Supplemental Information

Plk1 Phosphorylation of Mre11 Antagonizes the DNA Damage Response

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Supplemental Methods

Immunoblotting (IB) and immunoprecipitation (IP)

Cell lysates were prepared in TBSN buffer (20 mmol/L Tris-HCl, pH8.0, 1 mmol/L EDTA, 0.5 mmol/L Na_3VO_4, 5 mmol/L EGTA, 1% NP-40) supplemented with 150 mmol/L NaCl. For IP, lysates were incubated with antibodies in TBSN at 4˚C overnight, followed by 3 times wash with TBSN plus 500 mmol/L NaCl and 3 more times wash with TBSN plus 150 mmol/L NaCl.

Fluorescence activated cell Sorting (FACS) analysis

Cells in culture dishes were trypsinized, washed with PBS and fixed in 95% ice-cold ethanol for overnight. After centrifugation, the cells were resuspended in PBS and incubated with 50 mg/mL propidium iodide in the presence of 100 units/mL RNase A, followed by FACS analysis.

Repair Assays

To analyze I-Scel-induced GFP^+ frequencies in NHEJ reporter cells, we transfected I-Scel-expressing plasmid into reporter cells as described [1] to induce a break. To measure the repair by transient transfection, cells (2.5×10^4 cells/cm^2) were plated and transfected next day with 0.8 μg/mL of I-Scel plasmid mixed with 4 μL/mL of lipofectamine 2000 along with a variety of other vectors. The day prior to transfection, cells were incubated in 1.5 ml of medium without antibiotics. Three hours after transfection complexes were added, the medium was removed and replaced with medium containing antibiotics.

Irradiation

IR (2.2 Gy/min) was generated from a ^{60}Co γ-radiation source with an IBL 437C irradiator.

Colony formation assay

Cells (0.5×10^3) were seeded in 6-well plates and cultured in medium alone or containing different drugs for two weeks, with medium refreshment every 2 days. After culturing, cells were fixed in 10% formalin and stained with 0.5% crystal violet, and colony numbers were counted.

Supplemental references

1. Bennardo, N., et al., *Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair.* PLoS Genet, 2008. **4**(6): p. e1000110.

Supplemental figure legends

Figure S1. Plk1 suppresses DDR by inactivation of the ATM-Chk2 pathway. A, U2OS cells were processed as in Fig. 1A. Data are derived from the capture of at least 300 images of cells and amounts of foci per nucleus were shown. Error bars represent the standard deviation of foci numbers. Values are mean ± s.d. of three independent experiments. *: $P < 0.05$ (two-tailed unpaired t-test). B, U2OS cells were treated with/without 50 nmol/L BI2536 for 12 hours, and harvested for IF with p-ATM. For quantification, at least 300 cells were scored for p-ATM foci and percentages of cells with 5 or more foci are shown. Values are mean ± s.d. of three independent experiments. *: $P < 0.05$ (two-tailed unpaired t-test). Scale bars: 5 μm. C, U2OS cells were treated with 50 nmol/L BI2536 for 12 hours and harvested for IB with indicated antibodies. D, U2OS cells were treated with 50 nmol/L BI2536 for 12 hours and harvested for IB with p-Chk2. For quantification, at least 300 cells were scored for p-Chk2 foci and percentages of cells with 5 or more foci are shown. Values are mean ± s.d. of three independent experiments. *: $P < 0.05$ (two-tailed unpaired t-test). All bars represent 5 μm. E, U2OS cells were transfected with the indicated siRNA for 2 days and harvested for IB with indicated antibodies. F, U2OS cells were transfected with Plk1 constructs and irradiated with 5 Gy γ-ray and harvested for IB against Plk1 and β-actin. G, U2OS cells were transfected with Plk1 constructs and harvested for IB with indicated antibodies.

