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Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways

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Fanconi anaemia (FA) is a chromosomal instability disorder characterized by cellular sensitivity to DNA interstrand crosslinking agents and a high risk of cancer. Six of the eight proteins encoded by the known FA genes form a nuclear complex which is required for the monoubiquitination of the FANCD2 protein. FANCD2 complexes and colocalizes with BRCA1, but its presumptive role in DNA repair has not yet been clearly defined. We used yeast two-hybrid analysis to test for interaction between FANCD2 and 10 proteins involved in homologous recombination repair. FANCD2 did not interact with RAD51, the five RAD51 paralogs, RAD52, RAD54 or DMC1. However, it bound to a highly conserved C-terminal site in BRCA2 that also binds FANCG/XRCC9. FANCD2 and BRCA2 can be coimmunoprecipitated from cell extracts of both human and Chinese hamster wild-type cells, thus confirming that the interaction occurs *in vivo.* Formation of nuclear foci of FANCD2 was normal in the *BRCA2* mutant CAPAN-1 cells, which indicates that the recruitment of FANCD2 to sites of DNA-repair is independent of wild-type BRCA2 function. FANCD2 colocalized with RAD51 in foci following treatment with mitomycin C or hydroxyurea, and colocalized very tightly with PCNA after treatment with hydroxyurea. These findings suggest that FANCD2 may have a role in the cellular response to stalled replication forks or in the repair of replication-associated double-strand breaks, irrespective of the type of primary DNA lesion.

INTRODUCTION

Fanconi anaemia (FA) is an autosomal recessive genetic disorder characterized by progressive bone marrow failure, multiple congenital abnormalities and an increased risk of cancer. The cellular phenotype is characterized by chromosomal instability and hypersensitivity to DNA interstrand crosslinking (ICL) agents such as mitomycin C (MMC) and cisplatin. Currently, at least 11 complementation groups are known to exist (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L) (1,2), and the genes for all of these groups except FA-B, FA-I and FA-J have been identified (3–11). The FANCA, FANCC, FANCE, FANCF, FANCG and FANCL proteins have been shown to form a nuclear multiprotein complex, known as the FA core complex, which is required for the monoubiquitination of the FANCD2 protein (12–18). How the modified form of FANCD2 contributes to DNA repair is not yet clear. Recent studies have suggested a broader role for this protein in homologous recombination and double-strand break (DSB) repair (reviewed in 19). In particular, FANCD2 has been shown to coimmunoprecipitate with BRCA1 and NBS1, and to colocalize with these two proteins in DNA damage-induced nuclear foci (18,20). Also, FANCD2 is phosphorylated *in vivo* in an ATM-dependent manner following ionizing radiation (21), and chromosome pairing during the pachytene stage of meiosis is impaired in FANCD2$^{-/-}$ mice

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(22). Finally, whereas FANCD2 is activated relatively slowly after treatment with ICLs, activation is rapid in response to ionizing radiation, and occurs only in S-phase after DSBs have formed (23).

Homologous recombination repair (HRR) is thought to involve a number of proteins which include BRCA2, RAD51 and its five paralogs (XRCC2, XRCC3, RAD51B, RAD51C and RAD51D), RAD52 and RAD54 (24). We have investigated the possible role of FANCD2 in the HRR pathway by screening for direct interaction between FANCD2 and these 10 proteins in the yeast two-hybrid system. Positive interactions were investigated further by coimmunoprecipitation and colocalization studies in human and rodent cells.

