SUPPLEMENTARY MATERIALS

METHODS

Cell culture and transfection

All RNAi transfections were performed by using Oligofectamine (Invitrogen) transfection reagents. The MDC1 and CNTL (luciferase) siRNA used in this study (sequences: 5'-GUC UCC CAG AAG ACA GUG ATT-3'; and 5'-CGU-ACG-CGG-AAU-ACU-UCG-ATT-3' respectively) were purchased from MWG Biotech AG. All MDC1 siRNA experiments were validated with a siRNA smartpool against the MDC1 3'UTR (sequences: 5'-GAA CUC AGG GCG UUA GAA AUU; 5'-UGU CAU AAG UGG CAG CUA UUU-3'; 5'-GUU AUG GAG UGC UAC UAA AUU; 5'-GGA UUG GGG UAU AUU GAU AUU-3') purchased from Dharmacon. Cells were transfected with expression plasmids using Lipofectamin 2000 (Invitrogen) and Fugene 6 (Roche), according to manufacturers instructions. γ-irradiations were performed using a Faxitron X-ray cabinet equipped with a 1 mm aluminium filter to administer dose rates of either 0.44 Gy/min, or 0.27 Gy/min.

Immunofluorescence microscopy

Samples were processed according to standard procedures (Kolas et al, 2007), or pre-extracted for 5 minutes on ice with cytoskeletal buffer (10mM PIPES pH 6.8, 300mM sucrose, 50mM NaCl, 3mM EDTA, 0.5% Triton X-100, Protease Inhibitor Cocktail [Complete, EDTA-free; Roche]) before fixation in 2% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were immunostained as in (Kolas et al, 2007). Confocal images were acquired using an Olympus FV1000 Inverted microscope, equipped with 63X and 40X oil lenses. Images were acquired with Olympus FV1000 Software.

Generation of DNA DSBs by laser micro-irradiation

Cells were grown in glass-bottomed dishes and pre-sensitized by the addition of 5-bromo-2-deoxyuridine (BRdU) to the tissue culture medium to a final concentration of 10μM for 24 hours. Laser damage was generated using an Olympus FluoView 1000

confocal microscope equipped with a 405 nm laser diode (6mW) focused through a 60x UPlanSApo/1.35 oil objective to yield a spot size of approximately 0.5 μm. Laser settings were chosen as to generate a detectable damage response restricted to the laser path only in a presensitisation-dependent manner i.e. 50 laser scans with 45% AOTF (0.40 mW output). Operation of the UV laser was assisted by the FV1000-software.

Peptide pull-downs

Peptide pull-downs were carried carried out as in (Kolas et al, 2007) with the following biotinylated peptides: MDC1 S329 T331: Biotin-SGS-GFIDSDTDAEEERIP; MDC1 pS329 pT331: Biotin-SGS-GFID[pS]D[pT]DAEEERIP; and H2AX peptides (Stucki et al, 2005); except pull-downs with baculovirus-expressed and purified MRN (a gift from Tanya Paull) which were performed in the following buffer: 25 mM Tris pH 8, 150 mM NaCl, 10% Glycerol, 1 mM DTT, 0.1% BSA, 0.05% Tween-20, protease inhibitors (Complete, Roche).

CK2 *in vitro* phosphorylation

1μg of purified GST-fusion protein was incubated with 50 U of CK2 (NEB) in supplied buffers, and ATP was supplemented to 100 μM final concentration. Reactions were incubated for 2 h at 30°C. Reactions were scaled up or down as necessary. For interaction studies, GST-fusion kinase reactions were added to a 10x volume of high-salt bead buffer (BB: 300 mM NaCl, 50 mM Tris pH 8, 10% Glycerol, 1 mM DTT, 0.1% Tween-20, Protease inhibitor cocktail (Complete, Roche)), and fragments were captured on 15 μl of Glutathione Sepharose 4B (GE Healthcare) for 1 h at 4°C. Beads were then washed extensively in high salt BB before incubating in extracts/lysates, diluted 1:1 in low salt BB (as above, with final concentration adjusted to 150 mM NaCl) for pull-down experiments. After incubations, beads were washed extensively before sample preparation.

