A divalent FHA/BRCT-binding mechanism couples the MRE11–RAD50–NBS1 complex to damaged chromatin

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The MRE11–RAD50–NBS1 (MRN) complex accumulates at sites of DNA double-strand breaks in large chromatin domains flanking the lesion site. The mechanism of MRN accumulation involves direct binding of the Nijmegen breakage syndrome 1 (NBS1) subunit to phosphorylated mediator of the DNA damage checkpoint 1 (MDC1), a large nuclear adaptor protein that interacts directly with phosphorylated H2AX. NBS1 contains an FHA domain and two BRCT domains at its amino terminus. Here, we show that both of these domains participate in the interaction with phosphorylated MDC1. Point mutations in key amino acid residues of either the FHA or the BRCT domains compromise the interaction with MDC1 and lead to defects in MRN accumulation at sites of DNA damage. Surprisingly, only mutation in the FHA domain, but not in the BRCT domains, yields a G2/M checkpoint defect, indicating that MDC1-dependent chromatin accumulation of the MRN complex at sites of DNA breaks is not required for G2/M checkpoint activation.

Keywords: DNA double-strand breaks; chromatin; NBS1; MDC1; G2/M checkpoint

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INTRODUCTION

Nijmegen breakage syndrome (NBS) is a rare autosomal genetic disorder. NBS patients suffer from growth retardation, microcephaly, dismorphic features, immunodeficiency and predisposition to cancer, mainly lymphomas. Cells derived from NBS patients are radio-sensitive, show chromosomal instability and cell-cycle checkpoint, as well as apoptotic defects (van der Burg *et al*, 1996).

The NBS gene codes for a 754-amino-acid protein named NBS1 (p95; nibrin). It exists exclusively in a complex with two enzymes: MRE11, a structure-specific nuclease, and RAD50, an ATPase/adenylate kinase. Together, these three proteins form the MRE11–RAD50–NBS1 (MRN) complex, a conserved and essential DNA-damage response (DDR) factor that functions in many cellular processes involving DNA double-strand breaks (DSBs),

including DSB repair, checkpoint signalling, DNA replication, meiotic recombination and induction of apoptosis (Stracker *et al*, 2004; Difilippantonio & Nussenzweig, 2007).

The MRN complex accumulates at sites of DSBs in large microscopically discernible subnuclear structures, usually referred to as DNA-damage foci. The functional implication of this massive accumulation at sites of DSBs is not yet fully understood. We and others showed recently that focus formation by the MRN complex is mediated by a direct interaction between NBS1 and phosphorylated mediator of the DNA damage checkpoint 1 (MDC1), which is a large nuclear adaptor protein that specifically recognises phosphorylated H2AX (γ H2AX; Chapman & Jackson, 2008; Melander *et al*, 2008; Spycher *et al*, 2008; Wu *et al*, 2008). The interaction between NBS1 and MDC1 is dependent on the amino-terminal portion of NBS1 that contains the FHA domain and interacts directly with a constitutively phosphorylated acidic repeat region in MDC1, the SDT repeat (Chapman & Jackson, 2008; Melander *et al*, 2008; Spycher *et al*, 2008). The SDT repeat region is characterized by conserved patches of 8–10 amino acids comprising serine and threonine residues typically separated by an aspartate and embedded further in an acidic sequence environment. This SDT region (referred to as the SDTD region in some papers) interacts with the MRN complex in a phosphorylation-dependent manner. In human MDC1, six SDT motifs were identified and deletion of at least five of them leads to complete abrogation of MRN foci formation (Melander *et al*, 2008; Spycher *et al*, 2008). Analysis of NBS1 recruitment to sites of DSBs showed that on expression of an MDC1 version lacking the SDT regions, NBS1 only accumulates in micro-foci and is not found in the broader chromatin compartments usually covered by γ H2AX and MDC1 (Chapman & Jackson, 2008). This indicates that the MRN complex is recruited to DSBs in an MDC1-independent manner, but its sustained interaction with the DSB-flanking chromatin requires MDC1.

Interestingly, MDC1 and MRN exist in a complex even in undamaged cells. This interaction is dependent on the activity of the acidophilic casein kinase 2 (CK2), for which the SDT motifs form consensus phosphorylation sites (Spycher *et al*, 2008; Wu *et al*, 2008). Both serine and threonine residues in each SDT motif are phosphorylated by CK2 *in vivo* and only doubly phosphorylated pSDpT motifs are able to mediate the interaction with NBS1 (Melander *et al*, 2008; Spycher *et al*, 2008).

