SUPPLEMENTARY DATA – J.-Y. Park et al.

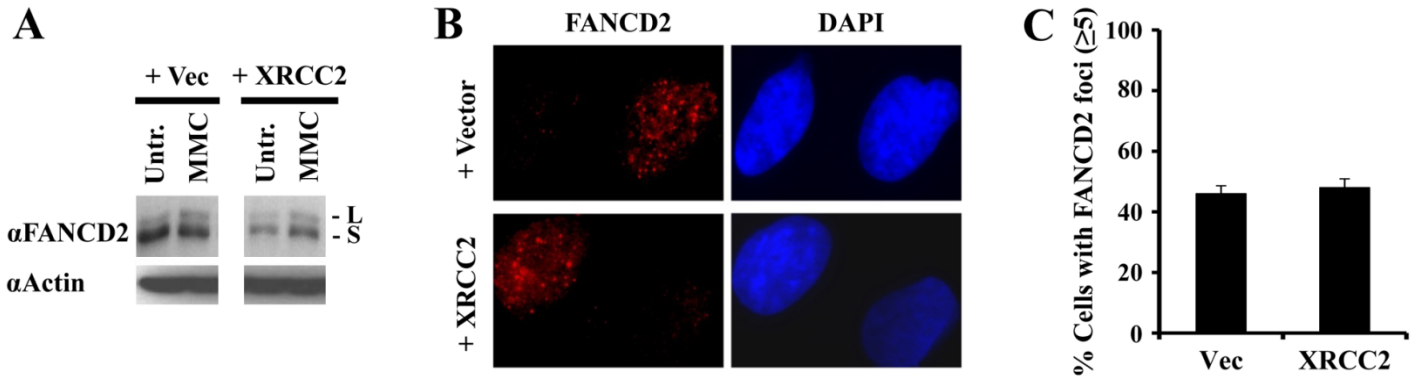

Figure S1. XRCC2-deficient cells have normal FANCD2 monoubiquitination and foci formation. (A) 900677AT fibroblasts containing only the vector have similar levels of monoubiquitinated FANCD2 (-L) as do cells that were reconstituted with XRCC2, both in untreated populations, or following treatment with 0.5 μM MMC (B-C). Examples (B) and quantification (C) of the assembly of FANCD2 foci in 900677AT cells, with or without reconstitution with XRCC2, following treatment with 0.5 μM MMC for 16 hr.

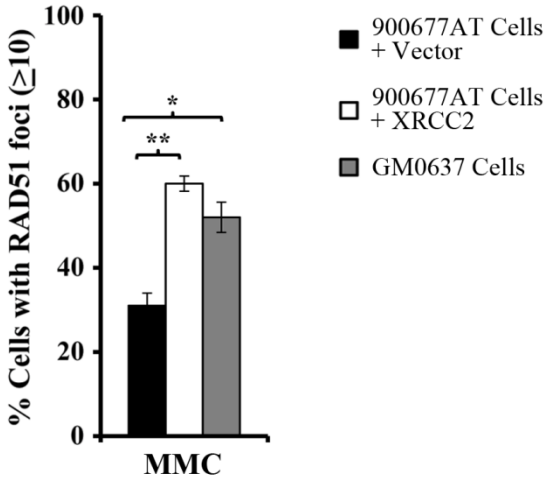

Figure S2. Reconstitution of 900677AT cells with XRCC2 restores RAD51 foci to levels similar to those in a WT reference line. Quantification of RAD51 foci in *XRCC2*$^{-/-}$ 900677AT cells, with or without reconstitution of XRCC2, or in SV40 Lg T antigen immortalized GM0637 fibroblasts from a non-diseased control, following treatment with 0.5 μM MMC for 16 hr. p values, as determined using Student's t-test: *$p<0.001$, **$p<0.0001$.

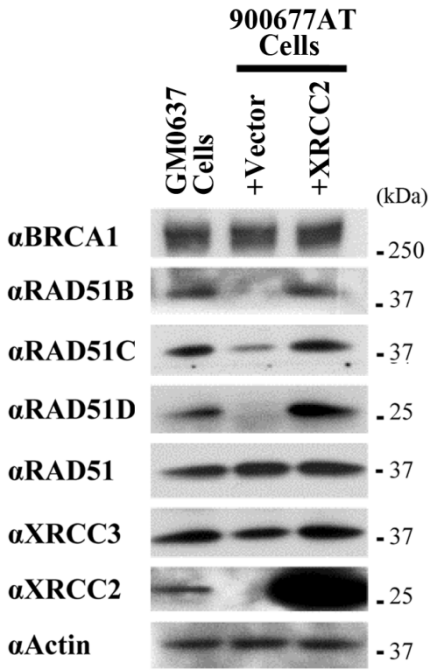

Figure S3. Comparison of the levels of BCDX2 RAD51 paralog proteins in 900677AT cells reconstituted with XRCC2 and in cells with wild type XRCC2. Extracts were prepared from untreated 293T control cells, or 900677AT cells reconstituted with the empty vector alone or with wild type XRCC2. The indicated proteins were detected on immunoblots using specific antibodies directed against each protein. Actin is shown as a loading control for the levels of proteins present in extracts.