Immunoprecipitation

Cells were lysed in Benzonase lysis buffer: 40 mM NaCl, 25mM Tris (pH 7.5), 2 mM $MgCl_2$, 0.5% NP-40, protease inhibitor cocktail (Complete EDTA-free, Roche), 25 μl

Benzonase (Novagen), and incubated 4°C for 10 min before adjusting salt to 450mM and incubating for a further 30 min. Lysates were clarified by centrifugation, and 1-2 mg of lysates were adjusted to the final buffer conditions: 150-200 mM NaCl, 25 mM Tris (pH 7.5), 10% Glycerol, 2mM EDTA, 1 mM DTT, 0.125% NP-40, Protease inhibitors (Complete, Roche). After incubation with 1-2μg antibody, immmune complexes were captured with Protein G Dynabeads (Dynal) for 1 h at 4°C, and beads extensive washed the above buffer.

Recombinant protein production & cell extracts

Recombinant proteins were expressed in *E. coli* strain BL21(DE3). Fusion proteins were purified on glutathione-sepharose 4B according to the batch method described in the GST manual (GE Healthcare). For IVT expressed protein fragments, 50μl TnT reactions were assembled according to manufacturer's instructions (Promega), plasmid DNA added, and reactions incubated for 90 min at 30°C. Nuclear extracts were prepared according to Dignam *et al.* (1983), or purchased (HeLa Nuclear extracts, Cilbiotech).

Plasmids

The GST-$SDTD_2$ and GST-$SDTD_6$ plasmids were generated by inserting a fragment corresponding to MDC1 amino acid residues (a.a.) 296-340 or 181-517 into pGEX-4T1. The siRNA-resistant $MDC1^{WT}$ expression plasmid was generated as described in (Kolas et al, 2007). The $MDC1^{SDTD\Delta}$ expression plasmid was generated in a similar manner, but the region encoding a.a. 197-506 was deleted by fusion PCR and replaced with a fragment encoding six serine-glycine repeats to form a flexible linker. HA-$SDTD_6$-NLS was generated by fusing a fragment corresponding to HA-tagged MDC1 a.a. 197-516, to the triple NLS sequence from peYFP-Nuc (Clonetech). To produce the HA-fNBS1 plasmid to use in *in vitro* TnT expression reactions (Promega), a fragment corresponding to NBS1 a.a. 1-348 was cloned into pEPEX-HA. Site-directed mutagenesis to generate derivatives of the above plasmids was carried out with Quikchange (Stratagene). Point-mutations predicted to abrogate NBS1 FHA and BRCT-dependent interactions were previously described (R28A, H45A) (Lukas et al, 2004; Zhao et al, 2002), or selected (K160M) due to sequence and predicted structural homology with other well

characterized BRCT domains.

Antibodies

We employed the following antibodies: mouse anti-53BP1 (clone 19, BD Biosciences), mouse anti-MDC1 (clone MDC1-50, Sigma-Aldrich), rabbit and sheep anti-MDC1 (Goldberg et al, 2003), mouse anti-BRCA1 (clone D9, Santa Cruz), anti HA mouse anti-GST (clone B4, Santa Cruz), rabbit anti-NBS1/p95 (Novus Biologicals), rabbit anti-53BP1 (Novus Biologicals), mouse anti-RAD50 (Genetex, clone 13B3), mouse anti-MRE11 (Genetex, clone 12D7), mouse anti-HA (Covance, clone HA.11), rabbit anti-Cyclin A (Santa Cruz), rabbit anti-SMC1 and anti-SMC1 pS966 (Bethyl). The MDC1 pSDpTD antiserum was raised against a phospho-peptide encoding MDC1 aa 325-335 in which both S329 and T331 are phosphorylated. Resulting antisera was then affinity purified against the phospho-peptide, following cross-depletion against a non-phosphorylated peptide equivalent (21st Century Biochemicals, MA).

Flow Cytometry

Plates containing ~1x10^6 cells were trypsinised and cells collected and pooled with mitotic cells obtained in prior washes. Cells were harvested and washed by centifugation at 500g in ice cold PBS, before fixing in 1% paraformaldeyde at 37°C for 10 min. Cells were subsequently fixed in a 10x volumes of methanol overnight at -20°C. Cells were harvested by centrifugation, washed 2x in PBS, then incubated in FACS incubation buffer (FIB: 0.5% BSA in PBS, 0.02% sodium azide) containing 1:30 dilution of indicated antibody for 1 hour. Cells were subsequently washed once in FIB before resuspending in 1:400 anti-rabbit/mouse Alexa 488 Dye (Molecular Probes) and incubated for 30 min. Cells were washed once more, then resuspended in 10 μg/ml propidium iodine, 0.25 mg/ml RNase A in PBS, before analysis using a FACS Calibur flow cytometer (BD Bioscience).

