).

We then performed a reciprocal analysis and tested the structural requirements of NBS1 to interact with the MDC1 N terminus. To this end, we coexpressed the HA-tagged N-terminal fragment of MDC1 spanning the first 1,100 amino acids (p1100) with various Myc-tagged variants of NBS1 and assessed their interaction by an immunoprecipitation assay. Consistent with a previous study that measured the interaction of NBS1 mutants with full-length MDC1 (Lukas et al., 2004a), we found that a point mutation within the NBS1-FHA domain ($NBS1^{R28}$) completely uncoupled NBS1 from the MDC1 N terminus (Fig. 1 E, lane 2). In contrast, neither disruption of the NBS1 C terminus responsible for interaction with MRE11 and ATM ($NBS1^{\Delta C}$) nor mutation of the three major phosphorylation sites targeted by the ATM kinase ($NBS1^{3A}$) had a measurable effect, and these mutants interacted with the MDC1 fragment as efficiently as the wild-type (WT) NBS1 (Fig. 1 E). Together, these data suggest that the MDC1 N terminus contains the necessary structural signature to allow recognition of MDC1 by the FHA domain of NBS1.

The SDT-rich region in the MDC1 N terminus mediates interaction with NBS1

Next, we subjected the N-terminal fragment of MDC1 to a bioinformatic analysis and searched for conserved motifs that may potentially mediate the interaction with NBS1. Interestingly, the region between amino acids 210 and 460 (that is, the part of MDC1 identified in the previous section as necessary and sufficient to bind NBS1) contained up to six acidic SDT motifs, some of which are conserved among different vertebrate species (Fig. 2 A and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708210/DC1). To test whether the SDT clusters influence the MDC1–NBS1 interaction, we generated another set of MDC1 fragments either spanning different parts of the SDT-containing region or harboring an in-frame deletion of the entire SDT region (Fig. 2 B). The fragment containing all six SDT repeats efficiently bound to endogenous NBS1 when expressed in the U2OS/shRNA cell line (Fig. 2 C, lane 4). Strikingly, even shorter fragments containing either three N-terminal (SDT1) or four C-terminal (SDT2) repeats still interacted with NBS1, albeit with a lower efficiency than the full-length SDT region (Fig. 2 C, lanes 5 and 6). Conversely, deletion of the entire SDT region completely abolished the ability of the MDC1 N terminus to bind NBS1 under these conditions (Fig. 2 C, lane 3). Together, these data indicate that the SDT repeats within the MDC1 N terminus might be involved in binding to NBS1, and we set out to further explore this possibility.

The SDT repeats are phosphorylated in vivo

We reasoned that if the SDT repeats mediate the MDC1–NBS1 interaction, the Ser/Thr residues within the repeats should be phosphorylated in vivo. To test this important prediction, we analyzed phosphorylation patterns of various forms of MDC1 (Fig. 3 A) immunopurified from U2OS cells metabolically labeled with [^{32}P]orthophosphate. As expected, the tryptic phosphopeptide map of endogenous MDC1 revealed considerable complexity, indicating that numerous phosphorylation events converge on these large adaptor proteins (Fig. 3 B). Strikingly, the majority of the phosphopeptide spots observed in the endogenous protein were preserved in the N-terminal fragment of MDC1 (Fig. 3 C), and some of the most prominent spots were clearly also resolved in the isolated SDT region (Fig. 3 E). Indeed, a systematic analysis of various deletion fragments and/or the SDT mutants in which the Ser/Thr residues were substituted to Ala confirmed that at least four of the prominent SDT clusters are subjected to phosphorylation in vivo (Table I). A typical example of such an assay is provided in Fig. 3 (F and G), where mutation of the second (amino acids 299/301) or the sixth (amino acids 453/455) SDT repeat eliminated the respective prominently labeled spot. Consistently, an N-terminal fragment of MDC1 terminating within the SDT region (at amino acid 400) lacked the spot corresponding to the sixth SDT (amino acids 453/455; Fig. 3 D). In addition, assessment of the spot charges after mutating single phosphoacceptor sites (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200708210/DC1) and a direct phospho–amino acid analysis of the prominently labeled SDT repeats (Ser299/Thr301 and Ser453/Thr455, respectively; Fig. S2 B) confirmed that both Ser and Thr residues were simultaneously phosphorylated. Together with recent results from the large-scale phosphoproteomic projects that independently confirmed phosphorylation of the SDT clusters (Table I), these data show that the SDT repeats within the MDC1 N terminus are indeed phosphorylated in vivo and suggest that the SDT-associated phosphates may serve as docking sites for the FHA domain of NBS1.

