Supplementary methods:

Robot-automated screen

The automated screen was performed using a liquid handling station (STAR; Hamilton Robotics). U2OS cells expressing a dominant-negative p53 in a tet-off system were reverse transfected with a custom designed siRNA library targeting 236 DNA damage repair genes (Applied Biosystems) and an siRNA library targeting the human kinome (Applied Biosystems). The libraries are comprised of three independent siRNA constructs per target, with each construct being individually spotted. In brief, 4 μl siRNA was added to 2.5 μl OptiMEM (Invitrogen) to each well of a 384-well plate (Corning). To this, a 6.4 μl OptiMEM/0.1 μl Oligofectamine mix was added and left to stand for 15 min after which 27 μl of cells was added to give a total cell density of 2,800 cells per well. The final concentration of siRNA was 100 nM. Cells were incubated for 48 h, followed by 6 Gy of ionizing radiation. 2 hours later Nocodazole was added and cells were incubated for 8 hours, followed by Hoechst staining (bisBenzimide H 33342; Sigma-Aldrich), 4% paraformaldehyde fixation, permeabilization, and phosphorylated H3Ser10 antibody staining, in conjunction with an Alexa Fluor 594 secondary antibody. Cells were left at 4°C in PBS before imaging. The cells were imaged on an IN Cell Analyzer 1000 (GE Healthcare), and 10 images per well were acquired using a 20× objective to count ~2,000 cells per well. The acquired images were then analyzed using the IN Cell Analyzer Workstation 3.5 software (GE Healthcare).

Screen data analysis

The siRNA scores (the percentage of H3Ser10p–positive cells) were normalized according to median sample score of each plate (i.e. excluding controls). To minimize the risk of misinterpretation of gene activity because of off-target effects of the siRNAs, we computed a statistical score that modeled the probability of a gene 'hit' based on the collective activities of multiple siRNAs (mainly 3) per gene using the statistical method redundant siRNA activity (RSA) analysis (König et al, 2007). Using the RSA method, we first ranked the siRNAs according to their score (excluding the controls). Afterwards, a p-value was calculated for each gene based on how likely it was to observe this distribution of siRNA ranks in the list by chance. The calculation of the p-values was based on the iterative hypergeometric distribution{König, 2007 #2436}. The genes were filtered according to their RSA-calculated p-value (5% significance threshold), and the two screen p-values for each gene were combined using Fisher's method (also known as Fisher's combined probability test). The entire data analysis was conducted using the statistical software ‘R’.

Oligonucleotide sequences:

The following oligonucleotide sequences were purchased from Sigma-Aldrich:

BRCA2-1: 5'CCAACUUUGUCCUUAACUA[dT][dT];

BRCA2-2: 5'GGAAUGUUCCCAAUAGUAG[dT][dT];

BRCA2-3: 5'GGCAAAUGUUGAAUGAUAA[dT][dT];

CHK1: 5'GGGAUAUUAAACCAGAAAA[dT][dT];

PALB2: 5'CUUAGAAGAGGACCUUAUU[dT][dT];

AURORA A: 5'GGCAACCAGUGUACCUCAU[dT][dT];

BORA: 5'CUAUGAGACUUCAGAUGUA[dT][dT];

Rad51#1: 5'UUGAGACUGGAUCUAUCAC[dT][dT];

RAD51#2: 5' GAGCUUGACAAACUACUUC[dT][dT];.

MISSION® siRNA Universal Negative Controls (Sigma-Aldrich) were used as negative controls.

Cell synchronization:

For cell synchronization by double thymidine block, cells were grown in medium containing 2mM thymidine for 20h to induce the 1st block, released into normal medium for 15 hours, and incubated again with 2mM thymidine for 20h for the 2nd block, followed by release into normal medium. BRCA2 siRNA transfection was performed after the release from the 1st block.