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No structural information of full-length NBS1 is yet available, but recent nuclear magnetic resonance (NMR) structural data suggested that besides the FHA domain, NBS1 might also feature a tandem BRCT domain at its N terminus (Xu *et al*, 2008). Similarly to FHA domains, tandem BRCT domains have been shown to act as phospho-specific protein–protein interaction modules (Glover *et al*, 2004).

Here, we present evidence that both the NBS1 FHA domain and the tandem BRCT domain interact specifically with phosphorylated MDC1. We show that single point mutations in key residues in both the FHA and the tandem BRCT domain of NBS1 disrupt the interaction with MDC1 and abrogate the accumulation and retention of the MRN complex at sites of DSBs. Surprisingly, only a mutation in the FHA domain induces a significant G2/M DNA-damage checkpoint defect, whereas mutation in the tandem BRCT domain does not. Thus, our findings indicate that MDC1-mediated accumulation of the MRN complex at sites of DSBs is not required for G2/M checkpoint activation and strongly suggest that the FHA domain of NBS1 might have additional, as yet unidentified, interaction partners that mediate G2/M checkpoint activation in response to DSBs.

RESULTS AND DISCUSSION

Both FHA and BRCT domains of NBS1 interact with MDC1

Until recently, sequence comparison and structure prediction algorithms indicated that the N-terminal region of NBS1 contained an FHA domain and one single BRCT domain (reviewed in D'Amours & Jackson, 2002). Three years ago, a second putative BRCT domain at the N terminus of NBS1 was discovered by means of a refined bioinformatic analysis (Becker *et al*, 2006). The existence of two BRCT domains downstream from the FHA domain at the NBS1 N terminus was partly confirmed by a recently published NMR structure of the second BRCT domain (Xu *et al*, 2008). Interestingly, there seems to be no spacer between the FHA domain and the putative tandem BRCT domain, indicating that these domains might form one single compact globular structure (Fig 1A). Moreover, conservation of key phospho-binding amino-acid residues in the BRCT tandem domain suggests that like the FHA domain, it might act as a phospho-specific protein–protein interaction module.

We and others have shown recently that the FHA domain of NBS1 associates directly with a constitutively phosphorylated region in MDC1, the SDT repeat region (Chapman & Jackson, 2008; Melander *et al*, 2008; Spycher *et al*, 2008). Mammalian MDC1 contains a total of six SDT motifs, and at least three of these are required for efficient MRN accumulation at sites of DSBs (Spycher *et al*, 2008). This might indicate that more than one binding site with affinity to the phosphorylated SDT region might exist in NBS1. Thus, we tested whether the intact NBS1 BRCT tandem domain was required for efficient association of NBS1 with the full-length phosphorylated SDT region. We phosphorylated (or mock-treated) the human glutathione-S-transferase (GST)-tagged MDC1 SDT fragment and assessed its ability to interact with *in vitro*-translated full-length NBS1 protein that carried point mutations in key residues in its phospho-binding FHA and BRCT tandem domains, respectively. As shown before, full-length wild-type NBS1 interacted efficiently with the phosphorylated SDT region of MDC1 (Fig 1B; Melander *et al*, 2008; Spycher *et al*, 2008). Interestingly, FHA domain single mutant

(R28A) and a BRCT tandem domain single mutant (K160M) also showed residual SDT-binding activity. However, a double phosphopeptide-binding mutant (R28A/K160M) failed to bind to the phosphorylated SDT region (Fig 1B). This indicates that both the FHA domain and the BRCT tandem domain are able to interact with the phosphorylated MDC1 SDT region *in vitro*.

NBS1 does not exist on its own in the nuclei of mammalian cells, as it is always associated with MRE11 and RAD50. Thus, our assay conditions with the *in vitro*-translated NBS1 do not accurately reflect a physiological situation where NBS1 is part of a heterotrimeric complex. Therefore, we co-expressed all three subunits of the MRN complex in insect cells and tested their binding affinity to the phosphorylated SDT region of MDC1. Also in the context of the intact MRN complex, wild-type NBS1 bound efficiently to the phosphorylated SDT region (Fig 1C). Surprisingly, neither the FHA mutant (R28A) nor the BRCT tandem domain mutant (K160M) was able to associate with the phosphorylated SDT region (Fig 1C). This indicates that when NBS1 exists in a heterotrimeric complex with MRE11 and RAD50, both the intact FHA domain and the BRCT tandem domain of NBS1 are essential for efficient association with phosphorylated MDC1. It is not clear why the NBS1 single mutants interacted with the phosphorylated SDT region when translated *in vitro* but did not in the context of the heterotrimeric MRN complex. However, it is possible that when NBS1 is an integral part of the MRN complex, its N-terminal phosphopeptide-binding region might be sterically less accessible so that efficient association with the SDT region is only possible when both the FHA domain and BRCT tandem domain are contributing to the interaction.