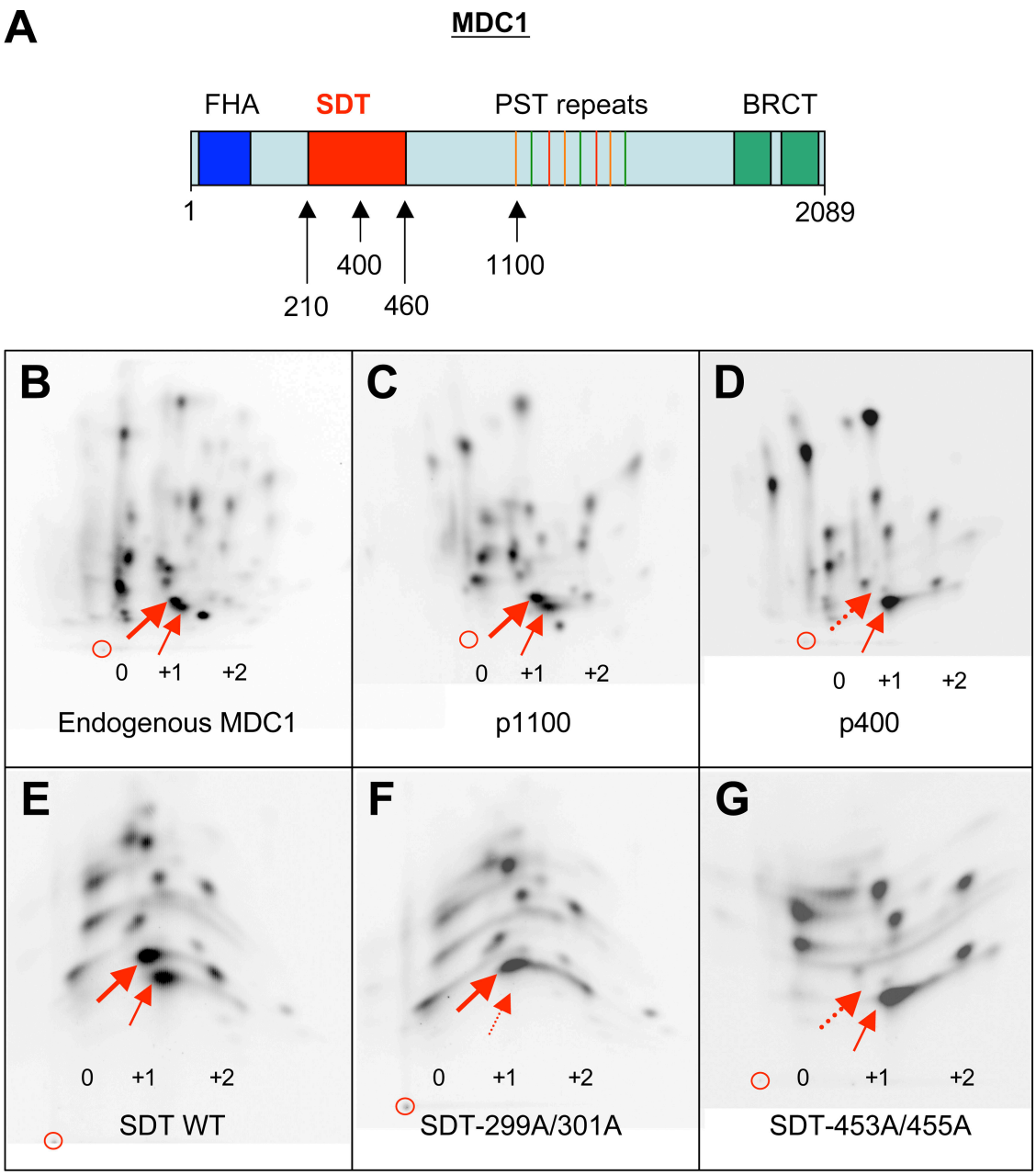

Figure 3. **MDC1 N terminus is phosphorylated in vivo, and some of the prominent phosphorylation sites include the SDT clusters.** (A) A schematic diagram of MDC1 and the position of the fragments used to identify phosphorylation sites in vivo. (B–G) Examples of SDT-associated Ser/Thr phosphorylations in vivo. U2OS cells either untransfected (B) or transfected with the indicated HA-tagged MDC1 constructs (C–G) were labeled with ^{32}P for 4 h. Lysates from labeled cells were immunoprecipitated with anti-MDC1 (B) or anti-HA (C–G) antibodies, and the isolated proteins were subjected to tryptic digestion. The resulting peptides were separated by electrophoresis followed by thin-layer chromatography and analyzed by phosphorimager. The spots indicated by arrows mark prominent phosphopeptides derived from Ser^{299}/Thr^{301} (thin arrows) and Ser^{453}/Thr^{455} (bold arrows). Open circles mark the loading spots, and numbers indicate the charge of the phosphopeptides. Dotted arrows indicate the absence of a phosphopeptide spot.

CK2 phosphorylates the SDT repeats and promotes interaction between the MDC1 N terminus and NBS1

To test this hypothesis, we first tried to identify the kinase that can phosphorylate the SDT repeats. Sequence analysis by the recently introduced NetworKIN approach (Linding et al., 2007) revealed that the SDT clusters strongly resemble consensus sites for the acidophilic protein kinase CK2. Therefore, from *Escherichia coli*, we purified a GST-tagged SDT fragment (spanning amino acids 181–480) and its variant in which the Ser/Thr residues in all six SDT repeats were changed to Ala (designated as 12A) and subjected these fragments to phosphorylation by CK2 in an in vitro kinase assay. Indeed, CK2 efficiently phosphorylated the WT SDT fragment in vitro (Fig. 4 A, lane 5), and this phosphorylation was inhibited by adding the CK2 inhibitor into the kinase reaction (Fig. 4 A, lane 8). Significantly, phosphorylation of the SDT-12A mutant was very inefficient (Fig. 4 A, lane 6), suggesting that the bulk of the CK2-mediated phosphorylations within the MDC1 N terminus was indeed targeted to the SDT repeats.

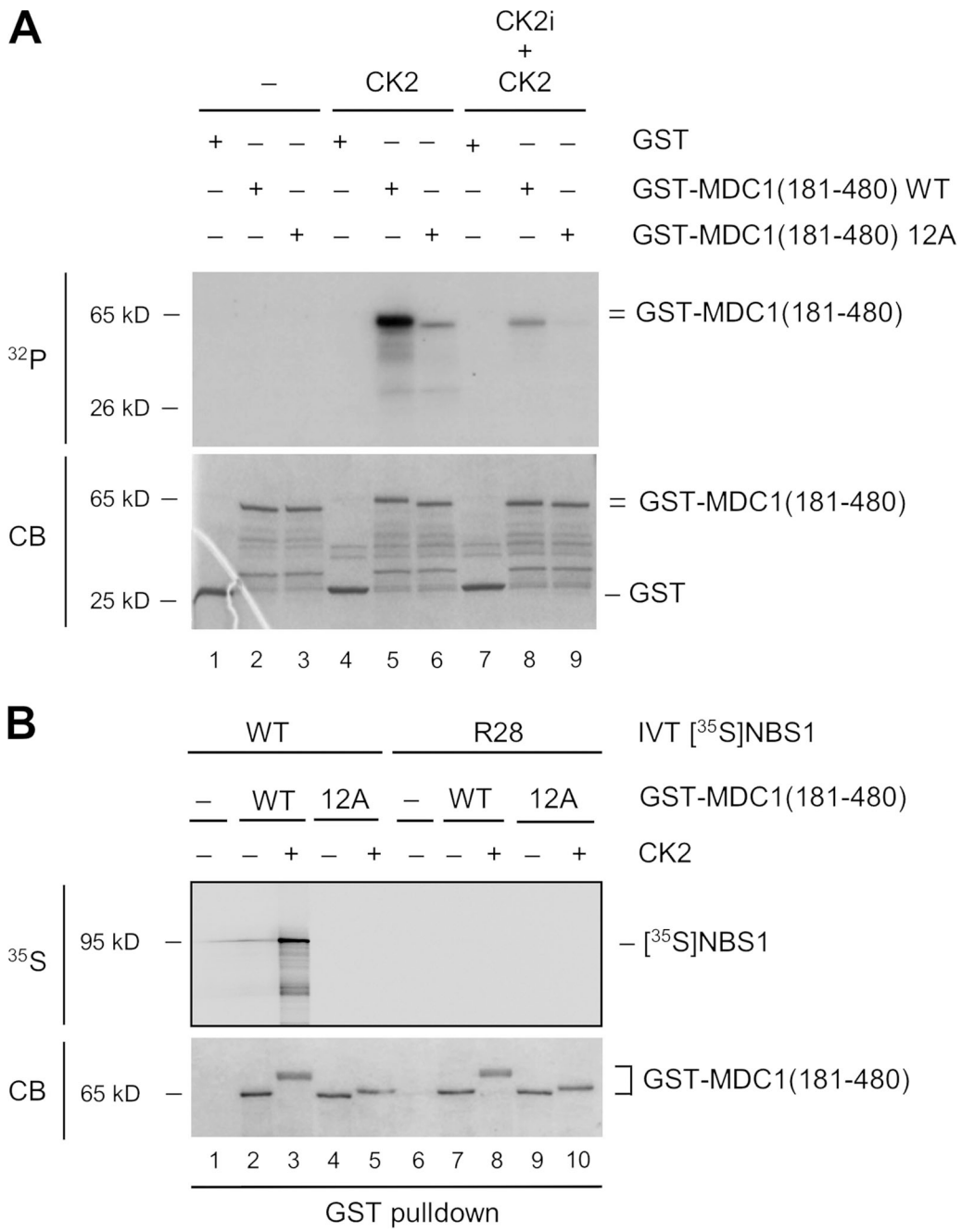

Figure 4. **Phosphorylation of the SDT clusters by CK2 promotes MDC1–NBS1 interaction in vitro.** (A) CK2 phosphorylates the SDT repeats of MDC1 in vitro. GST-MDC1 fragment spanning the entire SDT region (WT; amino acids 181–480) or its derivative in which all 12 potential CK2 sites were mutated to alanines (12A) was purified from *E. coli* and subjected to an in vitro kinase assay using a recombinant CK2 and ^{32}P-labeled ATP. Where indicated, 10 μM of the DMAT CK2 inhibitor (CK2i) was added to the reaction. After separation by SDS-PAGE, the gel was stained with Coomassie blue to validate the equal input of the GST-MDC1 fragments (bottom) and was dried and analyzed by phosphorimager (top). (B) The purified SDT region of MDC1 interacts with NBS1 in a phosphorylation- and CK2-dependent manner. GST-MDC1 fragments (as in A) were phosphorylated or not phosphorylated with recombinant CK2 (using nonradioactive ATP), captured on glutathione–Sepharose beads, and incubated with in vitro–translated ^{35}S-labeled NBS1 WT or its derivative with a point mutation within the FHA domain (R28) for 2 h. Bound complexes were resolved by SDS-PAGE. The gel was stained with Coomassie blue to validate equal input of the GST-MDC1 proteins (bottom), and the bound NBS1 was analyzed by phosphorimager (top). CB, Coomassie blue.

