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## COMPLEMENTATION OF HYPERSENSITIVITY TO DNA INTERSTRAND CROSSLINKING AGENTS DEMONSTRATES THAT *XRCC2* IS A FANCONI ANEMIA GENE

Jung-Young Park<sup>1</sup>, Elizabeth L. Virts<sup>2</sup>, Anna Jankowska<sup>2</sup>, Constanze Wiek<sup>3</sup>, Mohamed Othman<sup>4</sup>, Sujata C. Chakraborty<sup>5</sup>, Gail H. Vance<sup>5</sup>, Fowzan S. Alkuraya<sup>6,7</sup>, Helmut Hanenberg<sup>#2,3,8,\*</sup>, and Paul R. Andreassen<sup>#1,9,\*</sup>

<sup>1</sup> Division of Experimental Hematology & Cancer Biology, Cincinnati Children's Research Foundation, Cincinnati, OH 45229

<sup>2</sup> Wells Center for Pediatric Research, Riley Hospital for Children, Indiana University School of Medicine, Indianapolis, IN 46202

<sup>3</sup> Department of Otorhinolaryngology & Head/Neck Surgery, Heinrich Heine University 40225 Duesseldorf, Germany

<sup>4</sup> Department of Pediatrics, College of Medicine, King Saud University, Riyadh, 11211, Saudi Arabia

<sup>5</sup> Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202

<sup>6</sup> Department of Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, 11211, Saudi Arabia

<sup>7</sup> Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia

<sup>8</sup> Division of Pediatrics III, University Children's Hospital Essen, University Duisburg-Essen, 45122 Essen, Germany

<sup>9</sup> Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45229

# These authors contributed equally to this work.

### Abstract

**Background**—Fanconi anemia (FA) is a heterogeneous inherited disorder clinically characterized by progressive bone marrow failure, congenital anomalies, and a predisposition to malignancies.

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\* Co-corresponding authors: Helmut Hanenberg: Tel. +49 (201) 723-1053, Helmut.Hanenberg@uk-essen.de or Paul R. Andreassen: Tel. +1 (513) 636-0499, Paul.Andreassen@cchmc.org.

#### CONTRIBUTORS

JYP, ELV, AJ, CW, SCC, and GHV performed the studies. MO was responsible for clinical care. FSA provided cell lines. JYP, PRA and HH were responsible for study design. PRA, JYP and HH wrote the paper. CW and FSA edited the paper.

#### COMPETING INTERESTS

None declared.

**Objective**—Determine, based on correction of cellular phenotypes, whether *XRCC2* is a FA gene.

**Methods**—Cells (900677) from a previously identified patient with biallelic mutation of *XRCC2*, among other mutations, were genetically complemented with wild-type *XRCC2*.

**Results**—Wild-type *XRCC2* corrects each of three phenotypes characteristic of FA cells, all related to the repair of DNA interstrand crosslinks, including increased sensitivity to mitomycin C (MMC), chromosome breakage, and G2-M accumulation in the cell cycle. Further, the p.R215X mutant of *XRCC2*, which is harbored by the patient, is unstable. This provides an explanation for the pathogenesis of this mutant, as does the fact that 900677 cells have reduced levels of other proteins in the *XRCC2*-*RAD51B-C-D* complex. Also, *FANCD2* monoubiquitination and foci formation, but not assembly of *RAD51* foci, are normal in 900677 cells. Thus, *XRCC2* acts late in the FA-BRCA pathway as also suggested by hypersensitivity of 900677 cells to ionizing radiation. These cells also share milder sensitivities toward olaparib and formaldehyde with certain other FA cells.

**Conclusions**—*XRCC2/FANCU* is a FA gene, as is another *RAD51* paralog gene, *RAD51C/FANCO*. Notably, similar to a subset of FA genes that act downstream of *FANCD2*, biallelic mutation of *XRCC2/FANCU* has not been associated with bone marrow failure. Taken together, our results yield important insights into phenotypes related to FA and its genetic origins.

### Keywords

Fanconi anemia; *XRCC2*; *RAD51* paralogs; DNA interstrand crosslinks; Breast cancer susceptibility

## INTRODUCTION

Fanconi anemia (FA) is the most frequent inherited bone marrow failure (BMF) syndrome and is clinically characterized by progressive bone marrow failure, congenital anomalies, and a predisposition to acute myeloid leukemia and other malignancies.[1 2] The failure of the hematopoietic system on average manifests around 8 years of age, but this is highly variable, not only among different FA subgroups but also within the same subgroup, depending on the severity of the mutation.[3 4] In addition, as many as 30% of FA patients do not show any congenital abnormalities, which can include radial ray abnormalities, short stature, microcephaly, abnormal pigmentation and hypogonadism.[3] This further demonstrates that, phenotypically, FA is a pleiotropic disease.