Mass-spectrometric analysis

Gel slices were analysed by 1D LC/MS at the TCMR, Department of Biochemistry, University of Sussex, UK.

SUPPLEMENTARY FIGURES

LEGENDS

Fig S1. (A) Specificity of the anti-MDC1 S329/T331 phospho-specific antibody. Indicated amounts of MDC1 S329/T331 double-phosphorylated and non-phosphorylated peptide were spotted on nitrocellulose. Membrane was dried before blocking, then subsequently processed by standard western blotting protocols with anti-MDC1 pS329pT331 affinity purified antibody (concentration of 1:1000). **(B)** MDC1 pS329pT331 phosphorylation is constant during interphase. Asynchronous cultures of U2OS cells (panel 1) were fixed and probed with antibodies against Cyclin A, MDC1 (monoclonal), and MDC1 pS329pT331. Cells were then stained with an anti-rabbit/mouse 488nM fluorescent dye and DNA counter-stained with propidium iodide. Antigen levels were measured by fluorescence and plotted against DNA content by flow cytometry. Fluorescence was plotted on a log scale. **(C)** Demonstrates nuclear localization of HA-SDTD$_6$-NLS. 48 h following transfection cells were washed, fixed, and processed for immunofluorescence with anti-HA antibodies.

Fig S2. Protein levels of NBS1 in complemented NBS cell-lines. Lysates prepared from MRC5 control fibroblasts, NBS ILB1, and NBS ILB1 cells stably transfected with the indicated NBS1 expression constructs were immunoblotted with antibodies against NBS1 or SMC1 (loading control). Exogenous levels of NBS1 expression in NBS1 cell-line complemented with WT NBS1, may account for increased NBS1 recovery in corresponding lanes of Fig 3C.

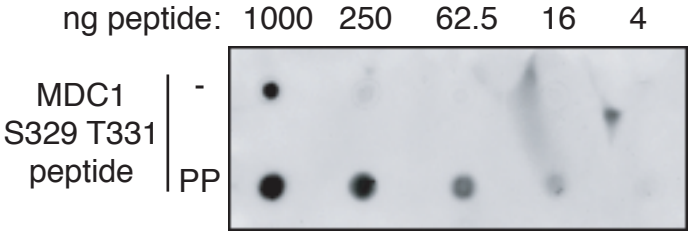
Fig S3. (A) siRNA mediated "knock-in" of MDC1^{WT} and MDC1$^{SDTD\Delta}$. U2OS cell-lines stably transfected with the indicated expression constructs were treated with two rounds of siRNA against MDC1. Lysates were prepared and experiments performed at 72 h after first transfection when endogenous MDC1 depletion was optimal. Experiments were discarded if MDC1-depletion < 90%. **(B)** MDC1-MRN interactions are dependent on the SDTD region of MDC1. Indicated expression constructs were transfected into HEK293

cells. After 48 h extracts were prepared with 200mM salt concentration, immunoprecipitated with anti-GFP antibodies, and immunoblotted. “INP”, input (5%). **(C)** The SDTD region of MDC1 mediates NBS1 IRIF formation. Endogenous MDC1-depletion was performed as in (A) and cells were either mock-treated, or treated with 5Gy γ-irradiation. 4 h following irradiation, cells were fixed and processed for immunofluorescence with MDC1 and NBS1 antisera. NBS1 foci were blind counted in at least 90 cells over 3 independent experiments. Error bars indicate standard error of the mean. *Indicates MDC1 siRNA resistant.

Fig S4. The MDC1 SDTD-MRN interaction is not required for BRCA1 IRIF. The indicated U2OS cell lines were treated with two rounds of control (luciferase) or MDC1-targetting siRNA for 72 h. Untreated cells, or cells treated with 5 Gy of X-rays **(A)** were processed for immunofluoresence 4 h following irradiation with anti-MDC1, and anti-BRCA1 antibodies. **(B & C)** are control-treated cells corresponding to irradiated cells in Fig 4 B & C respectively.