To test the impact of CK2-mediated phosphorylation of the MDC1 N terminus on its interaction with NBS1, we phosphorylated (or mock treated) the GST-tagged WT or the phosphorylation-deficient (12A) SDT fragments (using nonradioactive ATP in the kinase reaction) and assessed their ability to interact with an in vitro–translated ^{35}S-labeled NBS1. Efficient phosphorylation of the WT (but not the 12A) SDT fragment was confirmed by a marked mobility shift on the SDS gel (Fig. 4 B, bottom). Strikingly, only the CK2-phosphorylated but not the unmodified SDT region interacted with NBS1 in this

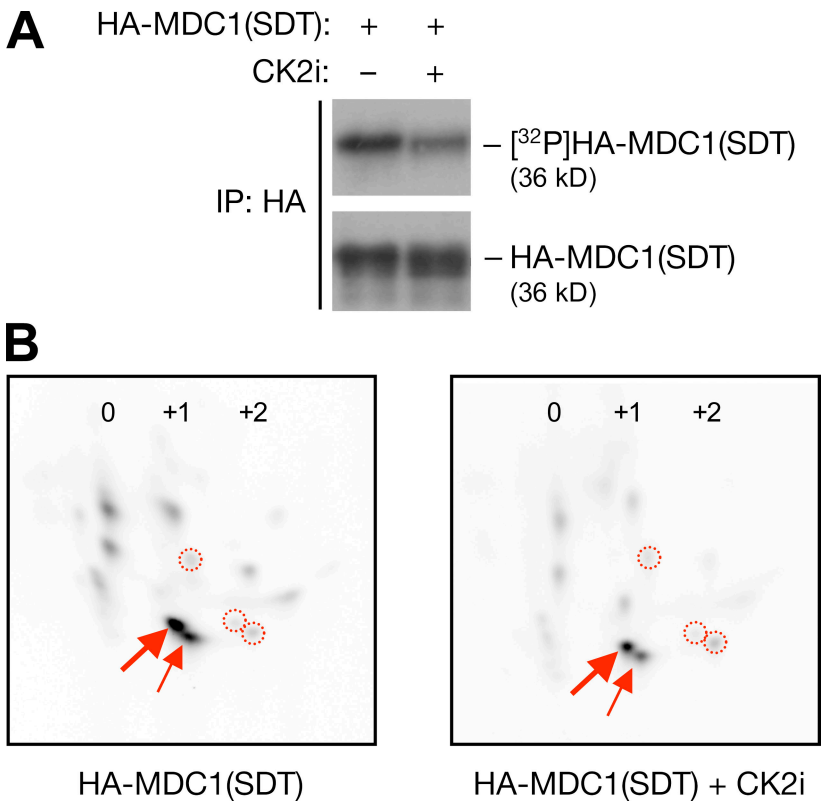

Figure 5. **Inhibition of CK2 reduces phosphorylation of the SDT repeats in vivo.** (A) Treatment of cells with a CK2 inhibitor reduces phosphate incorporation into the SDT-containing fragment of MDC1. U2OS cells were transfected with the HA-tagged fragment of MDC1 spanning the entire SDT region (amino acids 210–460) and labeled with ^{32}P for 4 h. Where indicated, 10 μM of the DMAT CK2 inhibitor (CK2i) was added to the culture medium for the entire labeling period. Lysates from labeled cells were immunoprecipitated with an anti-HA antibody and, after resolving the proteins on SDS-PAGE, were analyzed by the phosphorimager for the extent of ^{32}P incorporation (top). The equal input of proteins in each lane was subsequently verified by immunoblotting with the anti-HA antibody (bottom). (B) Inhibition of CK2 impairs phosphate incorporation into the dominant SDT repeats. The bands corresponding to the labeled SDT fragments from A were excised from the gel and subjected to tryptic digestion. The resulting phosphopeptides were separated by electrophoresis followed by thin-layer chromatography and analyzed by phosphorimager. Note that the ^{32}P incorporation into two prominent spots representing Ser^{299}/Thr^{301} (thin arrows) and Ser^{453}/Thr^{455} (bold arrows) is reduced to ∼50% in cells treated with CK2 inhibitor (CK2i). Dotted circles indicate spots whose labeling did not appreciably change after CK2 inhibition; the intensity of the ^{32}P signal associated with these spots was taken as a baseline to estimate the decrease in phosphate incorporation into the SDT repeats. Numbers indicate the charge of the phosphopeptides.

in vitro binding assay (Fig. 4 B, lane 3). Importantly, this interaction required the intact FHA domain of NBS1, as the R28 mutant of NBS1 failed to bind to the MDC1 N terminus even though this was efficiently phosphorylated (Fig. 4 B, lane 8). The SDT-12A mutant did not bind to any form of NBS1 under these conditions (Fig. 4 B, lanes 4, 5, 9, and 10).

To explore the emerging cross talk between CK2 and the N terminus of MDC1, we tested the impact of CK2 inhibition on SDT phosphorylation in cells. Indeed, preincubation of U2OS cells with a CK2 inhibitor significantly reduced the overall incorporation of radioactive phosphate to the ectopically expressed SDT fragment (Fig. 5 A). More importantly in this context, the ensuing phosphopeptide analysis revealed that this was accompanied by an ∼50% reduction of phosphate incorporation into two prominent SDT repeats (Ser299/Thr301 and Ser453/Ser455; Fig. 5 B). Thus, CK2 can at least partially phosphorylate the SDT repeats in vivo. To further support this conclusion, we immunopurified HA-tagged MDC1 N terminus (Fig. 6 A) or endogenous MDC1 (Fig. 6 B) from cells and subjected these immunocomplexes to an in vitro kinase assay (without any additional substrate). In both cases, MDC1 copurified with an autocatalytic kinase activity that was strongly attenuated by a specific CK2 inhibitor (Fig. 6, A and B). Interestingly, this MDC1-associated CK2 activity was clearly present in undamaged cells (Fig. 6, A and B; lane 1) and did not appreciably increase after exposing the cells to IR (Fig. 6 A, lane 2). Collectively, the data in Figs. 4–6 show that CK2 can phosphorylate the SDT repeats and suggest that this may contribute to trigger a productive interaction between the MDC1 N terminus and the FHA domain of NBS1.

The SDT repeats are required for MDC1–NBS1 interaction in vivo

To directly test whether the SDT repeats determine MDC1–NBS1 interaction in vivo, we expressed in U2OS cells the HA-tagged MDC1 N termini with progressively increasing mutations within the SDT repeats and assessed their ability to bind endogenous NBS1. The isolated mutations of the Ser/Thr residues within the second (amino acids 299/301) or the sixth (amino acids 453/455) SDT cluster had only marginal effects, and these mutants bound NBS1 almost as efficiently as the WT MDC1 N terminus (Fig. 7 A, lanes 1–3). Strikingly, a combination of these mutations (designated as 4A) markedly impaired the ability to bind NBS1 (Fig. 7 A, lane 4), and mutation of the Ser/Thr residues in all six SDT clusters (12A) abolished the MDC1–NBS1 interaction completely (Fig. 7 A, lane 5). Thus, consistent with the in vitro binding experiments described in Fig. 4, the integrity of the SDT repeats appears to be critical to support the interaction between MDC1 and NBS1 in cells. In addition, the progressive attenuation of this interaction achieved by increasing mutations of the SDT-associated Ser/Thr residues suggested that the SDT repeats (especially the dominant ones such as Ser299/Thr301 and Ser453/Thr455) bind NBS1 in a redundant fashion.