Flow cytometry

Cells were fixed 48 h after transfection in 70% ethanol and stained with rabbit anti–phospho-H3Ser10 (1:500; Millipore) and mouse anti–γ-H2AX (1:500; Millipore) for 1 h, followed by 1 h incubation with Alexa Fluor 488 anti–rabbit and 647 anti-mouse IgG (1:1000; Invitrogen). DNA was stained 0.1 mg/ml propidium iodide (PI) containing RNase for 30 min at 37°C or with Hoechst 33258 (1,5 mg/ml). Flow cytometry analysis was performed on FACSCalibur or LSRII flow cytometers (BD Biosciences) using CellQuest or Cellquest Pro software (Becton Dickinson). For EdU analysis, cells were pulsed for 10 min before IR with 10 mM EdU. EdU was detected with a Click-iT EdU Cell Proliferation Assay kit (Invitrogen), and DNA was stained with the DNA stain Cell Cycle 633 provided in the kit. The EdU-positive and -negative populations were gated, and the percentage of H3Ser10p–positive cells within each population was determined.

Immunoblotting

For immunoblotting, cells were lysed in radioimmunoprecipitation assay buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1% IgePal630, 1% deoxycholic acid [Na salt], 0.1% SDS, 1 mM PMSF, 5 μg/ml leupeptin, 1% aprotinin, 50 mM NaF, and 1 mM DTT) or in boiling lysis buffer (Tris HCl pH7.5, 2% SDS, 1mM Na_3VO_4) or 4xLSB buffer (200mM Tris-HCl pH6.8, 400mM DTT, 40% Glycerol, 8%SDS, 0.005% Bromphenolblue). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated in primary antibody diluted in 5% milk, followed by incubation with secondary antibody (peroxidase-labeled anti–mouse or –rabbit IgG; 1:10,000; Vector Laboratories).

Immunofluorescence

Cells were fixed for 5 minutes in methanol-acetone at -20ºC, and incubated with primary antibodies diluted 1:500 for 1h at room temperature, followed by incubation with secondary antibodies (goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568 diluted 1:1000) for 30 minutes at room temperature and mounting with Vectashield mounting medium containing DAPI (Vector Laboratories). Imaging was performed using a 63x 1.4 NA oil immersion objective on an Axio Imager Z1 microscope with ApoTome (Zeiss) using Axio Vision Release 4.8 software.

Live cell imaging was performed using a Deltavision Core microscope (Applied Precision) in combination with a UApo 40X/1.35 NA objective. Cells were filmed in Lab-Tek dishes (Nunc) using Leibovitz´s L-15 medium (Gibco).

Supplementary figure legends

S1) Left panel: Plot of the 'Strictly Standardized Mean Difference' (SSMD) for the DNA repair screens. For each plate id (x-axis) the calculated SSMD is plotted for both screen 1 (circles) and screen 2 (triangles). Right panel: Scatterplot showing gene log2-values for DNA repair screen run 1 versus run 2 with positive and negative controls highlighted in grey and red, respectively.

S2) HeLa cells, parental U2OS cells and p53-/- HCT116 cells (Control) and cells transfected with BRCA2 siRNA (BRCA2), were treated with 6 Gy of IR and 2 h later with Nocodazole for 8 h and analyzed by flow cytometry. The bar plot is the mean of the percentage of H3Ser10p–positive cells of three experiments. Error bars indicate s.e.m. (n=3), except for experiment performed in HeLa cells (n=2).

S3) S- and G2-phase cells overcome IR-mediated G2 arrest when BRCA2 is knocked down. (Compare 2h IR + 8h Noco siBRCA2 vs. siControl in the presence and absence of Aphidicolin). Flow cytometric analysis of U2OS p53dneg cells, transfected with Control or BRCA2 siRNA for 2 days and treated with 6 Gy of IR for 2, 5, and 10 h. Where indicated, Aphidicolin (Aph) was added immediately after IR to block S phase cells from cycling. In case of the untreated cells, Aphidicolin was added for 10 h. Where indicated, cells were treated with Nocodazole (Noco) for 8 h. The bar plot is the mean of the percentage of H3Ser10p–positive cells of two experiments.

S4) U2OS p53dneg cells were transfected with siRNAs targeting BRCA2 or RAD51 (2 different siRNAs), respectively, and after 2 days harvested for immunoblotting analysis or treated with 6 Gy of IR and 2 h later with Nocodazole for 8 h and subsequently harvested for flow cytometric analysis. Error bars indicate s.e.m. (n=3).

