SUPPLEMENTARY MATERIALS

METHODS

Cell extraction and protein purification

Hela nuclear extract was purchased from Cilbiotech (Mons, Belgium). MDC1-GST-SDT fragment was affinity purified on Glutathione-Sepharose (GE Healthcare Biosciences). Recombinant MRN purification from Sf9 cells was described (Spycher et al, 2008). For in vitro-translation of full-length NBS1, the TNT system (Promega) was used.

Microirradiation and single-cell analysis

In order to generate DSBs in defined nuclear volumes laser microirradiation was performed with a MMI CELLCUT system containing a 355 nm UVA laser (55 Hz, Molecular Machines & Industries, Switzerland) coupled to an Olympus IX71 microscope station and focused through an LUCPLFLN 40X objective. The MMICELLTOOLS software with MMIUVCUT plug-in assisted the laser operation using an energy output of 50%. Prior to laser irradiation, cells were grown on coverslips in cell culture dishes in the presence of 10 µM BrdU (Bromodeoxyuridine; Sigma) for 24 h. Coverslips were transferred into LabTek chamber slides (Nunc) and mounted on the microscope stage for irradiation. After irradiation, cells were placed back in the incubator for 30-60 min before fixation.

Biochemical analysis

For GST pull down assays, purified GST-fusion proteins (5 µg) were mixed with 1/5 volume of a standard TNT reaction and 5 µg of purified MRN, respectively.
Where indicated, GST fusion proteins were pre-treated with 100 U of CK2 (New England BioLabs). The mixture was incubated at 4°C for 30 min to allow binding. Glutathione sepharose beads were added and the suspension was incubated for further 60 min. The beads were washed with buffer (50mM Tris pH 7.5, 120 mM NaCl, 1 mM DTT, 0.2% NP-40) and resuspended in SDS loading buffer.

For co-immunoprecipitation, HEK 293T cells were co-transfected with a Flag-tagged fragment of MDC1 (1-800 aa) and Myc-tagged Nbs1 constructs. Cells were lysed in lysis buffer (25 mM Tris pH 7.5, 40 mM NaCl, 2 mM MgCl₂, 0.5 % NP-40, protease and phosphatase inhibitors, 25 U/ml benzonase (Novagen)) and incubated for 30 min at 4 °C. The concentration of NaCl was increased to 450 mM and incubated for another 30 min at 4 °C. After centrifugation, extracts were diluted to 100 mM NaCl, added to pre-blocked anti-flag(M2)-beads (Sigma) and incubated for 3 h at 4 °C. The beads were washed with IP-buffer (25 mM Tris pH 7.5, 100 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM DTT, 0.5 % NP-40, protease and phosphatase inhibitors) and resuspended in SDS loading buffer. All samples were analyzed by SDS PAGE and immunoblotting.

**Checkpoint analysis**

Cells were irradiated with a Faxitron X-ray cabinet at the indicated doses during the exponential growth phase. 1h later, cells were harvested, fixed with 70% ethanol/PBS and incubated over night at -20°C. After permeabilization with 0.25% Triton/PBS, cells were stained with anti-phospho-histone H3 (Upstate), followed by secondary anti-FITC (Jackson) or Alexa 700 (Invitrogen) antibodies and propidium iodide. Data were acquired with a Becton Dickinson flow cytometer (NSB...
fibroblasts) or a Beckham Coulter CyAn ADP 9 Color flow cytometer (U2OS YFP-BRCT cell line).
Supplementary Figure S1

(A) NBS1 expression profile of NBS-iLB1 fibroblasts stably transduced with wild type NBS1, R28A NBS1 and K160M NBS1.

(B) Two independent clones of NBS-iLB1 fibroblasts stably transduced with wild K160M mutant NBS1 were left untreated or irradiated with 1 Gy and 10 Gy. Cells were harvested 1 hour after irradiation, fixed with methanol and stained with an antibody against phosphorylated H3 (P-H3) and propidium iodine. The percentage of P-H3 positive cells was determined by FACS analysis. NBS-iLB1 parental cells and NBS1-iLB1 cells stably transduced with wild type NBS1 served as negative and positive controls, respectively. Error bars represent standard deviation.
**Figure S1**

**A**

<table>
<thead>
<tr>
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<th>NBS-iLB1</th>
<th>WT</th>
<th>R28A</th>
<th>K160M</th>
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<tr>
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**B**

Bar graph showing the percentage of H3-P cells across different IR (Gy) treatments with and without the presence of WT, K160M #2, and K160M #7. The x-axis represents IR (Gy) levels (0, 1, 10), and the y-axis represents the percentage of H3-P cells (0 to 100%). The graph includes bars for NBS-iLB1, + WT, + K160M #2, and + K160M #7 treatments.