The SDT repeats determine the retention of NBS1 at the DSB-modified chromatin

Having obtained biochemical evidence for a link between the phosphorylated N terminus of MDC1 and the FHA domain of NBS1, we set out to investigate the functional relevance of these observations for chromatin retention of NBS1 at the DSB-modified chromatin. To this end, we again used the U2OS-derived

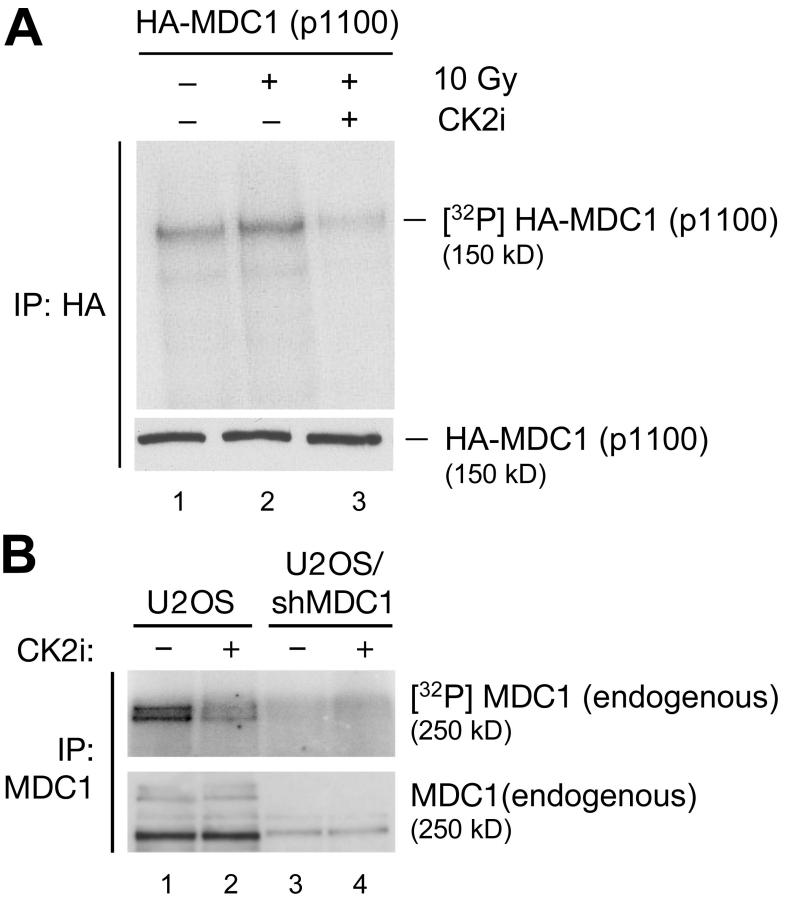

Figure 6. **MDC1 associates with CK2 activity.** (A) MDC1 N terminus copurifies with CK2 activity. U2OS cells were transfected with HA-tagged MDC1 N terminus (p1100). After 24 h, the cells were left untreated or were subjected to 10 Gy IR for 1 h. Cells were then lysed, and the MDC1 fragment was immunoprecipitated with an anti-HA antibody and assayed for the ability to undergo autophosphorylation in an in vitro kinase assay. Where indicated, the kinase reaction was supplemented with 10 μM of the DMAT CK2 inhibitor (CK2i). After SDS-PAGE and transfer to nitrocellulose, the extent of MDC1 phosphorylation was analyzed by phosphorimager (top), and the equal input of MDC1 in each reaction was validated by immunoblotting with the anti-HA antibody (bottom). (B) Endogenous MDC1 associates with CK2 activity. Lysates from asynchronously growing U2OS or U2OS/shMDC1 cells were subjected to immunoprecipitation with an MDC1-specific antibody and assayed for autophosphorylation of endogenous MDC1 (top). The equal input of MDC1 in each reaction was validated by immunoblotting with the anti-MDC1 antibody (bottom). Where indicated, 500 nM of the DMAT CK2 inhibitor (CK2i) was added to the kinase reaction.

cell line with stably depleted endogenous MDC1 (MDC1/shRNA; Fig. 1 D). To test the suitability of this cellular model for the in vivo reconstitution experiments with heterologous MDC1 alleles, we first confirmed that the endogenous MDC1 in these cells was indeed down-regulated to the degree that prevented any cytologically discernible accumulation of NBS1 in the laser-generated DSB tracks (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200708210/DC1). We then reconstituted this cell line with WT MDC1 or the phosphorylation-deficient variants in which the Ser and Thr residues were mutated in two (4A) or in all six (12A) SDT repeats. All of these MDC1 variants were generated in the context of full-length proteins, tagged with the HA epitope on the N terminus, and rendered insensitive to shRNA. Strikingly, although the WT and both phosphorylation-deficient forms of MDC1 avidly accumulated at the DSB-flanking chromatin, only the WT version was able to fully restore the retention of NBS1 in this compartment (Fig. 7 B, left). In contrast, cells expressing the MDC1-4A mutant retained only residual amounts of NBS1 at the DSB-modified chromatin (Fig. 7 B, middle), and MDC1-12A completely failed to support any cytologically discernible accumulation of NBS1 in these regions (Fig. 7 B, right). Importantly, these cell-based data closely matched the progressive loss of MDC1–NBS1 interactions in the pull-down assay using the analogous MDC1 phosphodeficient mutants (Fig. 7 A, compare lanes 1, 4, and 5). Together, these data suggest that the SDT-associated phosphorylations trigger productive interaction between MDC1 and NBS1 and facilitate retention of the latter protein at the DSB-modified chromatin.

Discussion

Our results elucidate the nature of one of the most proximal steps in the spatiotemporal evolution of DSB-modified chromatin. Specifically, although availability of the MDC1 adaptor protein and the NBS1-FHA domain were known to regulate MRN chromatin retention, the way these two factors communicate with each other remained elusive. Our findings mechanistically explain this conundrum by identifying the conserved SDT repeats in the MDC1 N terminus and by showing that their phosphorylation generates recognition sites for the NBS1-FHA domain. In addition, we identify CK2 as a candidate kinase that can phosphorylate the SDT repeats and show that the integrity of the SDT-associated phosphoacceptor residues is required for MRN retention at the sites of DNA damage.

At first glance, it appears surprising that CK2, a ubiquitous and constitutively active kinase, regulates MDC1, a protein that functions almost exclusively in stochastic events triggered by genotoxic stress in which it operates strictly locally at the sites of chromosomal lesions. To comprehend this seemingly unusual cross talk, it is important to realize that MDC1 and NBS1 interact already in unstressed cells (Goldberg et al., 2003; Stewart et al., 2003), and, as we show in this study, even this predamage interaction is at least partly regulated by CK2. Based on this observation, the first obvious scenario that comes to mind is that as a result of