Additionally, FA is typified by exquisite hypersensitivity of cells to DNA interstrand crosslinking (ICL) agents such as diepoxybutane (DEB) or mitomycin C (MMC).[2 5 6] Cells from FA patients also characteristically display other phenotypes that are associated with a defective response to DNA interstrand crosslinkers, including chromosomal abnormalities and cell cycle defects.[5 6] These cellular phenotypes extend across all FA genetic complementation groups.[1]

At present, germ-line defects in 19 mostly recessive genes are clinically associated with FA. *FANCR/RAD51*, *FANCS/BRCA1* and *FANCT/UBE2T* are the most recently identified FA

genes.[7-12] The products of these genes interact in different protein complexes that comprise the FA-BRCA pathway and, which together, are essential for the error-free removal of DNA interstrand crosslinks.[2 13]

The products of eight of the FA genes (*FANCA*–*B*–*C*–*E*–*F*–*G*–*L*–*M*) form the FA *core* complex. This large protein complex functions as an E3 ligase and, in combination with the FANCT/UBE2T E2 conjugase, culminates in the monoubiquitination and activation of FANCD2 and FANCI,[7-9 14-16] a central step in the FA pathway. Loss-of-function defects in any of these so called ‘early’ or ‘upstream’ genes leads to a defect in the monoubiquitination of FANCD2 and FANCI.[6 14-16]

The other eight FA genes are classified as ‘late’ or ‘downstream’ FA genes because cells that are deficient in these genes have intact FANCD2 monoubiquitination.[13] A subset of FA proteins, all of which are products of *late* FA genes, including BRCA1/FANCS, BRCA2/FANCD1, PALB2/FANCN and RAD51C/FANCO, have a common role in regulating the assembly of RAD51 foci.[10 17-19] Further, some of these late FA genes, including *FANCO/RAD51C*, *FANCR/RAD51* and *FANCS/BRCA1*, are *atypical*, as patients with germline mutations in these genes show FA-related malformations but do not seem to develop BMF.[8 10 18]

We previously reported a child with congenital abnormalities suggestive of FA. At the age of 2.5 years, the boy displayed normal bone marrow cellularity and normal PB values.[20] Based on positional mapping and whole exome sequencing, the child was found to have inherited, among other germ-line mutations, a premature biallelic stop gain mutation in *XRCC2*.[20] While these phenotypes suggest that *XRCC2* could be an FA gene, this hypothesis remains to be critically tested.

Here, on the basis of complementation of three different FA-related cellular phenotypes related to the repair of ICLs, we demonstrate that *XRCC2* is the 20<sup>th</sup> FA gene and is the second RAD51 paralog so identified. We also provide insight into the function of *XRCC2* in preventing FA by demonstrating that it is required to maintain levels of another FA protein, RAD51C, as well as other RAD51 paralogs that are normally present in the XRCC2-RAD51B-C-D complex. Taken together, including the absence of BMF in the patient with biallelic mutation of *XRCC2*, and the phenotypes of 900677 cells, our data suggests that *XRCC2* belongs to a subset of FA genes that are atypical and which function downstream in the FA-BRCA pathway.

## MATERIALS AND METHODS

Additional details on Materials and Methods are provided online as supplementary information

### Cell culture

Cells were grown as described previously,[21] except where noted otherwise.

### **Cloning and mutagenesis**

The R215X mutant of XRCC2 was generated using the QuikChange II Site-Directed Mutagenesis kit (Stratagene).[21]

### **Transfection and viral transduction**

900677AT cells were reconstituted with XRCC2 using a retroviral vector, as described previously.[9 18] For experiments involving the R215X mutant of XRCC2, cells were retrovirally transduced (pOZ) as described.[22]

### **Immunofluorescence microscopy**

Cells were grown on coverslips, fixed, permeabilized, washed, incubated with primary and secondary antibodies, and mounted, as described previously.[23]

Microscopy, collection of images, counting of three replicates per sample, and the generation of figures was as described previously.[23]

### **Immunoprecipitation**

Cell lysis and immunoprecipitation assays, performed with anti-Flag M2 Affinity Gel (Sigma) or specified antibodies, were as described previously.[21]

### **Antibodies**

Several commercially available primary antibodies were utilized: RAD51D (Novus), XRCC2 (Santa Cruz), BRCA1 (Millipore) and BRCA2 (Calbiochem). Anti-RAD51C, anti-XRCC3, anti- $\gamma$ H2AX, anti-HA, and anti- $\beta$  actin antibodies were as described elsewhere. [24]

Secondary antibodies for immunofluorescence microscopy and the detection of immunoblots using chemiluminescence (Amersham) were as described previously.[23]

### **DNA damage sensitivity assays**

For measurements of MMC, olaparib and formaldehyde sensitivity, cells were treated with MMC at doses ranging from 0-400 nM, olaparib at doses from 0-4  $\mu$ M, and formaldehyde at doses from 0-80  $\mu$ M. Relative survival was measured using a colorimetric assay as described.[21]

For measurements of sensitivity to IR, colony assays were performed on 900677AT cells, with or without correction with XRCC2, as described previously.[24]

### **Chromosome breakage analysis**

For assays of chromosome breakage in primary and Lg T-immortalized lines, cells were treated with DEB and MMC, respectively. Cells were trypsinized, washed, swollen, fixed, and dropped onto slides.[25]

The total number of aberrations per chromosome, including breaks, gaps and radials was counted as described.[26], and the percentage of cells with one or more radial chromosomes was also calculated.

### G2-M accumulation

To measure G2-M accumulation, cells were treated with 0.35 µg/ml of melphalan according to published protocol[27], fixed, stained with propidium iodide and treated with RNase A, gated, and analyzed using ModFit software as described.[22]

## RESULTS

Previously, we described a Saudi child born to healthy first cousin parents with a biallelic stop gain mutation in *XRCC2* (transcript NM\_005431), c.643C>T, predicted to encode p.R215\*.[20] This child showed intrauterine growth retardation, absent thumbs and radii, microcephaly, kidney malformations and other clinical features that are frequently observed in FA patients.[20] While these findings suggested the possibility that the patient had FA, mutations in other genes were also found. Thus, whether or not *XRCC2* is a FA gene was not determined.

