Supplemental Material to:

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DNA2 and EXO1 in replication-coupled homology-directed repair and in the interplay between HDR and the FA/BRCA network

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A. DNA replication

B. FancD2/FancI, Endonuclease, Unhooking of X-link

C. Translesion synthesis, Removal of unhooked X-link

D. DNA2 and/or EXO1 5’ to 3’ resection

E. RAD51 strand invasion

F. HR
Karanja Supplementary Figure S2

A

<table>
<thead>
<tr>
<th>pRESQ.shSCR</th>
<th>pRESQ.shDNA2'</th>
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<tbody>
<tr>
<td>Input</td>
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<tr>
<td>DNA2 IP</td>
<td>DNA2 IP</td>
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<tr>
<td>IgG</td>
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IB: DNA2

B

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<th>pRESQ.shDNA2'</th>
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<tr>
<td>pBabe.cDNA2</td>
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<tr>
<td>siCNTL</td>
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<td>siEXO1</td>
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DNA2

Actin
Karanja Supplementary Figure S3

Wildtype
pso2
dna2-1
pso2 dna2-1

500 μM Cisplatin
SUPPLEMENTAL INFORMATION

Supplemental Figure S1. Model for DNA End Resection During ICL Repair. Illustrating DNA2 Function(s) a. Cisplatin exposure creates interstand crosslinks. b. Approaching replication forks are unable to unwind past the lesions. c. FANCM/FAAP24 recognizes lesions and recruits the core 8 FA genes to monoubiquitinate FANCD2/I, which then localizes to foci with BRCA1, FANCN, FANCJ, Rad51, and PCNA. The forks are incised flanking the crosslink on the template of one chromatid 1, 2 probably by Mus81/Eme1 (3' cut) and XPF-ERCC1 (5' cut). c.-d. The crosslink is uncoupled, and the sister chromatid retaining the damage is repaired by TLS (translesion synthesis) and damage removal by NER (nucleotide excision repair) 1-3. The other sister chromatid contains a double-strand break, which is repaired in a Rad51-dependent step 4. Importantly, Rad51 associates before the incisions, suggesting that ssDNA and Rad51 filament stabilization occur even before DSB generation by the incisions steps, perhaps for fork protection 5. BRCA1 is also required early and is antagonized by 53BP1, which inhibits resection 6-9. e. Our data suggest that DNA2, and/or EXO1, function in resecting the nascent DNA either before or after the DSEs are formed, perhaps along with MRN and CtIP 10-12. f. DSB formation and further resection up to 700 bp from the cut site 4 allows formation of a RPA filament for checkpoint activation and BRCA2 mediated Rad51 filament assembly necessary or strand invasion and initiation of sister chromatid exchange and checkpoint activation, involving BRCA2, Rad51, FANCJ and FANCN. If the DSBs are not resected and repaired efficiently, steps e. and f., promiscuous NHEJ (non-homologous end joining) can lead to rearrangements, cell death and cancer, because NHEJ is elevated in FA cell lines and its suppression can alleviate some FA
defects\textsuperscript{13, 14}. Arrows indicate 3’ ends. FANCD2 interactions suggest the pathway may be more concerted than this linear model implies.

**Supplemental Figure S2. Depletion and Overexpression of DNA2.** Endogenous DNA2 was efficiently depleted using pRESQ.shDNA2’ (A) and robustly expressed (B) from a complementation plasmid harboring RNAi-resistant DNA2 cDNA, as we also showed previously\textsuperscript{15, 16}. Related to Figures 1C,D and 2.

**Supplemental Figure S3. Yeast pso2 dna2-1 Double Mutants are Dypersensitive to Displatin.** Yeast pso2, dna2-1 single mutants and pso2 dna2-1 double mutant were exposed to DMSO or cisplatin (500 µM, 2h) washed and grown for 6 days at room temperature. The pso2 dna2-1 double mutant was more sensitive to cisplatin than the single mutants, whereas, the pso2 mutant was slightly more sensitive to cisplatin than the wild type.

**SUPPLEMENTAL EXPERIMENTAL PROCEDURE**

**Immunofluorescence microscopy**

U2OS cells were grown for 1 day on poly-L-lysine coated coverslips and treated with 1 µM CPT for 1 h. Cells were pre-extracted for 6 min on ice with cytoskeletal buffer

\textsuperscript{2}
(10mM HEPES/KOH pH7.4, 300 mM Sucrose, 100 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100, 1mM PMSF, protease and phosphatase inhibitors) before fixation with 4% formaldehyde for 25 min at room temperature. After fixation cells were washed in PBS and blocked in 10% FBS/PBS before addition of primary antibodies (α-BrdU, α-RPA2 pS4/S8, α-Rad51, α-γH2Ax) diluted at 1:1000 in 10%FBS, 0.1% saponin in PBS. Cells were incubated at 4°C overnight then washed in 10%FBS/PBS and stained with the following secondary antibodies anti-rabbit IgG-Alexa Fluor 488, anti-mouse IgG-Alexa Fluor 594 or anti-BrdU Alexa Fluor 488 for 1h at room temperature. DNA was counterstained with 4’ 6’-diamidino-2-phenylindole (DAPI, 0.3 µM) and cover slips were mounted on slides with Vectashield mounting agent (Vector Laboratories). Images were acquired using Zeiss Axio epifluorescent microscope and processed with AxioVision Rel. 4.8 (Carl Zeiss) and Adobe Photoshop (Adobe) software.

