Supplementary information

Cyclin-dependent kinase targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination

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Supplementary methods

CDK kinase assays

CDK1/Cyclin B was from Millipore and purified MRN was a gift from T. Paull (University of Texas, Austin). Assays were performed in CDK1/Cyclin B buffer for 10 min at 37 °C in the presence of ^{32}P-γ-ATP.

Western blots and immunoprecipitations

Anti-NBS1 pSer-432 was generated as a rabbit polyclonal antibody (now available from Abcam). Other antibodies used: NBS1 (rabbit; Merck), MRE11 (rabbit; Novus), Cyclin A (rabbit; Santa Cruz), Histone H3 pSer-10 (rabbit; Millipore), ATM pSer-1981 (rabbit; Rockland), ATM (mouse; gift from Y. Shiloh, Tel Aviv), NBS1 pSer-343 (rabbit; Cell Signalling), CHK1 pSer-317 (rabbit; Bethyl), CHK1 (mouse; Santa Cruz), GFP (mouse; Roche), CDK1 (mouse; Oncogene), CDK2 (rabbit, Santa Cruz). NBS1 was immunoprecipitated with a mouse monoclonal antibody (Abcam; 2 μl/mg protein extract). GFP immunoprecipitations were performed with GFP-Trap® beads (Chromotek).

Transfections

GFP-CtIP (Sartori et al, 2007) was transfected in NBS-ILB1 cells using FuGENE HD (Roche) and following manufacturer's instructions. siRNA transfection was performed in U2OS cells using Lipofectamine RNAiMax (Invitrogen) following manufacturer's instructions. Sequences of the siRNAs used: luciferase 5′-cguacgcggaauacuucga-tt-3′, CDK1 5′-gggguuccuaguacugcaa-tt-3′, CDK2 5′-gccagaaacaaguugacgg-tt-3′.

Immunofluorescence

Primary antibody staining was for 1 h in 5% FBS in 1xPBS (NBS1, rabbit; Merck), γH2AX (mouse or rabbit; Upstate and Cell Signalling, respectively), RPA2 (mouse; Abcam). Secondary antibody staining was done with Alexa Fluor 488 or 594 (Molecular Probes) for 30 min. All incubations were at room temperature. To detect ssDNA, cells were labeled with BrdU for 36 h, washed, and cultured in the absence of BrdU for 24 h before the experiment. BrdU was detected under native conditions with an anti-BrdU antibody (mouse; GE Healthcare).

EdU incorporation assay

Cells were pulsed with 10 μM EdU for 30 min before collection. EdU detection was performed with Click-iT® EdU Flow Cytometry kit (Invitrogen) following manufacturer's instructions.

Quantification of mitotic cells

Identification of mitotic cells was by staining chromosomal DNA with propidium iodide combined with immunofluorescent detection of histone H3 pSer-10 (rabbit;

Millipore). When measuring mitotic entry after HU treatment, cells were incubated with 2 mM HU for 24 h. After extensive washing, cells were incubated in fresh medium containing nocodazole, and the proportion of mitotic cells at the indicated times was measured.

Cell survival assays

Cells were exposed to differing doses of IR or HU. For HU, cells were exposed to the drug for 24 h. After 10–14 days, colonies were stained with 0.5% crystal violet/20% ethanol, counted and normalized to plating efficiencies.

DNA repair assays

HR and NHEJ assays were as described previously (Pierce et al, 1999; Lou et al, 2004).