At the time of this current report, the boy is seven years old, shows no signs of BMF, and has normal hematological counts: hemoglobin 12.7 mg/dL, thrombocytes 397,000/dL, leukocytes 7,000/dL and an absolute neutrophil count of 2,100/dL.

To better understand the defect in 900677A cells, we quantified chromosome abnormalities induced by diepoxybutane in primary skin-derived fibroblasts from the patient in comparison to other primary fibroblast cell lines. The results (table 1) demonstrate that 900677A cells possess the characteristic hypersensitivity of FA cells towards DEB when compared to non-diseased normal fibroblasts (NL143889), similar to what has also been reported for *RAD51C*<sup>-/-</sup> cells.[18] However, as compared to *FANCA*<sup>-/-</sup> (GM16631) and *FANCC*<sup>-/-</sup> (GM16754) fibroblast cells with defects in 'early' FA genes, the 900677A fibroblasts were less sensitive to crosslinkers. There was also an increased level of spontaneous chromosome breakage in 900677A cells, as compared to the normal fibroblasts, similar to that observed in *FANCA*<sup>-/-</sup> and *FANCC*<sup>-/-</sup> cells.

Next, to better understand the pathogenicity of the *XRCC2* mutation, we assessed the levels of XRCC2 protein in SV40 large T-transformed 900677A (900677AT) cells. Although the c.643C>T mutation in *XRCC2* is predicted to encode a mutant XRCC2 protein truncated at position R215 (p.R215X), we could not identify any XRCC2 protein band by western blot using an antibody directed against the N-terminus of XRCC2 (figure 1A), neither full-length (34 kDa) nor truncated XRCC2 (23 kDa). By comparison, full-length XRCC2 was readily detected in T- antigen transformed HEK293 cells.

### Re-introduction of XRCC2 corrects all FA-associated cellular defects in 900677AT cells

In 2000, Joenje *et al.*[28] proposed more stringent criteria to define a new FA gene: either correction of FA-related cellular phenotypes following exogenous expression of the candidate FA gene/cDNA or identification of two or more unrelated patients with biallelic

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mutations in the same gene. Given that only a single patient in the Saudi family described above has been identified so far worldwide as having biallelic mutations in *XRCC2*, we focused on functional genetic correction of the 90067A cells. To this end, we expressed the wild-type (WT) *XRCC2* cDNA or a 5' green fluorescent protein (GFP)-fusion with *XRCC2* in 900677AT cells using retroviral vectors. Figure 1B shows that the retroviral expression vectors introduced *XRCC2*, and also EGFP-tagged *XRCC2* into cells, while no full-length *XRCC2* protein was detectable in control 900677AT fibroblasts transduced with the 'empty' vector (vec).

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We then examined transduced 900677AT fibroblasts for all three cellular phenotypes that are typical of FA cells: sensitivity to DNA interstrand crosslinking agents, ICL-induced chromosome instability, and ICL-induced accumulation in G2-M of the cell cycle [5 6]. First, we demonstrate that expression of *XRCC2*, either with or without N-terminal EGFP, corrected the cellular sensitivity to MMC (figure 1C). Next, we show that expression of WT *XRCC2* in 900677AT cells dramatically reduced chromosome instability, as compared to 900677AT cells containing the empty vector. This is seen both in the examples shown and by quantification of chromosomal abnormalities using two distinct measures (figure 1D). Re-expression of *XRCC2* corrected: 1) the total number of aberrations/cell, including breaks, gaps, and radial chromosomes, and 2) the percentage of cells with radial chromosomes. Additionally, the DNA interstrand crosslinker melphalan induced accumulation in G2-M of 900677AT cells that contained the vector alone, but this was corrected by re-expression of *XRCC2* (figure 1E). In summary, by each of three cellular phenotypes, 900677AT cells displayed a defective response to DNA interstrand crosslinking agents that is characteristic of FA. Importantly, in each case this pathological cellular phenotype was significantly corrected/complemented by expressing *XRCC2* in the cells.

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Next, to better understand the phenotype of human cells with a genetic deficiency for *XRCC2*, we examined the sensitivity of 900677AT cells to other DNA damaging agents (figure 2). Non-corrected 900677AT cells were particularly sensitive to IR, as compared to cells in which *XRCC2* had been re-introduced (figure 2A). Interestingly, while upstream FA proteins have a minimal role in mediating resistance to IR,[29] downstream FA proteins such as PALB2 and BRCA2 have a clear role.[24 30 31]

The *XRCC2* deficiency of 90677AT cells was also associated with increased sensitivity to the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib (figure 2B) and to formaldehyde (figure 2C). This is consistent with previous reports that cells from certain Fanconi anemia patients display increased sensitivity to either agent.[10 24 31-33]

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In summary, these studies clearly demonstrate that the biallelic mutation in *XRCC2*, c. 643C>T, which encodes p.R215X, is responsible for the FA-associated cellular defects in 900677 cells, as reintroduction of the WT protein corrects all pathological cellular phenotypes.