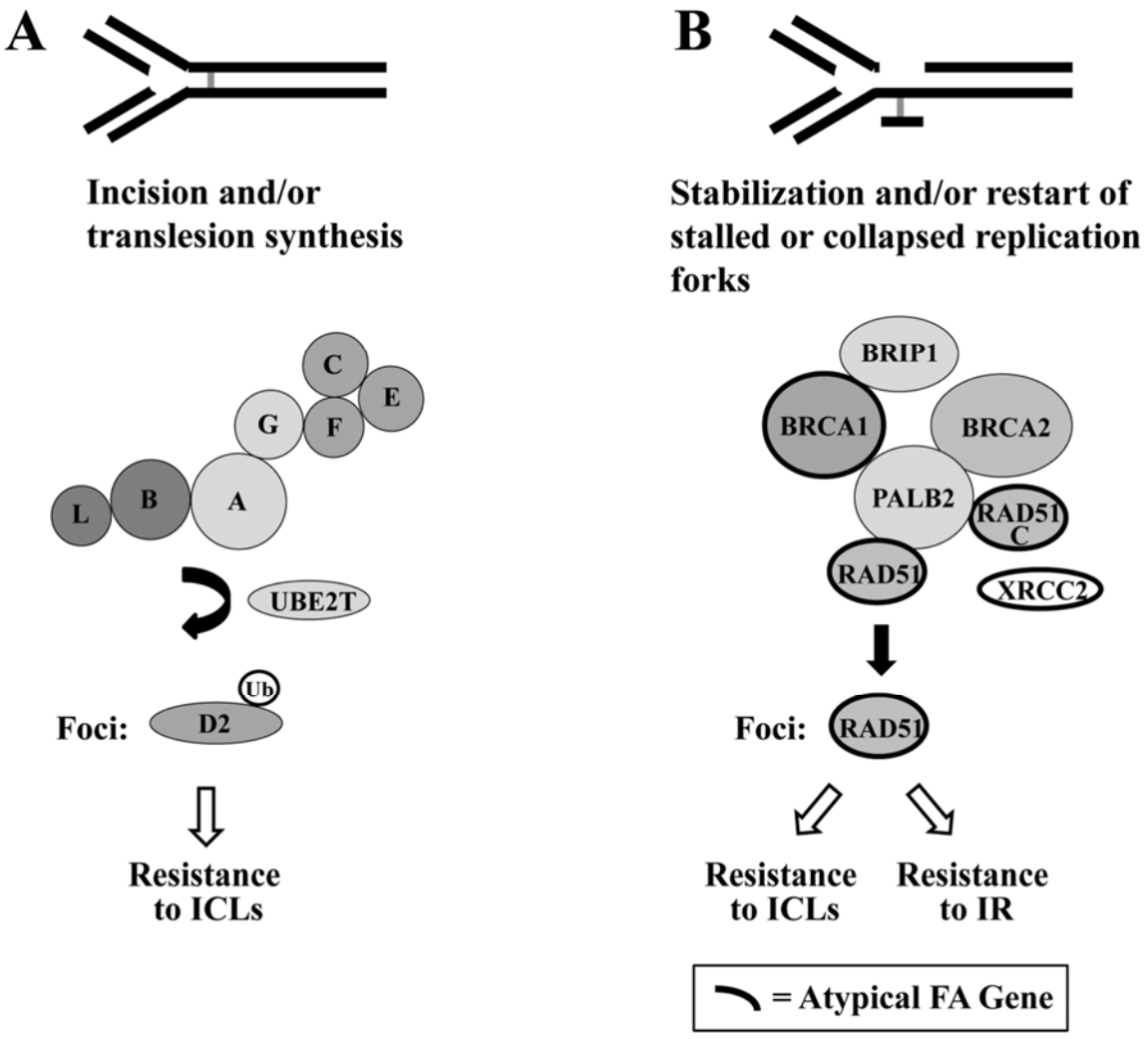

Figure S4. Early and late FA genes may function at different steps in the repair of ICLs, and are associated with distinct roles in the recruitment of FA proteins to damage foci. (A) Early FA proteins are components of two prominent protein complexes, the FA core complex, including FANC- A, B, C, E, F, G, and L, and the FANCD2-FANCI complex. These complexes cooperate with UBE2T in a pathway that culminates in the monoubiquitination of, and foci formation by, FANCD2. (B) In contrast, FA proteins classified as 'late' due to the fact they are not required for FANCD2 monoubiquitination, are instead required for the assembly of RAD51 foci in response to DNA damage. Several of these 'late' FA genes/proteins, including *XRCC2* as we show here, are associated with atypical FA since bone marrow failure has not been observed in patients with loss of function mutations in these genes. Atypical FA proteins are identified here by a thick/black outline. SLX4/FANCP and XPF/FANCQ, are also not required for FANCD2 monoubiquitination, but are not shown here for simplicity. SLX4 and XPF may have roles in ICL repair that are distinct from other proteins diagrammed here, specifically, in the unhooking and excision of the ICL.