**Western blotting and co-immunoprecipitation**

DNA2 and/or EXO1, depleted cells were treated with CPT (1 µM 1h) or CDDP (15 µM, 24 h). Cells were washed with PBS and lysed with TBS-Tx buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X100) containing 1 mM PMSF, protease and phosphatase inhibitors. Whole cell extract was recovered and protein concentration determined by Bradford assay. Laemmli buffer (0.5 M Tris-HCl pH6.8, 10% glycerol, 2% (w/v) SDS and 0.01% (w/v) bromophenol blue) was added and the extracts were boiled for 5 min on a heat block.

For co-immunoprecipitation from extracts of U2OS cells expressing endogenous levels of protein, 2 mg of total protein was incubated with 2.5 µl of α-Dna2, α-FANCD2
serum, or pre-immune serum and incubated for 1h at 4°C. Protein A bead slurry was added and incubated overnight at 4°C. Beads were boiled in Laemmli buffer before immunoblotting.

For co-immunoprecipitation from cells overproducing DNA2, HEK293T cells were transfected with pcDNA3.1-6His-4Myc-DNA2 plasmid \textsuperscript{17}. For testing the ability of endogenous FANCD2 to immunoprecipitate exogenous, purified DNA2, 2 µg of recombinant human FLAG-DNA2 \textsuperscript{18} was added to 2 mg of protein from HEK293T cells without DNA2 overexpression and incubated on ice for 4h. Both extracts were incubated with 2.5 µl of α-Dna2 or α-FANCD2 serum and pre-immune serum and incubated for 1h at 4°C. Protein A bead slurry was added and incubated overnight at 4°C. Beads were boiled in Laemmli buffer before immunoblotting.

To test whether DNA was required for the FANCD2/DNA2 interaction, DNaseI was added to the extract along with antibody \textsuperscript{16}. Effectiveness of DNA degradation was confirmed by gel electrophoresis.

**Pulse-field gel electrophoresis**

U2OS cells were treated with DMSO or CPT (1 µM, 2 h) in the presence of 10 µM DNA-PK inhibitor, NU7441. Cells were processed as previously described \textsuperscript{19}. Briefly, cells were harvested by trypsinization and agarose plugs of 2x10\textsuperscript{5} cells were prepared in disposable plug mold (Biorad). Plugs were incubated at 50°C for 48 h in lysis buffer containing 100 mM EDTA pH8.0, 10mM Tris-HCl pH8.0, 1% Sarkosyl, and fresh 1 mg/ml Proteinase K. Subsequently, plugs were washed in TE (20 mM Tris-HCl pH8.0,
50mM EDTA) and immobilized in 1% (w/v) agarose in 0.5% Tris-borate EDTA (TBE). Electrophoresis was performed using a Biorad CHEF DR II apparatus with the following parameters: 3 v/cm, 120 sec switch, 20 h at 14°C. Gel was stained with ethidium bromide (1µg/ml) and visualized using Gel Doc XR (Biorad).

**Metaphase chromosome spreads**

After depletion of DNA2 and/or EXO1, U2OS cells were treated with DMSO, CPT (1 µM, 1 h), or cisplatin (CDDP, 15 µM, 2h) washed in pre-warmed PBS and left to recover in fresh media for 2 h. Caffeine (2 mM, 5 h) was added to overcome the G2/M checkpoint and 1 h later Demecolcine (0.1 µg/ml, 4 h) was added to arrest the cells in metaphase. Cells were swelled in hypotonic solution, 0.8% sodium citrate, fixed with methanol/acetic acid (3:1) and dropped onto glass slides. After aging for 1 day at room temperature, chromosomes were stained with DAPI (0.3 µM). Coverslips were mounted on slides with Vectashield mounting agent (Vector Laboratories). Images were acquired using Zeiss Axio epifluorescent microscope and processed with AxioVision Rel. 4.8 (Carl Zeiss) and Adobe Photoshop (Adobe) software.

**Flow cytometry**

To assay for GFP expression U2OS cells integrated with the DR-GFP or SA-GFP reporter construct (Gunn et al., 2011, Pierce et al., 1999) were used. Briefly, DNA2 knockdown was performed as previously described. Vectors expressing I-Scel homing endonuclease or empty vector were transfected by electroporation and plated for 48 h. Cells were gently trypsinized, washed in PBS and analyzed in Becton-Dickinson
FACScan. During flow cytometry, dead cells were excluded by staining with 7-aminoactinomycin D (Invitrogen). Cells were gated by FL1, FL2, and FL3 (7-AAD) channels. From the viable cells, GFP+ cells were determined from the FL1 shift from the negative population (Pierce et al., 1999).

SUPPLEMENTARY REFERENCES