### The R215X mutant of *XRCC2* found in a FA patient is unstable

Since the R215X mutant of *XRCC2* was not detected in 900677AT cells (figure 1A-B), we assessed whether we could detect the truncated protein following stable expression in



U2OS- DR cells along with a N-terminal Flag-HA epitope tag (figure 3A). While truncated XRCC2 was clearly present, based upon faster migration, the levels were dramatically lower than for the XRCC2 WT protein. To test the hypothesis that the p.R215X mutant of XRCC2 found in an FA patient is unstable, we treated U2OS-DR cells expressing either the XRCC2 WT or R215X proteins with cycloheximide to inhibit new translation. As shown both on immunoblots (figure 3B) and on plots of protein band intensity (figure 3C), relative levels of the mutant XRCC2 R215X protein decreased more rapidly than the levels of WT XRCC2, thereby demonstrating that the XRCC2 R215X protein is indeed unstable with a shorter half-life. As further support for the increased turnover of R215X mutant protein, we show that inhibition of proteasome-mediated protein degradation with the MG132 inhibitor had a greater effect on levels of the XRCC2 R215X mutant than on levels of the WT protein, both on immunoblots (figure 3D) and in plots of band intensities (figure 3E). Thus, p.R215X may be pathogenic because of its relative instability.

### Identifying the role/position of XRCC2 in the FA-BRCA pathway

Posttranslational modification of FANCD2 by monoubiquitination is the central step in the FA pathway and is dependent on the normal function of ‘early’ FA gene products.[7-9 14] Further, the monoubiquitination of FANCD2 is required for the assembly of FANCD2 into nuclear foci.[14] Thus, as a first step toward determining the position of XRCC2 in the FA-BRCA pathway, we examined the levels of FANCD2 monoubiquitination and foci formation in 900677AT cells with or without re-expression of WT XRCC2 protein (online supplementary figure 1). Monoubiquitinated FANCD2 was detected as a slower migrating band on immunoblots in untreated samples in both corrected and uncorrected 900677AT cells. Further, increased levels of monoubiquitinated FANCD2 were induced by MMC in 900677AT cells regardless of the status of XRCC2. Additionally, the assembly of FANCD2 foci in response to MMC was unchanged by proficiency or deficiency for *XRCC2* (online supplementary figure 1B-C). Thus, different from FA core complex components, and FANCD2 and FANCI, XRCC2 does not function *early* in the FA-BRCA pathway.

To determine whether XRCC2 may instead function as a *late* FA protein, we then examined RAD51 foci in 900677AT cells that were defective for XRCC2 (figure 4). As demonstrated in the example shown (figure 4A) and by quantification (figure 4B), *XRCC2*<sup>-/-</sup> fibroblasts had a pronounced defect in the assembly of RAD51 foci, either in untreated populations or following exposure to MMC. This defect was correctable by complementation with *XRCC2*. In contrast, in the examples shown in figure 4A, the assembly of  $\gamma$ H2AX foci in response to MMC was similar in 900677AT cells either with or without restoration of XRCC2. Thus, 90677AT cells have a specific defect in the assembly of RAD51 foci.

Reconstitution of 900677AT cells with XRCC2 restored RAD51 foci to levels similar to those seen in fibroblasts from a control, wild-type individual (online supplementary figure 2). Importantly, while early proteins in the FA pathway have a minimal role in promoting the assembly of RAD51 foci, cells with loss of function mutations in late FA genes, such as *PALB2* and *BRCA2*, display marked deficiencies,[19 34] as do 900677AT cells. Thus, taken together, our results strengthen the hypothesis that XRCC2 functions in the FA-BRCA

pathway downstream of monoubiquitinated FANCD2 as a 'late' FA protein. The hypersensitivity of 900677AT cells to IR (Figure 2A) is also consistent with this conclusion.

### **The interaction of XRCC2 with RAD51C, which is also a late and atypical FA protein, is missing in 900677AT cells**

To better understand the function of XRCC2 as a FA protein, we sought to determine whether its interaction with another FA protein, RAD51C/FANCO,[18] is disrupted in 900677AT cells (figure 5). This hypothesis was based on the fact that XRCC2 and RAD51C are both RAD51 paralogs which are known to interact.[35 36] *XRCC2* and *RAD51C* also display further similarity by having a common role in promoting the assembly of RAD51 foci.[37]

Consistent with previous reports,[35 36] we found that RAD51C co-immunoprecipitated with XRCC2 in extracts from 900677AT cells that had been corrected by re-introduction of XRCC2 (figure 5A). As shown in figure 5B, the consequence of the c.643C>T mutation in 900677AT cells, which leads to undetectable XRCC2 protein, is that interaction with RAD51C cannot occur. As noted above (figure 5A), the XRCC2-RAD51C interaction was restored by re- introduction of *XRCC2*. Strikingly, we also found that RAD51C protein levels, while clearly detectable, were markedly decreased in XRCC2-deficient 900677AT cells, as compared to their genetically-corrected counterparts (figure 5B).

Given that RAD51B-C-D and XRCC2 form a complex together,[35 36] we then determined the effect of mutation of *XRCC2* in 900677AT FA cells on these other RAD51 paralogs (figure 5C). Importantly, each component of the BCDX2 complex was dramatically reduced in uncorrected cells, either with or without treatment with MMC, and XRCC2 and RAD51D were absent (figure 5C). Reconstitution of 900677AT cells with XRCC2 restored the levels of interacting RAD51 paralogs, such as RAD51B and RAD51C, to similar levels as in a normal fibroblast control, GM0637 (online supplementary figure 3). Of note, RAD51C but not XRCC2 can form a separate complex with another RAD51 paralog, XRCC3.[35 36] Proficiency or deficiency for XRCC2 had no clear effect on the levels of XRCC3, or on the levels of two other DNA double-strand break (DSB) repair-related FA proteins, RAD51 and BRCA1 (figure 5C). In summary, XRCC2 appears to have a specific function in maintaining the BCDX2 complex, which includes another late and atypical FA protein, RAD51C.