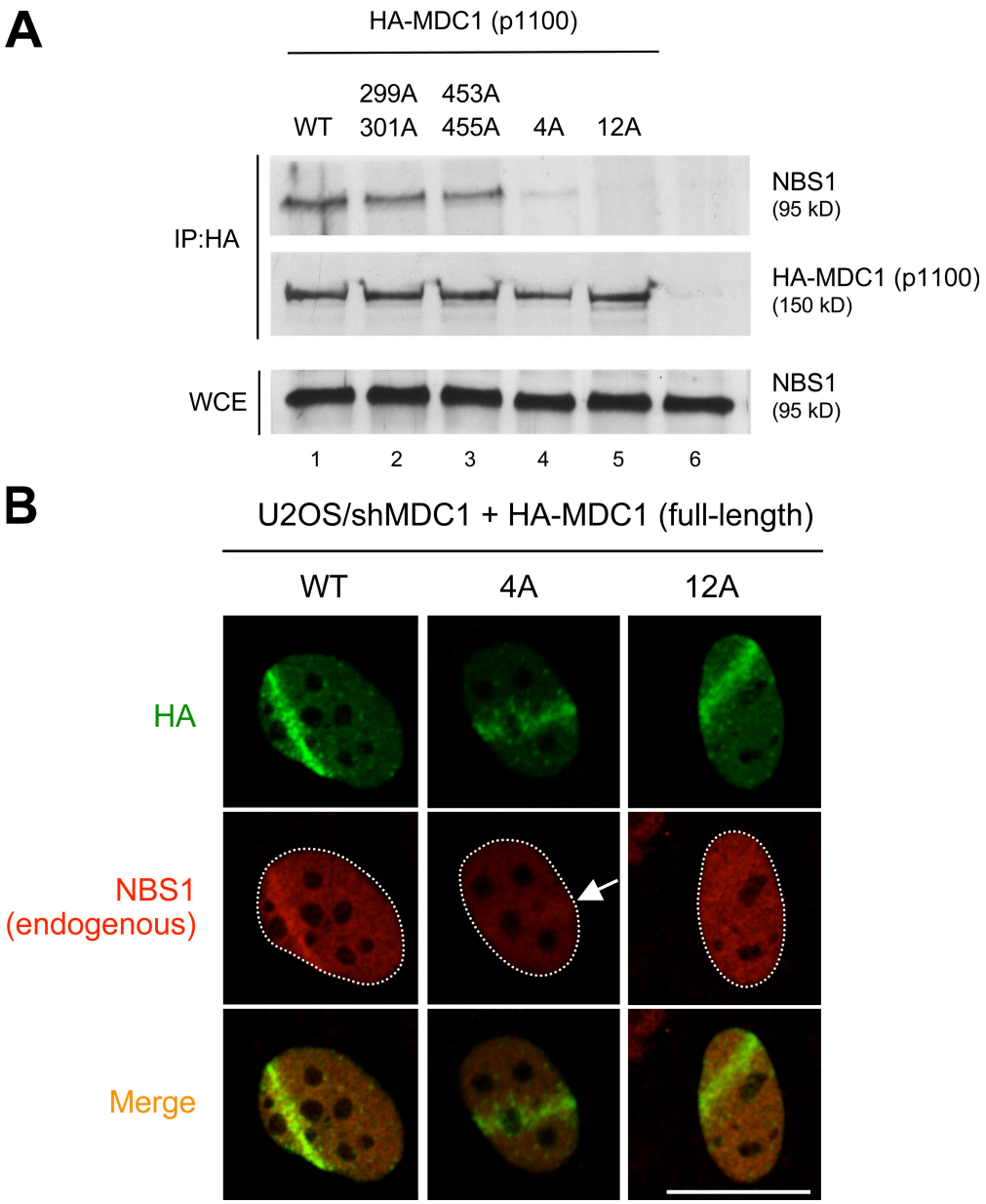

Figure 7. **Disruption of the SDT phosphoacceptor sites impairs MDC1–NBS1 interaction and abrogates the retention of NBS1 at the DSB-modified chromatin.** (A) Disruption of the SDT sites progressively inhibits MDC1–NBS1 interaction. U2OS cells were transfected with the HA-tagged MDC1 fragments spanning the intact N terminus (WT) and its derivatives where the indicated residues were substituted to alanines. The 4A fragment combines alanine substitutions of Ser299, Thr301, Ser453, and Thr455. In the 12A fragment, all of the 12 potential CK2 sites within the SDT region were mutated to alanines. After 24 h, lysates were prepared, immunoprecipitated with an anti-HA antibody, and analyzed by immunoblotting with the anti-NBS1 (top) and anti-HA (middle) antibodies. The immunoblotting of NBS1 from the whole cell extracts (bottom) validates an equal input of the lysate in each reaction. WCE, whole cell extract. (B) Disruption of the SDT sites impairs the sustained retention of NBS1 at the sites of DNA damage. MDC1/shRNA cells were transiently transfected with expression plasmids coding for the WT or the SDT-deficient 4A and 12A phosphorylation-deficient mutants of MDC1, respectively (the same mutations as in A but generated in the context of siRNA-insensitive full-length HA-MDC1). After 24 h, cells were subjected to laser microirradiation to generate local DSBs. After an additional 1 h, the cells were fixed and immunostained with antibodies to HA (to locate the DSB tracks) and to endogenous NBS1. The arrow indicates the residual amount of NBS1 retained in the DSB tracks in cells containing the MDC1-4A mutant. Bar, 10 μm.

its constitutive phosphorylation by CK2, MDC1 forms a stable holocomplex with MRN. In this model, the MRN focus formation simply reflects the propensity of MDC1 (as a fourth subunit of the MRN complex) to avidly interact with γ-H2AX. Although simple and attractive, several pieces of evidence suggest a more subtle way of regulation. First, MRN clearly has functions that do not require MDC1 (Petrini and Stracker, 2003; Falck et al., 2005; Jazayeri et al., 2006), and a strong affinity trap on SDT-phosphorylated MDC1 would likely reduce MRN's flexibility and limit its accessibility to nuclear compartments where it operates independently of MDC1. Indeed, kinetic analysis of NBS1 and MDC1 mobility in living cells revealed marked differences between these two proteins: although both NBS1 and MDC1 become retained at the DSB sites (a feature that determines formation of the cytologically