As an intact NBS1 FHA domain and a BRCT tandem domain seem to be essential for interaction with the MDC1 SDT region, we next asked if both of these domains were also involved in complex formation with MDC1 in mammalian cell extracts. We co-expressed a Flag-tagged 800-amino-acid N-terminal fragment of MDC1 (containing the SDT region) with Myc-tagged full-length NBS1 wild type and a mutant derivative, respectively, and tested their association by co-immunoprecipitation. Significantly, only wild-type NBS1 interacted with the MDC1 fragment in extracts prepared from the transfected cells, whereas neither the FHA and the BRCT tandem domain single-mutants (R28A; K160M) nor the double mutant (R28A/K160M) showed any significant binding activity towards MDC1 (Fig 1D).

The BRCT domains of NBS1 are required for MRN foci

Next, we investigated whether the K160M mutation in the BRCT tandem domain would also compromise the accumulation of the MRN complex at sites of DSBs, as observed earlier for the FHA domain mutant R28A (Cersaletti & Concannon, 2003; Lukas *et al*, 2004). We generated NBS1-ILB1 fibroblast cell lines stably transduced with wild-type and mutant NBS1 (supplementary Fig S1A online). Then, we assessed nuclear foci formation of NBS1 in these cell lines by immunofluorescence microscopy. In NBS1-ILB1 parental fibroblasts, no NBS1 staining was observed (Fig 2A, top row). However, 81% of the cells stably transduced with wild-type NBS1 showed focal accumulation of NBS1 1 h after irradiation at 5 Gy. By contrast, only 20% of cells stably transduced with R28A NBS1 and 13% of cells stably transduced with K160M NBS1, had a focal NBS1 staining pattern (Fig 2A), thus indicating that sustained interaction of

Fig 1 | Both the FHA domain and the tandem BRCT domain of NBS1 are required for the interaction with the phosphorylated SDT region of MDC1 *in vitro*. (A) Schematic representation of full-length human NBS1 and its domain composition. The enlarged area shows a sequence alignment of the FHA and BRCT domains of human, mouse and *Xenopus* NBS1. The putative secondary structure of the first (amino-terminal) BRCT domain is indicated by pale colours. The secondary structure of the second (carboxy-terminal) BRCT domain (indicated by bright colours) was derived from Xu *et al* (2008). Phospho-interacting amino acids are highlighted in yellow. (B) Purified MDC1 GST-SDT fragment was preincubated with CK2 and ATP. The fragment was then incubated with *in vitro*-translated ³⁵S-labelled NBS1 wild type or mutants for 1 h, washed and resolved by SDS-PAGE and autoradiography. (C) Purified MDC1 GST-SDT fragment was preincubated with CK2 and ATP. The fragment was then incubated with purified MRN complex where the NBS1 subunit was either wild type or contained a point mutation in the FHA domain (R28A) or in the BRCT tandem domain (K160M). Bound proteins were separated by SDS-PAGE followed by immunoblotting. The blots were probed with a polyclonal antibody against NBS1. (D) Human embryonic kidney 293T cells were transiently transfected with Flag-tagged MDC1(800) fragment and Myc-tagged NBS1 wild type and mutants, as indicated. Flag antibodies were used for co-immunoprecipitation and Myc antibodies for western blot analysis. CK2, casein kinase 2; GST, glutathione-S-transferase; IP, immunoprecipitation; MDC1, mediator of the DNA damage checkpoint 1; MRN, MRE11-RAD50-NBS1 complex; NBS1, Nijmegen breakage syndrome 1; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; wt, wild-type.

MRN complex with damaged chromatin requires the phosphopeptide-binding capacity of both the FHA and tandem BRCT domains of NBS1.

To develop these findings further, we used UV-laser micro-irradiation to induce DSBs in subnuclear volumes (Lukas *et al*,

2004). Under these conditions, wild-type NBS1 accumulated throughout the micro-irradiated nuclear compartments (Fig 2B). However, both the R28A and K160M mutation prevented binding of NBS1 to the γH2AX-coated areas, except for a small fraction of the protein scattered along the irradiated path

Fig 2 | The BRCT tandem domain of NBS1 is required for focal accumulation of the MRN complex at sites of DSBs *in vivo*. (A) NBS-ILB1 fibroblasts and NBS-ILB1 fibroblasts stably transduced with wild-type, R28A or K160M mutant NBS1, respectively, were irradiated at 5 Gy. The irradiated cells were incubated for 1 h, fixed with methanol and probed with the indicated antibodies. Cells were then analysed by confocal microscopy and nuclear foci-positive cells were counted for statistical evaluation. (B) NBS-ILB1 fibroblasts stably transduced with wild-type, R28A or K160M mutant NBS1, respectively, were micro-irradiated as described in Methods. The irradiated cells were incubated for 1 h, fixed with methanol and probed with the indicated antibodies. Cells were then analysed by confocal microscopy. DAPI, 4',6-diamidino-2-phenylindole; MRN, MRE11-RAD50-NBS1 complex; NBS1, Nijmegen breakage syndrome 1.