## **DISCUSSION**

Here, we identify *XRCC2* as the 20th FA gene, which should therefore be labeled as *FANCU*. In fact, XRCC2 perfectly fits the profile of an atypical *late* FA gene as will be summarized below. Historically, prior to the discovery of any FA genes, FA patients were defined clinically as presenting with the classical triad of congenital abnormalities, bone marrow failure and malignancies.[38] Cells from these patients also displayed characteristic chromosomal breakage and hypersensitivity in response to DNA interstrand crosslinking agents.[1 5 6] Subsequently, genes with loss-of-function mutations in FA patients that presented with the triad described above were labeled as 'FA genes' since they complemented the cellular FA phenotype, specifically hypersensitivity to ICLs. However, in the last few years, bi- or monoallelic germ-line defects have been identified in patients with



cellular ICL hypersensitivity and increased chromosomal breakage that do not show the complete triad of clinical features which are classically associated with FA. Patients with defects in these *atypical* FA genes, also called FA-like genes, including *BRCA1/FANCS*, *RAD51/FANCR* and *RAD51C/FANCO*, and as we show here, *XRCC2/FANCU*, have congenital abnormalities characteristic of FA but do not develop bone marrow failure.[10 11 18]

Each gene that is associated with FA, whether it be typical or atypical FA, encodes a protein that has an essential function in an elaborate mechanism that repairs DNA interstrand crosslinks (ICLs). This is shown schematically in online supplementary figure 4. Prominent steps in the repair of ICLs include recognition of the crosslink, incision to unhook the lesion, translesion synthesis to restore duplex DNA, and HR to restart the replication fork.[2 6] Proteins of the FA core complex, and UBE2T/FANCT, are involved early in the repair process and are required for the assembly of FANCD2 and FANCI into nuclear foci.[14-16] In contrast, homologous recombination occurs at later steps in the repair of ICLs and utilizes the products of other FA genes, such as *BRCA1/2*, *PALB2* and *RAD51*. Related to their function in HR, these proteins are instead involved in the assembly of the RAD51 recombinase into DNA damage foci and are also required for resistance to IR.[39] Thus, defects in the the assembly of RAD51 foci observed in 900677AT cells position *XRCC2* as a late FA protein.

Given the heterogeneity in clinical phenotypes,[3 4] the defining features that are common to all FA complementations groups are cellular hypersensitivity to DNA interstrand crosslinking agents and the fact that this can be corrected by re-expression of the appropriate gene/protein [2 5 6]. Ultimately, this hypersensitivity to ICLs reflects the common function for FA proteins in repairing this type of DNA lesion.

### **Biallelic mutation of *XRCC2* is responsible for all phenotypes in the patient's cells**

*XRCC2* is a *bona fide* FA gene by the accepted standard[28] of correcting three different phenotypes of 900677 cells that are related to cellular sensitivity to ICLs. Further, *XRCC2*-deficient 900677 fibroblasts also have a modest increase in sensitivity to olaparib and formaldehyde. Increased sensitivity to PARP inhibitors, including olaparib, has been observed for FA cells with inactivation of other late FA genes, especially *BRCA1/2*, *PALB2* and *RAD51C*.[10 24 31] Notably, the increased sensitivity of 900677AT cells to formaldehyde, which can be corrected by re-expression of *XRCC2*, is also consistent with our having identified them as FA cells. Sensitivity to formaldehyde and other aldehydes has been observed in a variety of cell lines with deficiencies in FA proteins.[32 33 40-43] 900677 cells, with or without complementation by *XRCC2*, display no defect in FANCD2 monoubiquitination or foci formation. Since monoubiquitination of FANCI by the FA core complex is essential for FANCD2 monoubiquitination and assembly into foci,[15 16] FANCD2 is a reliable indicator that *XRCC2* does not function early in the FA-BRCA pathway, but instead functions late. In support of this conclusion, *XRCC2*-deficient 900677AT cells display cellular hypersensitivity to either ICLs and IR, similar to cells with deficiencies for other late FA genes. Importantly, these cellular phenotypes are correctable by re-expression of *XRCC2*.

### **XRCC2 interacts with and stabilizes RAD51B, RAD51C/FANCO, and RAD51D**

The mutant R215X protein is detected at decreased levels as compared to the full-length protein. This appears to result from protein instability rather than nonsense-mediated decay of mRNA, since the truncating mutation is in the last exon of *XRCC2*.<sup>[44]</sup>

Given their deficiency for *XRCC2*, 900677AT cells lack the interaction of *XRCC2* with another FA protein, *RAD51C*. We also show, in figure 5, that all components of the BCDX2 complex, including *RAD51C*, are destabilized when WT *XRCC2* is missing in 900677AT cells. These effects may explain the pathogenicity of the p.R215X mutant.

Our hypothesis is that the deficiency of 900677AT cells for *XRCC2* results in the destabilization of *RAD51D*, since these proteins directly interact.<sup>[45]</sup> The fact that RNAi-mediated depletion of the *XRCC2* protein results in dramatically decreased levels of *RAD51D* is consistent with this hypothesis.<sup>[46]</sup> Instability of *RAD51D* then likely leads to destabilization of its direct binding partner, *RAD51C*.<sup>[45]</sup> Destabilized *RAD51C* could, in turn, lead to instability of *RAD51B*, since these proteins also directly interact.<sup>[45]</sup> Consistent with a primary effect of *XRCC2* deficiency on *RAD51D* stability, *RAD51D* was absent in 900677AT cells, in contrast to *RAD51B* and *RAD51C*, which were still detectable, albeit at lower levels (figure 5).