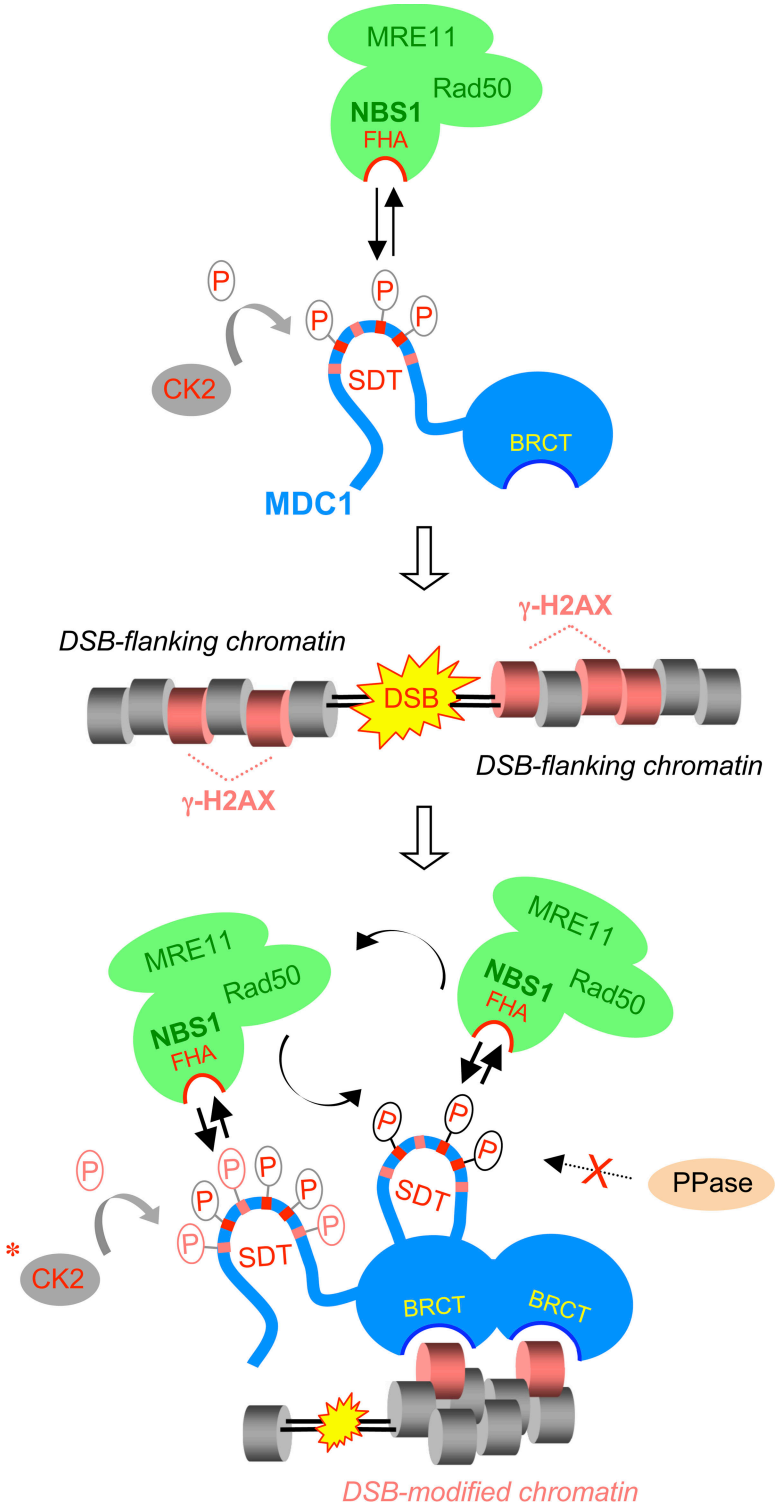

Figure 8. **A hypothetical model of the MDC1–MRN interaction before and after DNA damage.** In undamaged cells, CK2 targets the SDT-rich N terminus of MDC1. Phosphorylated SDTs are recognized by the FHA domain of NBS1 and allow dynamic interaction between MDC1 and the MRN complex (top). DSBs trigger the phosphorylation of H2AX (middle) followed by its recognition by the MDC1-associated BRCT domains (bottom). The resulting concentration of phosphorylated SDT repeated in the DSB-flanking chromatin allows immediate recruitment of the MRN complex to this compartment. CK2 (potentially together with another acidophilic kinase; indicated by an asterisk) may then stimulate MRN retention at the DSB sites by phosphorylating additional SDT repeats. Alternatively, the SDT-associated phosphates might be stabilized by inhibition of a DSB-associated protein phosphatase (PPase). Both mechanisms are compatible with a dynamic MRN exchange between distinct DSB-generated subcompartments but prevent its dilution in the undamaged nucleoplasm. See Discussion for an additional explanation.

discernible nuclear foci), the residence time of MDC1 at DSBs was nearly one order of magnitude longer compared with NBS1. As a result, although MDC1 becomes immobilized at the DSB-flanking chromatin, NBS1 remains highly mobile, a feature compatible with its flexible exchange among distinct DSB-generated subcompartments (Lukas et al., 2003, 2004a). Thus, the interaction between MDC1 and NBS1 is dynamic, and these two factors do not appear to form a rigid holocomplex.

Given these findings, the new data presented in this study, and similar results reached by Spycher et al. (see p. 227 of this issue), our current model describing the mechanism of MRN chromatin retention is as follows. In undamaged nuclei (Fig. 8, top), CK2 phosphorylates the SDT repeats of MDC1. Combined with the ability of the NBS1-FHA domain to recognize phosphorylated SDTs, this allows constitutive yet highly dynamic MDC1–MRN interaction, which remains amenable to

further regulation and prevents sequestration of any of these factors in rigid aggregates. The latter feature could be crucial to allow the rapid recognition of DNA breaks by the MRN complex, a process that is independent of MDC1 (see Introduction). After DSB generation (Fig. 8, bottom), the most proximal chromatin modification includes phosphorylation of the H2AX C terminus (γ-H2AX). Although several DSB regulators contain phosphate interaction motifs and domains, the MDC1-BRCT domain appears to interact with γ-H2AX with the highest affinity (Stucki et al., 2005), resulting in a rapid coating of the γ-H2AX–primed chromatin by MDC1. At this point, the MDC1 N terminus becomes important for the ensuing events on the DSB-flanking chromatin (Fig. 8, bottom). We propose that constitutive phosphorylation of the SDT repeats (translated to a continuous generation of binding sites for the NBS1-FHA domain) is likely the most efficient means to avoid any delay in recruiting MRN to the modified chromatin rapidly evolving around the incipient DBS lesions. Indeed, integration of a constitutive signal is consistent with the simultaneous recruitment of MDC1 and NBS1 to DSBs (Lukas et al., 2004a; Mailand et al., 2007) and suggests that in vertebrates, the SDT repeats of MDC1 coevolved with MRN to prime the latter for the fastest possible arrival at the sites of harmful chromosomal lesions.

Although our data and the complementary results obtained by Spycher et al. (2008) strongly implicate CK2 in phosphorylating the MDC1 N terminus, we cannot exclude the possibility that other acidophilic kinases contribute to regulate the MDC1–NBS1 interaction. Most notably in this regard, we show in Fig. 5 that chemical inhibition of CK2 reduced but did not abolish phosphorylation of the prominently phosphorylated SDT repeats (the maximum reduction of phosphate incorporation to these repeats was ∼50% compared with CK2-proficient cells). Unlike complete genetic disruption of the dominant SDT repeats (Fig. 7 B), this degree of the SDT phosphorylation impairment was not sufficient to prevent accumulation of NBS1 at the DSB sites (our unpublished data).

As alluded to in the preceding paragraphs of this section, this model poses an important conceptual question: how is constitutive and ubiquitous phosphorylation of the MDC1 N terminus transformed to a signal that locally and transiently enhances chromatin affinity for NBS1? The answer to this question is not trivial, but several scenarios come to mind (Fig. 8, bottom). First, it is possible that just the sheer increase in the local density of MDC1 (and the corresponding phosphates on the SDT repeats) may be sufficient to delay MRN at the DSBs to an extent that still allows its dynamic exchange between distinct DSB-generated subcompartments yet prevents its dilution in the undamaged parts of the nucleus. Second, although CK2 activity does not increase after DNA damage, one can envisage that DSBs may locally regulate (inhibit) chromatin-associated phosphatases and thereby decrease the rate of SDT-phosphate turnover. In principle, this may also increase local density of phosphorylated SDT repeats and thereby stabilize the interaction between the MDC1 N terminus and the NBS1-FHA domain. Third, an alternative (but not mutually exclusive) model is based on our observation that the MDC1 N terminus harbors a cluster of six SDT sites and that at least some of them contribute to the MDC1–NBS1 interaction in a cooperative fashion (Fig. 7). Thus, it is possible that although only a subset of the SDT repeats generates the basal level of MDC1–MRN interaction in undamaged cells, the DSB-associated MDC1 undergoes additional conformational changes in its N terminus that may allow phosphorylation of the entire SDT region and, thereby, locally increase the interaction platform for NBS1 and indeed the entire MRN complex. Further studies will be required to discriminate among these scenarios.

Finally, we note that the role of CK2 in the DNA damage response is not unprecedented. The most striking parallel with our findings has been provided by Loizou et al. (2004), who reported that CK2 phosphorylates XRCC1, an adaptor protein involved in single-strand DNA break repair. This and the subsequent studies (Clements et al., 2004; Bekker-Jensen et al., 2007; Iles et al., 2007; Kanno et al., 2007) showed that CK2-mediated phosphorylation was required for the interaction of XRCC1 with other repair proteins such as polynucleotide kinase, aprataxin, and XIP1 (the latter protein is also known as APLF and PALF, respectively). It is noteworthy that all of these proteins interacted with XRCC1 by their FHA domains, which is in striking analogy to the MDC1–NBS1 interaction described in this study. In addition, an exciting conceptual parallel between the CK2-XRCC1 and CK2-MDC1 interplay is provided by the fact that in both cases, CK2 targets large scaffold proteins that by themselves do not posses enzymatic activity but rather appear to integrate upstream signaling to organize other proteins at the vicinity of DNA lesions. The fact that CK2 targets different adaptor proteins engaged in distinct DNA repair processes (single-strand breaks and DSBs) suggests that phosphorylation of these molecular adaptors coevolved to increase the efficiency of the genome surveillance machinery. Although the recent genetic data strongly support the role of MRN chromatin retention for survival and genomic stability after DSB-generating insults (see Introduction), the CK2-mediated phosphorylation of XRCC1 facilitates the single-stranded break repair by promoting the recruitment of polynucleotide kinase and aprataxin and by protecting XIP1 from proteolytic degradation (Clements et al., 2004; Loizou et al., 2004; Bekker-Jensen et al., 2007; Iles et al., 2007; Kanno et al., 2007). When combined together, the previous findings and our new data support the emerging model that one important function of CK2 is to increase the efficiency of genome surveillance by targeting interaction matchmakers and thereby promoting the local concentration and/or stability of DNA damage regulators at sites of genetic lesions.