(Fig 2B, enlarged areas). This indicates that phospho-specific binding of both the NBS1 FHA domain and the BRCT tandem domain to the MDC1 SDT region is essential for efficient

accumulation and retention of the MRN complex in damaged nuclear areas.

G2/M checkpoint does not require BRCT domains of NBS1

We proposed previously that MDC1-mediated accumulation of the MRN complex in chromatin regions flanking DSBs was required for efficient activation of the G2/M DNA-damage checkpoint. This was on the basis of the observation that point mutations in the FHA domain that disrupt its phospho-specific binding show partial G2/M checkpoint defects both in human and mouse cells (Difilippantonio *et al*, 2005, 2007; Spycher *et al*, 2008). If this interpretation was correct, we would predict that the K160M mutation in the NBS1 BRCT tandem domain also leads to a G2/M checkpoint defect similar to the R28A FHA mutation, because MDC1-binding and chromatin accumulation are as severely compromised in the K160M mutant as they are in the R28A mutant (see above). Surprisingly, however, we found that several independent clones of NBS fibroblasts stably transduced with K160M NBS1 activated the G2/M checkpoint almost as efficiently as wild-type NBS1 (Fig 3; supplementary Fig S1B online). This indicates that MDC1-binding and MDC1-mediated accumulation of the MRN complex at sites of DSBs are not required for activation of the G2/M checkpoint.

MRN foci formation is not required for the G2/M checkpoint

To verify the aforementioned conclusion, we exploited an earlier observation that overexpression of a C-terminal fragment of MDC1 comprising its γ H2AX-binding C-terminal BRCT domains yielded a strong dominant-negative effect on the accumulation and retention of the DDR proteins at sites of DSBs (Stucki *et al*, 2005). We reasoned that if our conclusion was correct, we should not observe a G2/M checkpoint defect on overexpression of the MDC1 BRCT domains. To test this, we used a U2OS cell line carrying a stably integrated, tetracycline-regulated, expression cassette directing the expression of the MDC1 tandem BRCT domain fused to yellow fluorescent protein (YFP). As observed previously (Stucki *et al*, 2005), induction of YFP-BRCT expression by the tetracycline analogue doxycycline (DOX) completely abrogated MRN accumulation at sites of DSBs, as reflected by both NBS1 foci formation (Fig 4A) and UV-laser micro-irradiation (Fig 4B). However, induction of YFP-BRCT expression did not trigger a measurable G2/M checkpoint defect after 1 and 3 Gy of irradiation, respectively (Fig 4C). Significantly, however, down-regulation of endogenous MDC1 in this cell line still yielded a significant G2/M checkpoint defect, irrespective of whether YFP-BRCT expression was induced or not, thus supporting the previous observation that MDC1 is required for G2/M checkpoint activation (Lou *et al*, 2003, 2006; Stewart *et al*, 2003). These data thus support our conclusion that MDC1-mediated accumulation and retention of the MRN complex at sites of DSBs is not required for activation or maintenance of the G2/M checkpoint response.

Speculation

Here, we present a unique divalent FHA/BRCT-binding mechanism that couples the MRN complex to γ H2AX-enriched chromatin regions that mark sites of DSBs, and we show for the first time to our knowledge that phospho-binding activities of both the NBS1 FHA domain and the BRCT tandem domain are essential for focal accumulation of the MRN complex at sites of DSBs *in vivo*. It is

Fig 3 | Mutation in the tandem BRCT domain of NBS1 does not yield a G2/M DNA-damage checkpoint defect. NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild-type, R28A or K160 mutant NBS1, respectively, were left untreated or irradiated at 1 and 10 Gy. Cells were harvested 1 h after irradiation, fixed with methanol and stained with an antibody against phosphorylated H3 (P-H3) and propidium iodide. The percentage of P-H3-positive cells was determined by fluorescence-activated cell sorting analysis. In this graph, three independent experiments (each performed in triplicate) are summarized. The error bars represent the standard deviation. NBS1, Nijmegen breakage syndrome 1.