SUPPLEMENTARY INFORMATION

EXTENDED MATERIALS AND METHODS

Cell culture

900677A (primary) and 900677AT (large T antigen immortalized) fibroblasts with a genetic deficiency for *XRCC2*, and derivative lines, and HEK293T, U2OS-DR, and GM0637 cells, were grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were irradiated as described previously.[1] Stock solutions of cycloheximide (10 mg/ml in H_2O; Sigma), MG132 (10 mM in DMSO; Calbiochem), olaparib (10 mM in DMSO; Sigma), MMC (3 mM in 50% ethanol; Sigma), and HU (500 mM in medium; Sigma) were kept at -20^{o}C.

Cloning and mutagenesis

Human XRCC2 cDNA (SC116732) was purchased from Open Biosystems. The R215X mutant of XRCC2 was generated using the QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Transfection and viral transduction

900677AT cells were reconstituted with XRCC2 or a neomycin phosphotransferase containing control S91 gammaretroviral vector for selection with G418/geneticin, as described previously.[2 3] Alternatively, for experiments involving the R215X mutant of XRCC2, cells were retrovirally transduced (pOZ) with the mutant or XRCC2-WT along with a N-terminal Flag-HA tag and selected by IL-2 beads as described previously.[4]

Immunofluorescence microscopy

Cells were grown on coverslips coated with poly-D-lysine, fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 3 min. Following washes with PBS, slides were incubated with primary and secondary antibodies, washed, and mounted with a coverglass, as described previously.[5]

Microscopy, collection of images, counting of three replicates per sample, and the generation of figures were as described previously.[5]

Immunoprecipitation

Cells were lysed in NETN 420 buffer supplemented with protease inhibitors and PMSF as described previously.[1] Immunoprecipitations were performed with anti-Flag M2 Affinity Gel (Sigma) or specified antibodies as described previously.[1]

Antibodies

The following primary antibodies were utilized: RAD51D (Novus, NB100-166, rabbit), XRCC2 (Santa Cruz, sc-5895, goat and sc-365854, mouse), BRCA1 (Millipore, 07-434, rabbit) and BRCA2 (Calbiochem, OP95, mouse). Anti-RAD51C (mouse), anti-XRCC3 (rabbit), anti-γH2AX (mouse), anti-HA (mouse), anti-β actin (mouse) antibodies were as described elsewhere.[6]

Secondary antibodies for immunofluorescence microscopy included FITC conjugated donkey anti-mouse or anti-rabbit IgG and Rhodamine B-conjugated donkey anti-mouse or anti-rabbit IgG antibodies (Jackson Immunoresearch) as described previously.[5] For

immunoblotting, signals from HRP-linked secondary antibodies (Amersham) were detected by chemiluminescence (Amersham) as previously described.[5]

DNA damage sensitivity assays

For measurements of MMC, olaparib and formaldehyde sensitivity, cells were plated into 96 well plates in triplicate at $2X10^3$ cells/well. The next day, cells were treated with MMC at doses ranging from 0-400 nM, olaparib at doses from 0-4 μM, and formaldehyde at doses from 0-80 μM. Medium was aspirated and fresh medium without any of these DNA damage agents was added after overnight incubation, and cells were incubated an additional 3-4 days. Relative survival was measured using a colorimetric assay as described.[1]

For measurements of sensitivity to IR, 900677AT cells, with or without correction with XRCC2, were plated into 6 cm dishes in triplicate after irradiating with doses ranging from 0-8 Gy. Cells were incubated for 10 days and the number of colonies/dish was counted as described.[6]

Chromosome breakage analysis

For assays of chromosome breakage in primary cells, cultures in T75 flasks in DMEM medium containing 20% fetal bovine serum, as well as penicillin-streptomycin and L-glutamine, were exposed to 0, 0.01 or 0.1 μg/ml diepoxybutane (DEB) for 48 hr. Cells were accumulated in mitosis by treatment with 0.2 mg/ml colcemid for the last 7-8 hr. Metaphase spreads were prepared, G banded, and analyzed under a brightfield microscope.

For assays of Lg T-immortalized lines, cells were grown in a T25 flask and were treated with 300 nM MMC for 72 hr in the dark, and arrested in mitosis with a final concentration of 200

ng/ml of colcemid (Gibco) for 3 hr. Cells were collected by trypsinization, washed with medium containing serum, and treated with 50 mM KCl for 10 min at 37ºC for swelling. Cells were subsequently fixed, dropped onto slides, and stained with Giemsa as described,[7] and visualized using a microscope.

The total number of aberrations per chromosome, including breaks, gaps and radials was counted as described.[8] The percent of cells with one or more radial chromosomes was also calculated. Each count consisted of 3 sets of 30 metaphases each which was utilized for a mean ± standard deviation.

G2-M accumulation

To measure G2-M accumulation, cells were treated with 0.35 μg/ml of melphalan, fixed, stained with propidium iodide and treated with RNAse A, gated, and analyzed using ModFit software as described.[4]

REFERENCES FOR EXTENDED MATERIALS AND METHODS

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