Materials and methods

Cell culture and gene transfer

The human osteosarcoma cell line U2OS (American Type Culture Collection) was cultured in DME (Invitrogen) supplemented with 10% FCS, 50 U/ml penicillin, and 50 mg/ml streptomycin. The U2OS stable derivative expressing the MDC1-targetting shRNA (U2OS/shRNA) was described previously (Bekker-Jensen et al., 2006). All transfections were performed with FuGENE6 (Roche) according to the manufacturer's instructions, and cells were harvested after 24 h.

Plasmids

HA-tagged human MDC1 (pcDNA3-neo) was a gift from D. Stern (Yale University, New Haven, CT). HA-MDC1 fragments were generated by PCR and subcloned into a pcDNA4/TO (Invitrogen) plasmid. To the shorter

N-terminal MDC1 fragments, a triple nuclear localization signal was added by cloning the fragments into pCMV-myc/nuc (Invitrogen) vector and thereafter subcloning back into pcDNA4/TO by PCR. Myc-NBS1-pCMV plasmids were described previously (Horejsi et al., 2004). The QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to generate point mutations; deletions were made using the Phusion Site-Directed Mutagenesis kit (New England Biolabs, Inc.) and performed according to the manufacturer's instructions. Plasmid constructs were verified by sequence analysis.

Antibodies, chemicals, and immunochemical techniques

HA antibodies F7 and Y11 were purchased from Santa Cruz Biotechnology, Inc. The rabbit Nbs1 and Myc antibodies originated from Abcam. Rabbit and sheep polyclonal MDC1 antibodies were provided by S. Jackson (Gurdon Institute, Cambridge, UK). The specific CK2 inhibitor InSolution DMAT was obtained from EMD and was used in concentrations specified in the figure legends. For immunoprecipitation, cells were lysed, and proteins were extracted in a high salt buffer (20 mM Tris, pH 7.5, 400 mM NaCl, 0.5% NP-40, 1 mM EDTA, and protease and phosphatase inhibitor mixture). After centrifugation of the lysates, an equal volume of the same buffer without NaCl was added to produce a final NaCl concentration of 200 mM. Kinase assays and immunoblotting were performed as previously described (Falck et al., 2005; Mailand et al., 2006) except for the MDC1-associated kinase assay, in which no exogenous substrate was added.

In vivo labeling and phosphopeptide mapping

U2OS cells transfected with the indicated constructs were labeled for 4 h in phosphate-free DME with 20 mM Hepes, pH 7.2, and 5% dialyzed phosphate-free DME with 1 mCi/ml ^{32}P (PBS43; GE Healthcare). Two-dimensional phosphopeptide and phospho–amino acid mapping were performed as described previously (Blume-Jensen et al., 1995; Hansen et al., 1996). Tryptic phosphopeptides were separated on an electrophoresis system (HTLE-7000; CBS Scientific Company, Inc.) at 2,000 V for 30 min at 14°C. The second dimension chromatography was performed in isobutyric acid buffer for 14–16 h. Separated peptides were subjected to phosphorimager analysis (FLA-3000; Fuji).

CK2 kinase assay

1 µg of purified GST or GST-MDC1 fragments was phosphorylated with 100 U of recombinant CK2 kinase in the presence of 10 µCi γ-[^{32}P]ATP for 15 min at 37°C according to the manufacturer's instructions. The reaction was stopped by the addition of Laemmli sample buffer, and phosphorylated proteins were visualized by resolving the samples on SDS-PAGE followed by autoradiography.

In vitro binding assay

2 µg of purified GST-MDC1(181–480) fragments were left untreated or phosphorylated with recombinant CK2 (New England Biolabs, Inc.) for 30 min at 37°C in the presence of 1 mM ATP. The GST proteins were then bound to glutathione–Sepharose beads (GE Healthcare), and the beads were washed three times in EBC buffer (Mailand and Diffley, 2005). The beads were then incubated with in vitro–translated ^{35}S-labeled Nbs1 for 2 h at 4°C, washed four times with EBC buffer, resuspended in Laemmli sample buffer, and resolved by SDS-PAGE and autoradiography.

Laser microirradiation and microscopy

Induction of localized DSBs by microirradiation was performed as described previously (Lukas et al., 2003, 2004a; Bekker-Jensen et al., 2005, 2006). In brief, U2OS cells and their derivatives were grown on glass coverslips in the presence of 10 µM BrdU for 24 h and subsequently exposed to the pulsed UVA laser (λ = 337 nm) along a narrow track spanning the entire nuclear diameter. After an additional hour, the cells were fixed in 4% formaldehyde and subjected to immunostaining. Images were acquired with a confocal microscope (LSM 510; Carl Zeiss, Inc.) through a plan-Neofluar 40× NA 1.3 oil immersion objective (Carl Zeiss, Inc.). For dual color imaging, secondary antibodies coupled to AlexaFluor dyes with excitation wavelengths of 488 and 568 nm were used. Image acquisition and basic image processing were performed with LSM software (Carl Zeiss, Inc.).

Online supplemental material

Fig. S1 shows sequence alignment of the SDT-rich MDC1 regions in several vertebrate species. Fig. S2 provides in vivo evidence for the simultaneous phosphorylation of Ser and Thr residues within the individual SDT repeats. Fig. S3 shows that constitutive down-regulation of MDC1 by shRNA impairs retention of NBS1 at the sites of DNA damage. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708210/DC1.

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References

Bartek, J., and J. Lukas. 2007. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr. Opin. Cell Biol.* 19:238–245.

Beausoleil, S.A., M. Jedrychowski, D. Schwartz, J.E. Elias, J. Villen, J. Li, M.A. Cohn, L.C. Cantley, and S.P. Gygi. 2004. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. USA.* 101:12130–12135.

Bekker-Jensen, S., C. Lukas, F. Melander, J. Bartek, and J. Lukas. 2005. Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. *J. Cell Biol.* 170:201–211.

Bekker-Jensen, S., C. Lukas, R. Kitagawa, F. Melander, M.B. Kastan, J. Bartek, and J. Lukas. 2006. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J. Cell Biol.* 173:195–206.

Bekker-Jensen, S., K. Fugger, J.R. Danielsen, I. Gromova, M. Sehested, J. Celis, J. Bartek, J. Lukas, and N. Mailand. 2007. Human Xip1 (C2orf13) is a novel regulator of cellular responses to DNA strand breaks. *J. Biol. Chem.* 282:19638–19643.

Blume-Jensen, P., C. Wernstedt, C.H. Heldin, and L. Ronnstrand. 1995. Identification of the major phosphorylation sites for protein kinase C in kit/stem cell factor receptor in vitro and in intact cells. *J. Biol. Chem.* 270:14192–14200.

Cerosaletti, K.M., and P. Concannon. 2003. Nibrin forkhead-associated domain and breast cancer C-terminal domain are both required for nuclear focus formation and phosphorylation. *J. Biol. Chem.* 278:21944–21951.

Cerosaletti, K., and P. Concannon. 2004. Independent roles for nibrin and Mre11-Rad50 in the activation and function of Atm. *J. Biol. Chem.* 279:38813–38819.

Cerosaletti, K., J. Wright, and P. Concannon. 2006. Active role for nibrin in the kinetics of atm activation. *Mol. Cell. Biol.* 26:1691–1699.

Clements, P.M., C. Breslin, E.D. Deeks, P.J. Byrd, L. Ju, P. Bieganowski, C. Brenner, M.C. Moreira, A.M. Taylor, and K.W. Caldecott. 2004. The ataxia-oculomotor apraxia 1 gene product has a role distinct from ATM and interacts with the DNA strand break repair proteins XRCC1 and XRCC4. *DNA Repair (Amst.).* 3:1493–1502.

D'Amours, D., and S.P. Jackson. 2002. The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat. Rev. Mol. Cell Biol.* 3:317–327.

Difilippantonio, S., A. Celeste, O. Fernandez-Capetillo, H.T. Chen, B. Reina San Martin, F. Van Laethem, Y.P. Yang, G.V. Petukhova, M. Eckhaus, L. Feigenbaum, et al. 2005. Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. *Nat. Cell Biol.* 7:675–685.