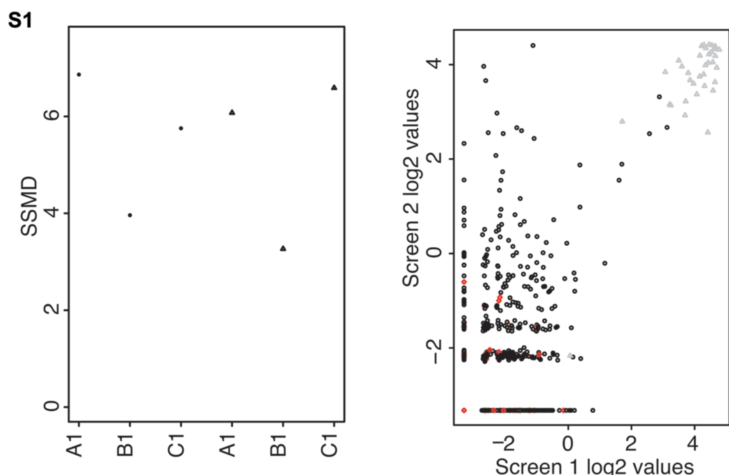
S5) Knockdown efficiency analysis of U2OS p53dneg cells transfected or co-transfected with siRNAs targeting BRCA2, PALB2, BORA and AURORA A, respectively. Cells were harvested for Western blot analysis two days after transfection. Anti-Actin antibody was used as a loading control.

S6) Immunoblot of U2OS p53dneg cells depleted for PALB2 for 2 days and harvested at 1, 2 and 5 h after 6 Gy of IR. Anti-Vinculin antibody was used as a loading control.

Supplementary movie files: Live cell imaging of U2OS p53dneg cells two days after transfection with Control (“siControl 6Gy.mov”) or BRCA2 (“siBRCA2 6Gy.mov”) siRNA. The cells were irradiated

with 6 Gy of IR immediately prior to the imaging process. The movies represent a time span of 12 hours.