Taken together, the association of *XRCC2* with Fanconi anemia suggests the possibility that a key function of the *XRCC2* protein in the FA-BRCA pathway is to stabilize the other members of the BCDX2 complex, all of which function in HR.<sup>[37]</sup> Interestingly, since *RAD51D* encodes a protein that directly interacts with the two other *RAD51* paralogs that are FA proteins, *RAD51C/FANCO* and *XRCC2/FANCU*, *RAD51D* appears to be an important candidate FA gene.

### **Atypical FA genes**

As our *XRCC2* patient is still younger than the median age of onset of bone marrow failure in FA patients, which is 7.6 years of age,<sup>[3]</sup> the vast majority of whom have defects in early FA genes, it is possible that hematopoietic defects will simply arise later. Also, it should be noted that while the patient initially displayed increased chromosome breakage in blood cells,<sup>[20]</sup> tests to exclude somatic reversion as an explanation for the absence of hematologic phenotypes have not been performed recently.

All the same, loss-of-function mutations in *BRCA1*, *RAD51*, *RAD51C*, and now *XRCC2*, all of which are late FA genes, have not been associated with bone marrow failure.<sup>[10 11 18]</sup> On this basis, we designate these genes, including *XRCC2*, as *atypical* FA genes. In this context, it should be noted that bone marrow failure is not uniformly observed even in 'typical' FA patients with mutations in early FA genes.<sup>[3]</sup>

Our classification of *XRCC2* as an atypical FA gene advances the understanding of the FA-BRCA pathway in multiple ways. First, our results have yielded a more complete knowledge of genes that cause FA when mutated. Second, these results further suggest that atypical FA genes, including *BRCA1/FANCS*, *RAD51C/FANCO*, *RAD51/FANCR*, and now *XRCC2/FANCU*, encode proteins that function late in ICL repair. Members of this subgroup of FA

genes are defined by a common role in regulating RAD51 foci, HR, and resistance to IR.[10 18 37 47]

The fact that heterozygous mutation of *XRCC2* is also associated with a predisposition to breast cancer[48] is consistent with the conclusion that *XRCC2* is an atypical late FA gene. Heterozygosity for two other atypical, and late, FA genes, *BRCA1* and *RAD51C*, is also associated with an increased risk of developing breast cancer, unlike heterozygosity for early FA genes.[48-51]