Chapman & Jackson Fig S1

A



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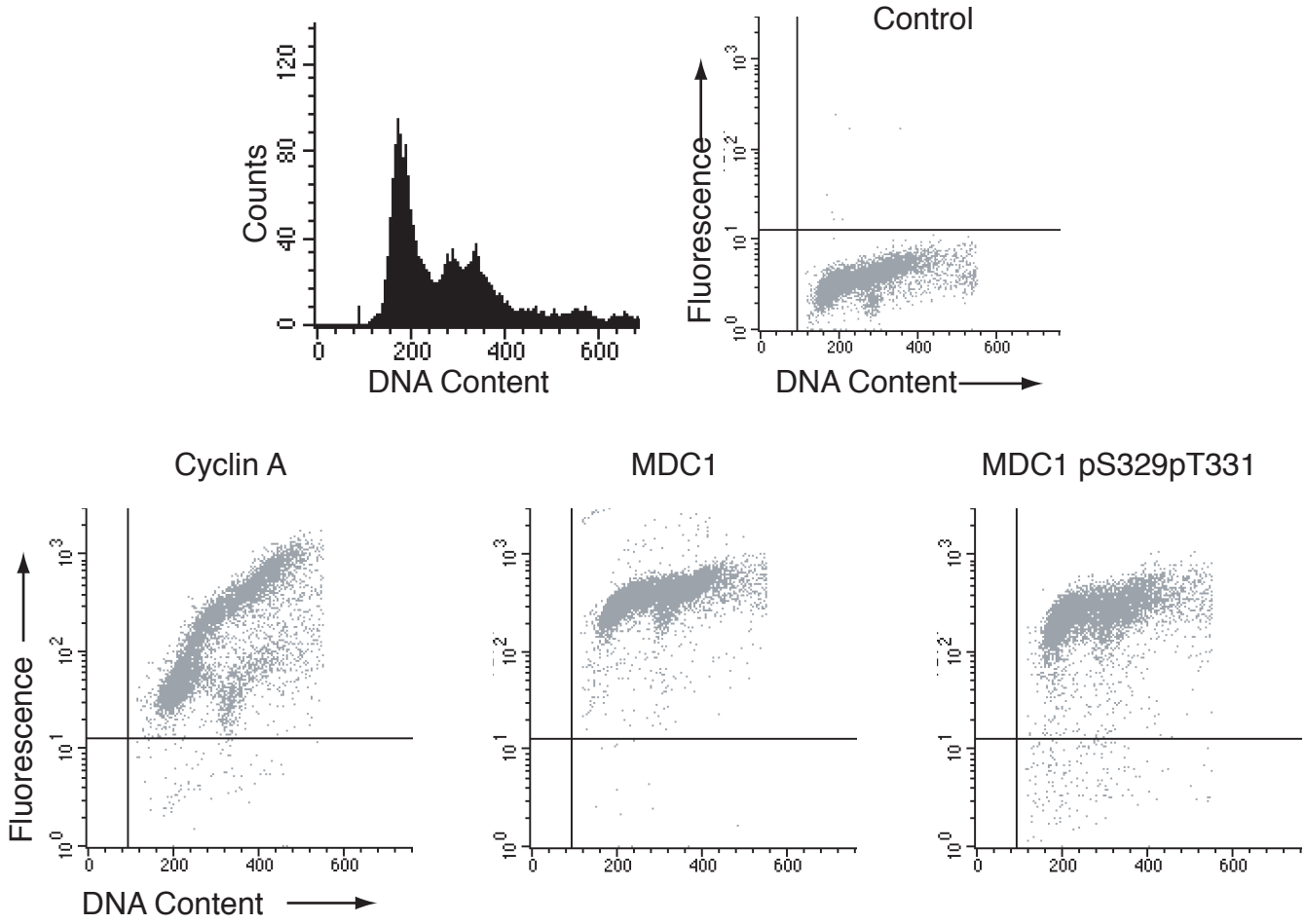