Replication restart assays

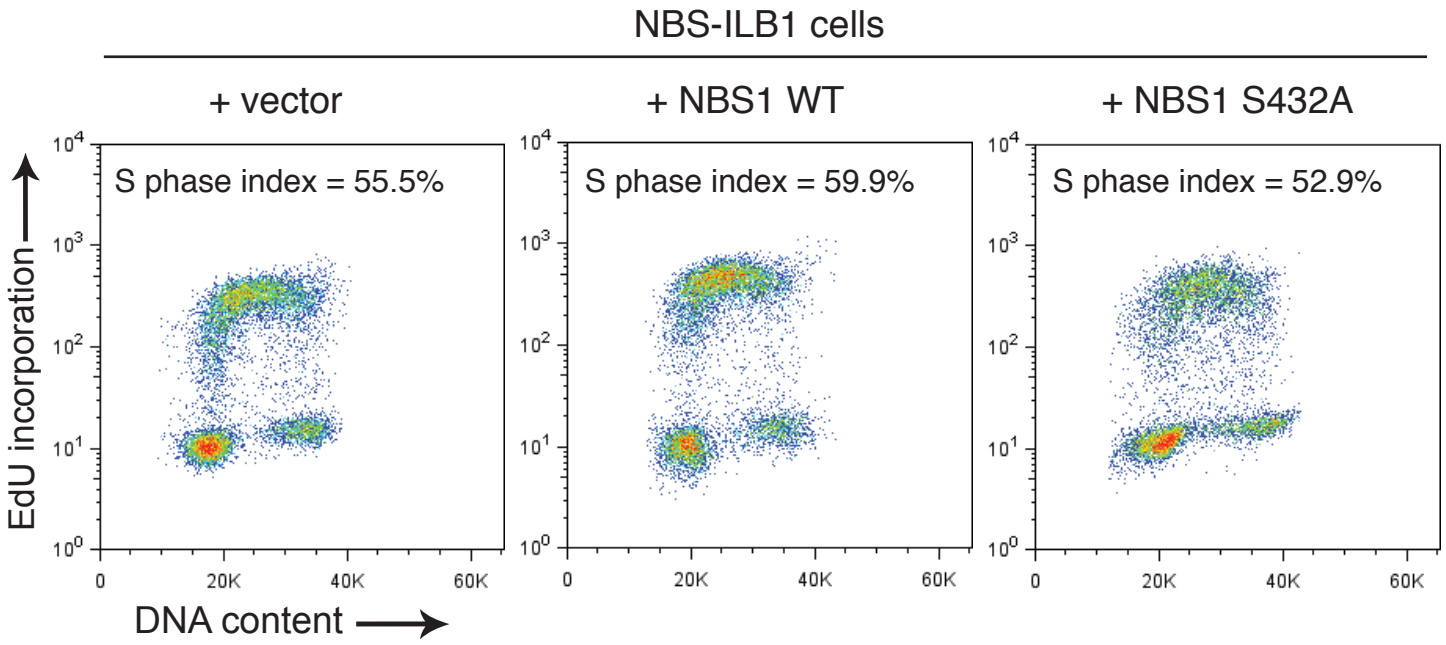
Replicative cells were marked by pulse labelling for 30 min with 5-chloro-2-deoxyuridine (CldU 50 μM) then arrested by treating with aphidicolin (10 μM) or HU (2 mM) for 6 h. After drug removal, cells were treated with 5-iodo-2-deoxyuridine (IdU 50 μM) for 30 min (aphidicolin treatment) or 60 min (HU treatment) so that replication restart after stalling could be visualised. CldU and IdU detection was as described previously (Stiff et al, 2005) with anti-BrdU antibodies from Abcam and Becton-Dickinson that cross-react with the indicated halogenated nucleotide. Replication recovery is represented by the overlap of CldU and IdU labelling.

Supplementary figure legends

Figure S1: EdU incorporation is similar in all used cell lines. The *y* axis represents intensity of EdU signal (EdU incorporation). The *x* axis represents DNA content as measured by intensity of 4',6-diamidino-2-phenylindole (DAPI) signal.

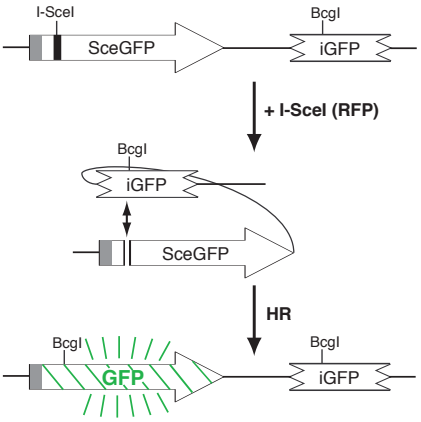
Figure S2: HR assay. **(A)** Schematic of the HR assay system introduced in NBS-ILB1 cells. **(B)** Only cells positive for RFP (indicating transfection with the I-*Sce*I endonuclease) and GFP (indicating recombination between the GFP fragments) are counted as HR events in the assay. **(C)** Representative images from one of the HR assays. Percentages indicate the amount of HR events.

FALCK et al SUPP. FIGURE S1



FALCK et al SUPP. FIGURE S2

A



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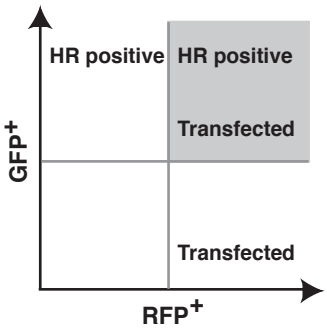

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