unknown why such a divalent binding mechanism has evolved, but it is interesting to note that mutation in the NBS1 FHA domain triggers a G2/M checkpoint defect, whereas mutation in the BRCT tandem domain does not. This suggests that besides phosphorylated MDC1, the NBS1 FHA domain might have an additional, as yet unidentified, binding partner that mediates G2/M checkpoint activation in response to DSBs. While this paper was under revision, it was shown that *Schizosaccharomyces pombe* Ctp1, a protein that is involved in the resection of DSBs in the S and G2 phases of the cell cycle, interacts directly with the yeast Nbs1 FHA domain in a mechanism that involves CK2-dependent phosphorylation of SDT-like motifs in Ctp1 (Lloyd *et al*, 2009; Williams *et al*, 2009). Thus, CtlP, the human orthologue of Ctp1, might be a promising candidate for an additional NBS1 interaction partner. CtlP was shown previously to be required for efficient induction of the G2/M DNA-damage checkpoint (Yu & Chen, 2004). Furthermore, human CtlP also contains a region that comprises several conserved CK2 consensus sites; indeed, this region is phosphorylated efficiently by CK2 *in vitro* (F.H. & M.S., unpublished observation). However, whether or not these putative CK2 sites in CtlP interact with human NBS1 to mediate the G2/M DNA-damage checkpoint remains to be established.

METHODS

Cell lines and plasmids. NBS-iLB1 cells stably expressing wild-type and K160M mutant NBS1 were generated by retroviral transduction. The YFP-BRCT-expressing U2OS cell line was described by Stucki *et al* (2005). The human MDC1 GST-SDT construct was described by Spycher *et al* (2008). The MDC1(800)

Fig 4 | Experimental uncoupling of the MRN complex from damaged chromatin does not trigger a G2/M checkpoint defect. (A) Nuclear foci formation of NBS1 in inducible U2OS YFP-BRCT-overexpressing cells after irradiation at 5 Gy. Non-induced cells (top) and YFP-BRCT-expressing cells (bottom). (B) Microlaser-induced DNA-damage recruitment analysis of NBS1 in inducible U2OS YFP-BRCT-overexpressing cells. Non-induced cells (top) and YFP-BRCT-expressing cells (bottom). (C) Overexpression of the MDC1 BRCT domains does not trigger a G2/M checkpoint defect. Expression of YFP-BRCT fusion protein was induced 8 h before irradiation (+DOX). Mock-induced cells acted as the control (-DOX). Depletion of endogenous MDC1 by siRNA (siM) partly abrogated the G2/M checkpoint regardless of whether or not MDC1 is proficient for γ H2AX binding. The error bars represent the standard deviation. DAPI, 4',6-diamidino-2-phenylindole; DOX, doxycyclin; MDC1, mediator of the DNA damage checkpoint 1; MRN, MRE11-RAD50-NBS1 complex; NBS1, Nijmegen breakage syndrome 1; siRNA, small interfering RNA; YFP, yellow fluorescent protein.

fragment was cloned into a modified pcDNA3.1-Flag mammalian expression vector (Invitrogen, Eugene, OR, USA). Myc-NBS1 was subcloned into a pFastBac transfer vector (Invitrogen) to

generate recombinant NBS1 baculoviruses and into pLPCX (Clontech, Mountain View, CA, USA) to generate retroviral particles, respectively. Point mutations were introduced by using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA).

Single-cell analysis. DSBs in defined nuclear volumes were induced by laser micro-irradiation using an MMI CELLCUT system containing a 355 nm UVA laser (55 Hz; Molecular Machines & Industries, Glattpburg, Switzerland). Cells were stained with antibodies against human Nbs1 (Novus, Littleton, CO, USA) and γ H2AX (Upstate, Temecula, CA, USA). Images were captured by using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 40 $\times$ (oil immersion, NA 1.25) objective.

Biochemical analysis. GST pulldown assays were performed by mixing 5 μ g of GST-fusion proteins with a standard TNT reaction and 5 μ g of purified MRN, respectively. For co-immunoprecipitation, human embryonic kidney 293T cells were co-transfected with a Flag-tagged fragment of MDC1 (1–800 amino acids) and Myc-tagged Nbs1 constructs. Anti-Flag(M2)-beads (Sigma-Aldrich, St Louis, MO, USA) were used to immunoprecipitate proteins from total cell extract. All samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting.

G2/M checkpoint analysis. G2/M checkpoint analysis of NBS fibroblasts was performed as described by Spycher et al (2008). See the supplementary information online for details.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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