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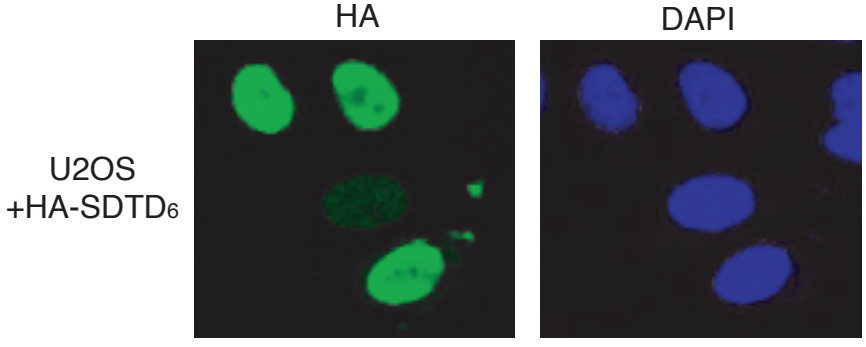
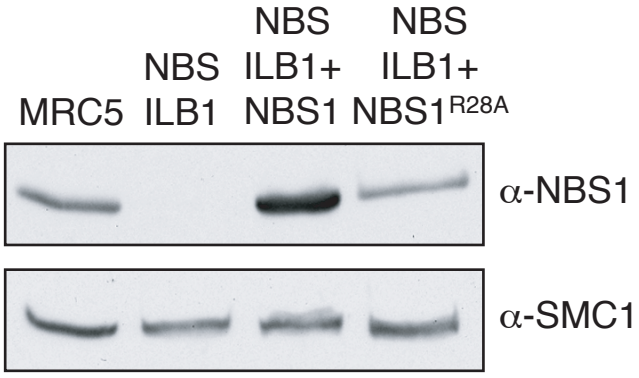
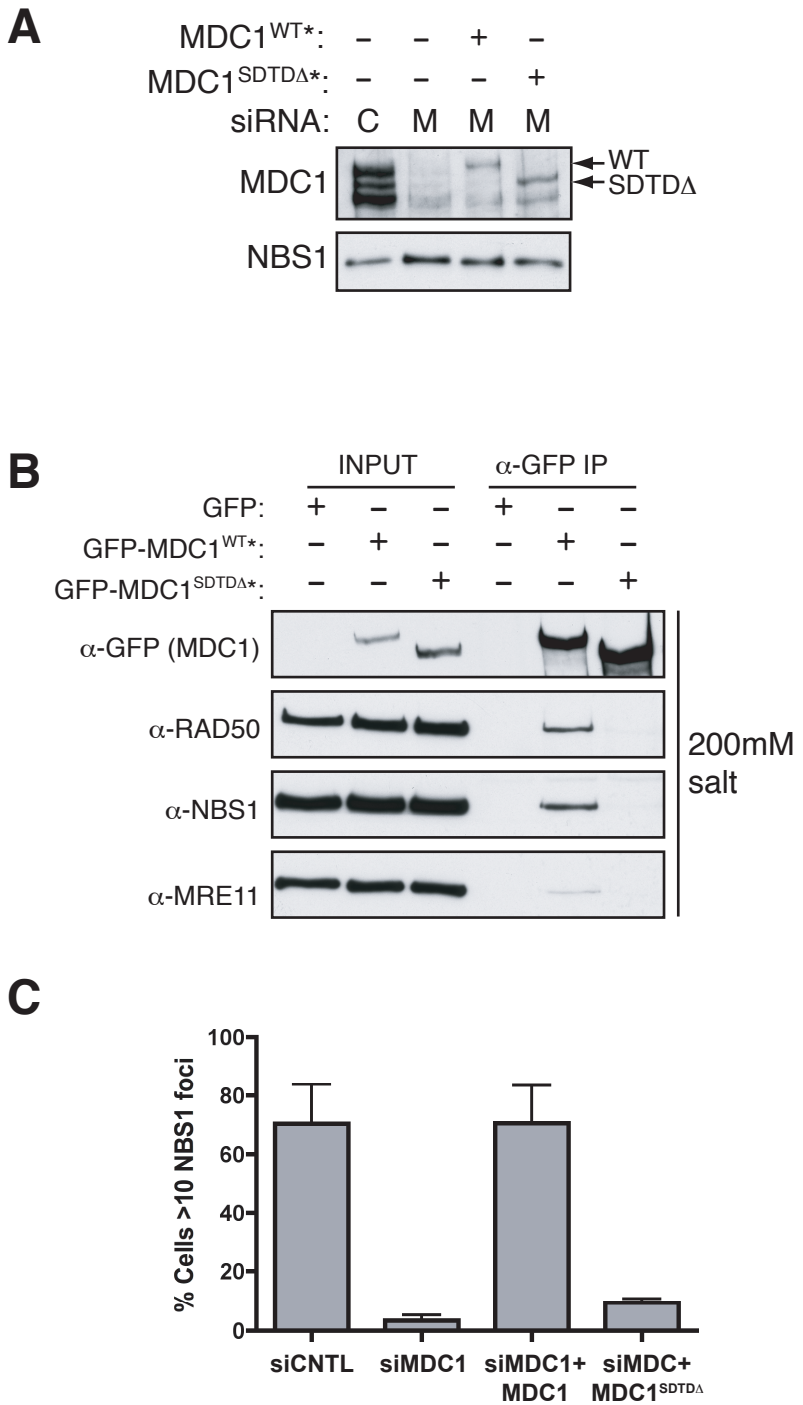


Chapman & Jackson Fig S2



Chapman & Jackson Fig S3



Chapman & Jackson Fig S4