Supplementary excel sheet: Lists of the kinases and DNA repair factors that were targeted during the two RNAi screens, including the percentage of Histone H3 Serine 10 phosphorylated cells after IR and Nocodazole treatment. Note that a number of non-kinases were included in the kinome library to serve as general controls (this includes E2F transcription factors).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SSMD
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C1
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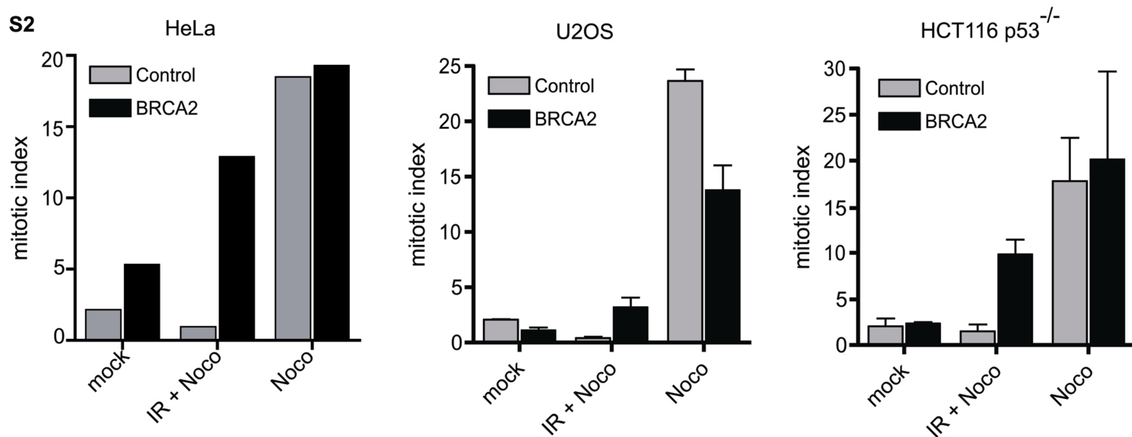
Screen 2 log2 values
Screen 1 log2 values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BRCA2
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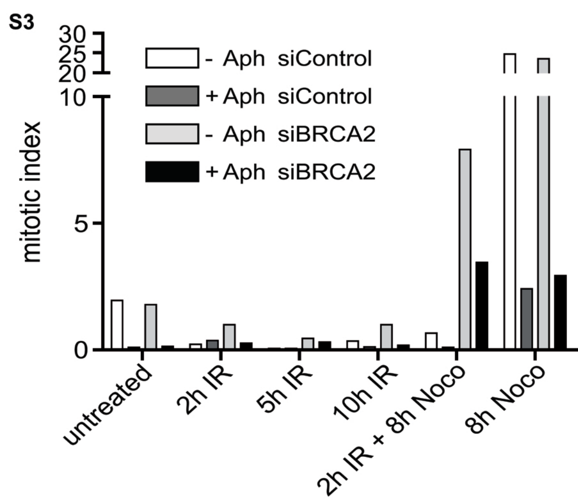
mock
IR + Noco
Noco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mitotic index
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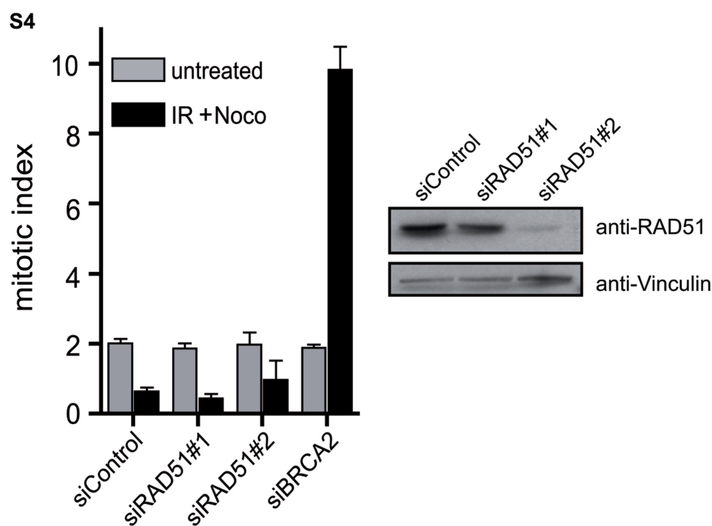
8h Noco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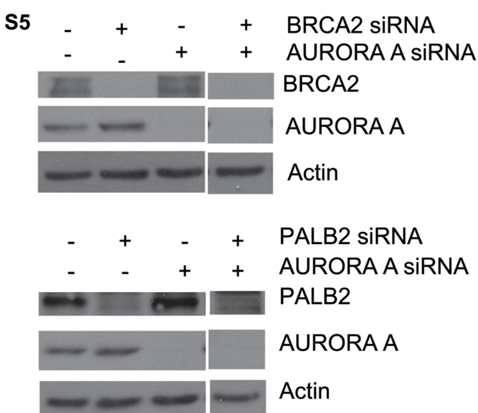
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BRCA2
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Actin
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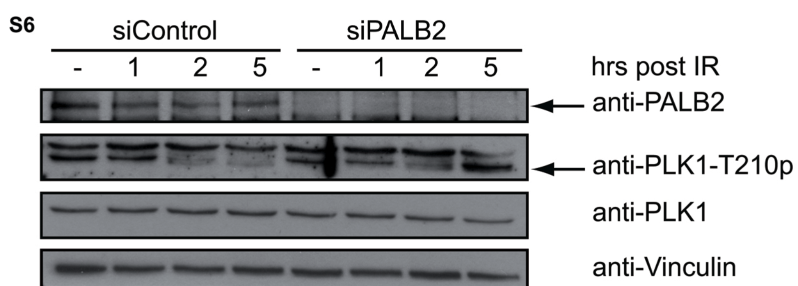
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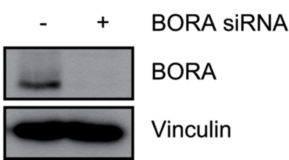
BORA siRNA
BORA
Vinculin