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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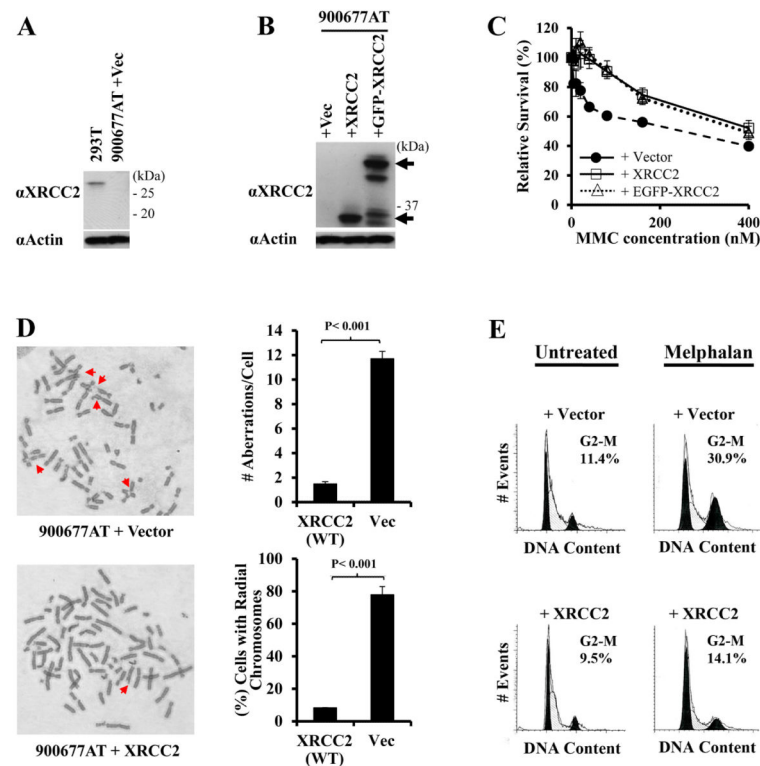
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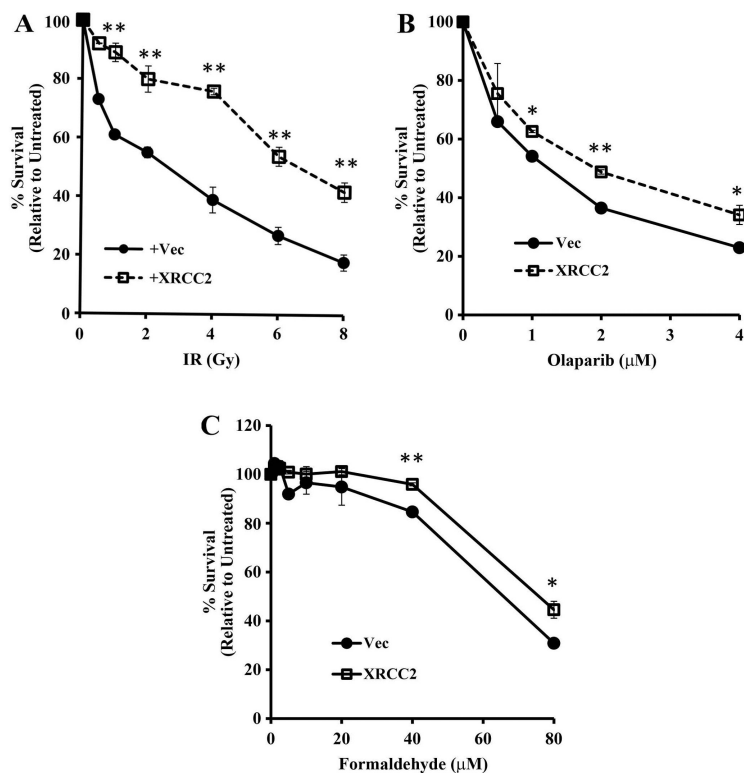
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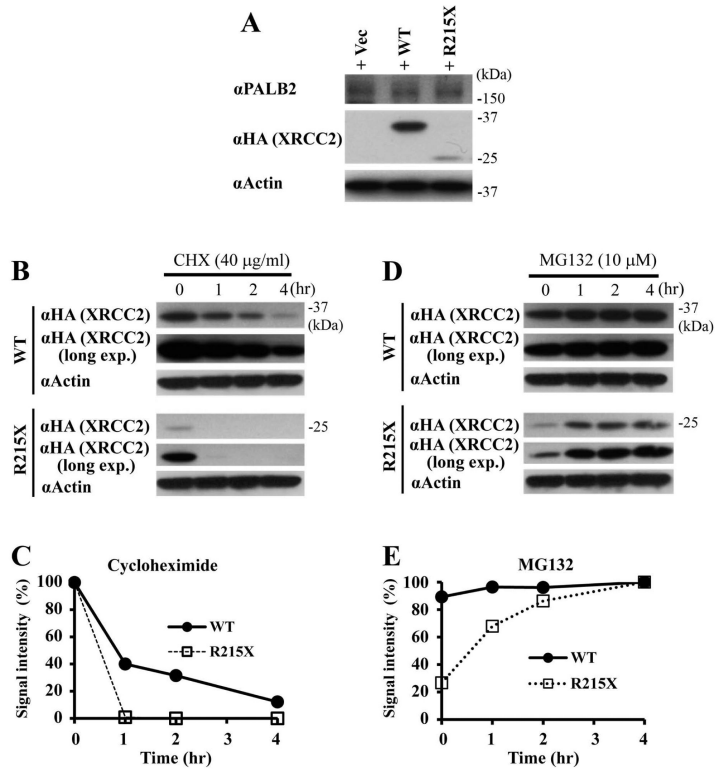


**Figure 1. Complementation with *XRCC2* corrects cellular phenotypes associated with sensitivity to DNA interstrand crosslinking agents that are characteristic of FA cells**

(A) *XRCC2* was detected in extracts from a non-diseased individual (293T), but neither full-length (~34 kDa) nor a truncated form of *XRCC2* (expected at ~23 kDa) was detected in extracts from 900677AT cells containing vector alone. (B) Retroviral expression of *XRCC2*, or a GFP-*XRCC2* fusion protein, restored *XRCC2* in 900677AT fibroblasts. Arrows indicate the position of the GFP-*XRCC2* (above) and *XRCC2* (below) proteins on immunoblots. Some degradation of GFP-*XRCC2* is evident. Actin is shown as a loading control. Molecular weight markers are indicated in (A-B). (C) Relative survival of 900677AT fibroblasts immortalized with SV40 large T antigen, and which contained the lentiviral vector, *XRCC2*, or GFP-*XRCC2*, following treatment with a range of concentrations of MMC. Values were normalized to levels in untreated populations for each cell line. (D) Examples are shown of chromosome aberrations in 900677AT immortalized fibroblasts reconstituted with vector or with *XRCC2* following treatment with 300 nM MMC for 72 hr (left). The total number of aberrations/metaphase cell, including breaks, gaps, radial and ring chromosomes, and the percentage of metaphase cells with one or more radial chromosomes, is shown for each line (right). (E) Histograms showing the cell cycle distribution in 900677AT cells, with or without correction for *XRCC2*, in untreated populations or following treatment with 0.35  $\mu$ g/ml melphalan. Curve fits for the G1 peak (left) and G2-M peak (right) were determined using ModFit and are shown in black in each histogram. The percentage of cells in G2-M is indicated on each histogram.