Difilippantonio, S., A. Celeste, M.J. Kruhlak, Y. Lee, M.J. Difilippantonio, L. Feigenbaum, S.P. Jackson, P.J. McKinnon, and A. Nussenzweig. 2007. Distinct domains in Nbs1 regulate irradiation-induced checkpoints and apoptosis. *J. Exp. Med.* 204:1003–1011.

Essers, J., W. Vermeulen, and A.B. Houtsmuller. 2006. DNA damage repair: anytime, anywhere? *Curr. Opin. Cell Biol.* 18:240–246.

Falck, J., J. Coates, and S.P. Jackson. 2005. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature.* 434:605–611.

Fernandez-Capetillo, O., A. Lee, M. Nussenzweig, and A. Nussenzweig. 2004. H2AX: the histone guardian of the genome. *DNA Repair (Amst.).* 3:959–967.

Goldberg, M., M. Stucki, J. Falck, D. D'Amours, D. Rahman, D. Pappin, J. Bartek, and S.P. Jackson. 2003. MDC1 is required for the intra-S-phase DNA damage checkpoint. *Nature.* 421:952–956.

Hansen, K., M. Johnell, A. Siegbahn, C. Rorsman, U. Engstrom, C. Wernstedt, C.H. Heldin, and L. Ronnstrand. 1996. Mutation of a Src phosphorylation site in the PDGF beta-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. *EMBO J.* 15:5299–5313.

Horejsi, Z., J. Falck, C.J. Bakkenist, M.B. Kastan, J. Lukas, and J. Bartek. 2004. Distinct functional domains of Nbs1 modulate the timing and magnitude

of ATM activation after low doses of ionizing radiation. *Oncogene.* 23:3122–3127.

Iles, N., S. Rulten, S.F. El-Khamisy, and K.W. Caldecott. 2007. APLF (C2orf13) is a novel human protein involved in the cellular response to chromosomal DNA strand breaks. *Mol. Cell. Biol.* 27:3793–3803.

Jazayeri, A., J. Falck, C. Lukas, J. Bartek, G.C. Smith, J. Lukas, and S.P. Jackson. 2006. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* 8:37–45.

Kanno, S., H. Kuzuoka, S. Sasao, Z. Hong, L. Lan, S. Nakajima, and A. Yasui. 2007. A novel human AP endonuclease with conserved zinc-finger-like motifs involved in DNA strand break responses. *EMBO J.* 26:2094–2103.

Kastan, M.B., and J. Bartek. 2004. Cell-cycle checkpoints and cancer. *Nature.* 432:316–323.

Kobayashi, J., H. Tauchi, S. Sakamoto, A. Nakamura, K. Morishima, S. Matsuura, T. Kobayashi, K. Tamai, K. Tanimoto, and K. Komatsu. 2002. NBS1 localizes to gamma-H2AX foci through interaction with the FHA/BRCT domain. *Curr. Biol.* 12:1846–1851.

Kobayashi, J., A. Antoccia, H. Tauchi, S. Matsuura, and K. Komatsu. 2004. NBS1 and its functional role in the DNA damage response. *DNA Repair (Amst.).* 3:855–861.

Lee, J.H., B. Xu, C.H. Lee, J.Y. Ahn, M.S. Song, H. Lee, C.E. Canman, J.S. Lee, M.B. Kastan, and D.S. Lim. 2003. Distinct functions of Nijmegen breakage syndrome in ataxia telangiectasia mutated-dependent responses to DNA damage. *Mol. Cancer Res.* 1:674–681.

Linding, R., L.J. Jensen, G.J. Ostheimer, M.A. van Vugt, C. Jorgensen, I.M. Miron, F. Diella, K. Colwill, L. Taylor, K. Elder, et al. 2007. Systematic discovery of in vivo phosphorylation networks. *Cell.* 129:1415–1426.

Loizou, J.I., S.F. El-Khamisy, A. Zlatanou, D.J. Moore, D.W. Chan, J. Qin, S. Sarno, F. Meggio, L.A. Pinna, and K.W. Caldecott. 2004. The protein kinase CK2 facilitates repair of chromosomal DNA single-strand breaks. *Cell.* 117:17–28.

Lou, Z., K. Minter-Dykhouse, S. Franco, M. Gostissa, M.A. Rivera, A. Celeste, J.P. Manis, J. van Deursen, A. Nussenzweig, T.T. Paull, et al. 2006. MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. *Mol. Cell.* 21:187–200.

Lukas, C., J. Falck, J. Bartkova, J. Bartek, and J. Lukas. 2003. Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. *Nat. Cell Biol.* 5:255–260.

Lukas, C., F. Melander, M. Stucki, J. Falck, S. Bekker-Jensen, M. Goldberg, Y. Lerenthal, S.P. Jackson, J. Bartek, and J. Lukas. 2004a. Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. *EMBO J.* 23:2674–2683.

Lukas, J., C. Lukas, and J. Bartek. 2004b. Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. *DNA Repair (Amst.).* 3:997–1007.

Lukas, C., J. Bartek, and J. Lukas. 2005. Imaging of protein movement induced by chromosomal breakage: tiny 'local' lesions pose great 'global' challenges. *Chromosoma.* 114:146–154.

Mailand, N., and J.F. Diffley. 2005. CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell.* 122:915–926.

Mailand, N., S. Bekker-Jensen, J. Bartek, and J. Lukas. 2006. Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. *Mol. Cell.* 23:307–318.

Mailand, N., S. Bekker-Jensen, H. Faustrup, F. Melander, J. Bartek, C. Lukas, and J. Lukas. 2007. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell.* 131:887–900.

Nyberg, K.A., R.J. Michelson, C.W. Putnam, and T.A. Weinert. 2002. Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* 36:617–656.

Olsen, J.V., B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen, and M. Mann. 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell.* 127:635–648.

Petrini, J.H., and T.H. Stracker. 2003. The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends Cell Biol.* 13:458–462.

Shiloh, Y. 2003. ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer.* 3:155–168.

Stewart, G.S., B. Wang, C.R. Bignell, A.M. Taylor, and S.J. Elledge. 2003. MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature.* 421:961–966.

Stracker, T.H., J.W. Theunissen, M. Morales, and J.H. Petrini. 2004. The Mre11 complex and the metabolism of chromosome breaks: the importance of communicating and holding things together. *DNA Repair (Amst.).* 3:845–854.

Stracker, T.H., M. Morales, S.S. Couto, H. Hussein, and J.H. Petrini. 2007. The carboxy terminus of NBS1 is required for induction of apoptosis by the MRE11 complex. *Nature.* 447:218–221.

Stucki, M., and S.P. Jackson. 2006. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst.).* 5:534–543.

Stucki, M., J.A. Clapperton, D. Mohammad, M.B. Yaffe, S.J. Smerdon, and S.P. Jackson. 2005. MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell.* 123:1213–1226.

Spycher, C., E.S. Miller, K. Townsend, L. Pavic, N.A. Morrice, P. Janscak, G.S. Stewart, and M. Stucki. 2008. Constitutive phosphorylation of MDC1 physically links the MRE11–RAD50–NBS1 complex to damaged chromatin. *J. Cell Biol.* 181:227–240.

Tauchi, H., J. Kobayashi, K. Morishima, S. Matsuura, A. Nakamura, T. Shiraishi, E. Ito, D. Masnada, D. Delia, and K. Komatsu. 2001. The forkhead-associated domain of NBS1 is essential for nuclear foci formation after irradiation but not essential for hRAD50[middle dot]hMRE11[middle dot]NBS1 complex DNA repair activity. *J. Biol. Chem.* 276:12–15.

Zhao, S., W. Renthal, and E.Y. Lee. 2002. Functional analysis of FHA and BRCT domains of NBS1 in chromatin association and DNA damage responses. *Nucleic Acids Res.* 30:4815–4822.

Zhou, B.B., and S.J. Elledge. 2000. The DNA damage response: putting checkpoints in perspective. *Nature.* 408:433–439.