Figure S2. Plk1 phosphorylation of Mre11 is required for inactivation of the ATM-Chk2 pathway. A, endogenous Plk1 phosphorylates endogenous Mre11 at S649. HeLa cells were treated with nocodazole (50 ng/mL) for 24 hours, collected by mitotic shake-off, and incubated for additional 60 minutes in medium containing Plk1 inhibitors [BI2536 (100 nmol/L), BI6727 (100 nmol/L), or GSK461364 (100 nmol/L)], Aurora A inhibitor [Aurora inhibitor I (50 nmol/L)], or CDK1/CDK2 inhibitor [RO-3306 (10 μmol/L)], followed by IB against anti-pS649-Mre11. B, U2OS cells were depleted of Mre11 with RNAi, transfected with the RNAi-resistant Mre11-S649A for 2 days and exposed to 1 Gy of IR, followed by incubation with BI2536 for 1 hour. C, U2OS cells were depleted of Mre11 with RNAi, transfected with the RNAi-resistant Mre11-S649A for 2 days, treated with 2 Gy IR, incubated with BI2536 for 1 hour, and harvested for γ-H2AX staining. At least 300 cells were counted for γ-H2AX foci and percentages of cells with 5 or more foci are shown. Values are mean ± s.d. of three independent experiments. *: $P < 0.05$. All bars represent 5 μm.

Figure S3. Mre11 is not involved in normal mitosis. A, HeLa cells were depleted of Mre11 or Plk1, and harvested for FACS analysis (top) or IB (bottom). B, U2OS cells were blocked with the DTB protocol to arrest at the G1/S boundary, released for the indicated times, and harvested for FACS. C, inhibition of Plk1 activity arrested cells in mitosis. U2OS cells were treated with BI2536 with indicated times, and harvested for FACS analysis. D, Mre11-S649 phosphorylation affects S688 phosphorylation. HeLa cells were transfected with GFP-Mre11 constructs (WT, S649A, S649D or S688D), and harvested for anti-pS649-Mre11 or anti-pS688-Mre11 IB. E, U2OS cells were synchronized with the DTB and released for 7 hours, followed by treatment with doxorubicin for 1 hour. Upon doxorubicin removal, cells were released for additional 2 hours ± caffeine ± TBCA and harvested for phospho-H3 (p-H3) staining. F, Plk1 binds to Mre11. 293T cells were co-transfected with GFP-Mre11 and Flag-Plk1$_{1\text{-}405}$ (Plk1 kinase domain) or Flag-Plk1$_{403\text{-}603}$ (Plk1 PBD domain) and harvested for anti-Flag IP, followed by IB.

Figure S4. Mre11 phosphorylation at S649/S688 has no impact on the MRN complex stability. A, schematic representation of Mre11 with the DNA-binding domains located at the C terminus. B, end-labelled DNA probes were incubated with different concentrations of Mre11. Reaction products were separated and visualized as described under "Methods". C, U2OS cells were depleted of Mre11 with siRNA for 2 days, exposed to IR and harvested. D, U2OS cells were depleted of Mre11 with siRNA and reconstituted with RNAi-resistant Mre11 constructs. E and F, Mre11-S649/S688 phosphorylation is dispensable for its interaction with Rad50 and Nbs1. E, 293T cells expressing GFP-Mre11 were subjected to anti-GFP IP. F, 293T cells expressing GFP-Mre11 (WT, 2A, 2D) were subjected to anti-GFP IP. G, HCT116 cells were transfected with different GFP-Mre11 constructs for 24 hours and harvested for anti-Nbs1 IF. H, left panel, a diagram for the assay based on the EJ5-GFP reporter, which contains two tandem recognition sites for I-SceI endonuclease. Right panel, NHEJ reporter cells were co-transfected with siRNA to deplete Mre11 or control together with I-SceI and Mre11 (WT or S649D/S688D) expressing plasmids. Percentages of I-SceI-induced GFP$^+$ cells were measured. Bars represent the means of triplicates.