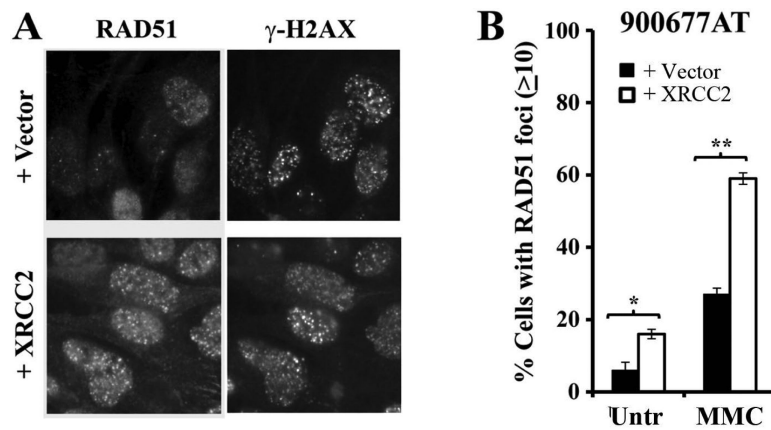


**Figure 2. XRCC2 promotes cellular resistance to multiple DNA damaging agents, in addition to MMC**  
 (A-C) Resistance of *XRCC2*<sup>-/-</sup> 900677AT cells, with or without reconstitution of XRCC2, to a range of doses of IR (A), the PARP inhibitor olaparib (B), and formaldehyde (C). Values for each cell line and type of DNA damage are relative to untreated populations for each cell line and that particular treatment. p values, as determined using Student’s t-test: \*p<0.01, \*\*p<0.0001.

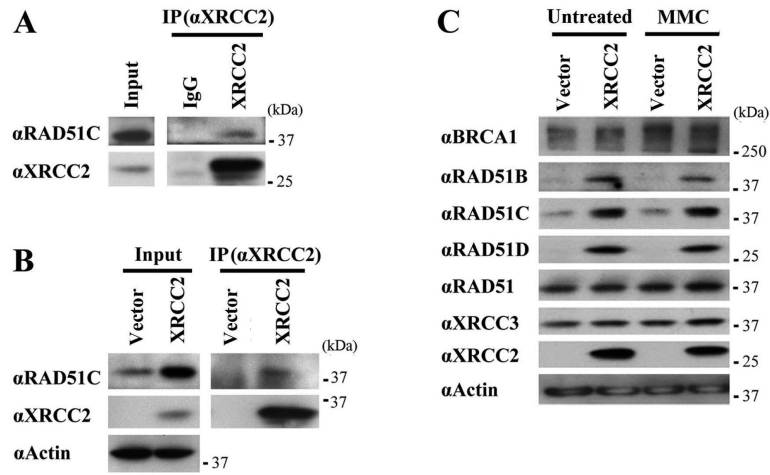


**Figure 3. The R215X truncation mutant of XRCC2, which is found in a Fanconi anemia patient, is unstable**

(A) As compared to WT XRCC2, the R215X truncation mutant of XRCC2, found in a FA patient is expressed at low levels in U2OS-DR cells. (B) The R215X mutant of XRCC2 turns over more rapidly than the WT form, as shown by treatment with cycloheximide (CHX). Given the low levels of XRCC2-R215X, long exposures (long exp.) are shown for comparison to the WT form. (C) Signal intensity after treatment with cycloheximide is shown for the WT and R215X forms of XRCC2 relative to the untreated lane. (D) Inhibition of proteasome-mediated protein degradation in U2OS-DR cells with MG132 has a greater effect on the R215X form of XRCC2 than on the WT. (E) Quantification of the effect of MG132 on the levels of WT and R215X XRCC2 relative to the levels at 4 hr of treatment. Flag-HA-XRCC2 was utilized throughout the figure and actin is shown as a loading control.



**Figure 4. XRCC2 promotes the assembly of RAD51 foci similar to other late FA proteins**  
 (A) Representative images of RAD51 foci detected in *XRCC2*<sup>-/-</sup> 900677AT cells, with or without reconstitution with XRCC2, using indirect immunofluorescence microscopy, following treatment with 0.5  $\mu$ M MMC for 16 hr.  $\gamma$ H2AX foci demonstrate the assembly of DNA damage foci in both cell lines irrespective of the status of XRCC2. (B) Quantification of RAD51 foci in *XRCC2*<sup>-/-</sup> 900677AT cells, with or without reconstitution of XRCC2, either in untreated populations or following exposure to MMC.



**Figure 5. XRCC2 maintains the levels of other proteins in the BCDX2 complex, and 900677AT cells have a diminished interaction of the XRCC2 and RAD51C FA proteins**  
 (A) The interaction of RAD51C with XRCC2 was detected in immunoprecipitates performed using antibodies against XRCC2 or a non-specific control (IgG) with extracts derived from 900677AT cells in which XRCC2 had been re-introduced. (B) Levels of RAD51C that immunoprecipitated with XRCC2 using anti-XRCC2 antibodies in extracts from untreated XRCC2 proficient (XRCC2) or deficient (Vector) 900677AT cells. (C) The levels of various DNA damage response proteins, including each of the somatic RAD51 paralogs, in XRCC2<sup>-/-</sup> 900677AT cells either with or without exogenous expression of XRCC2. Cells were either left untreated or were incubated with 0.5  $\mu$ M MMC for 16 hr, and each protein was detected using the indicated antibody. Actin is shown as a loading control for the levels of proteins present in extracts (B-C).

**Table 1**

Spontaneous and DEB-induced chromosome breaks in 900677A and other lines.

Sample	Dose	Mean Breaks/Cell
GM16631 ( <i>FANCA</i> -/-)	0 mg/ml	0.18
	0.01 mg/ml	1.52
GM16754 ( <i>FANCC</i> -/-)	0 mg/ml	0.10
	0.01 mg/ml	1.44
900677A ( <i>XRCC2</i> -/-)	0 mg/ml	0.22
	0.01 mg/ml	0.48
	0.1 mg/ml	2.35
NL143889 ( <i>Normal Fibr.</i> )	0 mg/ml	0.00
	0.01 mg/ml	0.04
	0.1 mg/ml	0.15

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