RESULTS

Yeast two-hybrid analysis

FANCD2 was tested for direct interaction with the RAD51, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, BRCA2, RAD52, RAD54 and DMC1 proteins using the yeast two-hybrid system. The plasmids used in the yeast two-hybrid assays were functional, since previously reported self-interactions or heterologous interactions were observed, as judged by expression of reporter genes (*ADE2, HIS3* and *LacZ*) (Fig. 1A). However, neither RAD51, nor the RAD51 paralogs, or RAD52, RAD54 and DMC1 interacted directly with FANCD2 (Fig. 1A). FANCD2 was also tested for direct interaction with BRCA2 using a series of fragments spanning the entire coding region of BRCA2 (25). FANCD2 was found to interact with the C-terminal region (amino acids 2118–2566) of BRCA2 (Fig. 1B and C) in both cotransformation and mating experiments. This segment of BRCA2 is one of two regions that also interacts with FANCG (25); it did not interact with several control constructs including SV40 T-antigen or with other FA proteins (Fig. 1C) (25), showing that the interactions with FANCD2 and FANCG were specific. The binding site in BRCA2 was further refined to amino acid residues 2350–2545 (Fig. 1B and C), which we have also shown to be involved in the interaction of BRCA2 with FANCG (25). FANCD2 did not interact with any other of the BRCA2 fragments covering the rest of the protein (data not shown). The FANCD2 construct which interacted with BRCA2 encodes amino acid residues 1–1102 of the FANCD2 protein (Fig. 1B and C), but a FANCD2 construct lacking the first 325 amino acids of the N-terminus of the protein failed to interact with BRCA2 (Fig. 1B and C) indicating that this region is required for the interaction. A FANCD2 construct spanning amino acid residues 1–1102 with an in-frame deletion of residues 248–359 (FANCD2 1–1102 delTD) also failed to interact with BRCA2 (Fig. 1C), confirming the importance of the N-terminus of FANCD2 for this interaction. This region is also involved in binding FANCE (26), so the binding sites for FANCE and BRCA2 may overlap.

FANCD2 coimmunoprecipitates with BRCA2

The FANCD2/BRCA2 interaction observed in the yeast two-hybrid system was further investigated by testing for co-immunoprecipitation of the endogenous proteins in extracts from mammalian cells. Cells were treated with MMC to induce DNA damage at a concentration (20 nM), which allows >90% survival of wild-type mammalian cells in clonogenic assays (27). BRCA2 was immunoprecipitated with FANCD2 using a FANCD2 antibody in both human and Chinese hamster wild-type cells (Fig. 2A). Coimmunoprecipitation failed to occur in the human FA-G cell line EUFA673 and in the hamster FA-G mutant NM3. It was also absent in FA-G EUFA143 cells transduced with empty pMMP vector, but was restored in FA-G EUFA143 cells transduced with pMMP vector containing the FANCG cDNA. The interaction of FANCD2 with BRCA2 was not dependent on the damage induced by MMC since coimmunoprecipitation also occurred in untreated cells (Fig. 2B).

BRCA2 is not required to recruit FANCD2 into foci

CAPAN-1 cells express only a single allele of the *BRCA2* gene which is itself truncated within BRC7 of the BRCA2 protein (28). BRCA2 in this cell line thus lacks the FANCD2 binding site as determined by our yeast two-hybrid analysis. In order to determine whether FANCD2 binding to BRCA2 is required for the formation of FANCD2 foci, we treated CAPAN-1 cells with MMC and analysed the formation of FANCD2 foci. FANCD2 focus formation in these cells was found to be normal (Fig. 3A); the number of cells displaying more than five FANCD2 foci was 13% prior to MMC treatment and 40% following MMC treatment, which is very similar to that seen in HeLa cells (Table 1).

FANCD2 colocalizes with RAD51 and PCNA following DNA damage

A major role of BRCA2 is thought to be in controlling the activity of the RAD51 recombinase (29–31) with which it interacts directly (32,33). In keeping with this idea, the formation of DNA damage-induced RAD51 foci has been shown to be critically dependent on BRCA2 (34). In order to determine whether FANCD2 and RAD51 are involved in the same DNA damage-response pathway, we tested for their colocalization following treatment with MMC in HeLa cells using confocal immunofluorescence microscopy. HeLa cells were treated with 50 ng/ml MMC and stained for endogenous FANCD2 and RAD51 using affinity purified antibodies. Consistent with the findings of Taniguchi *et al.* (35), FANCD2 and RAD51 showed some degree of colocalization in untreated cells (Fig. 3B and Table 1), presumably in cells that are in S-phase (35). Here we also show that the degree of colocalization increases substantially following treatment with MMC (Fig. 3B and Table 1), suggesting that FANCD2 and RAD51 complexes co-operate in mounting a cellular response to DNA damage with ICL agents.

In order to determine whether FANCD2 might have a more general role in the repair process, rather than one limited specifically to the repair of ICLs, we tested for its ability to colocalize with RAD51 following treatment with the replication block inducing agent, hydroxyurea (HU). Replication stalling by HU treatment is thought to result in replication fork collapse, which may lead to the formation of a DSB (36). HeLa cells were treated with 3 mM HU and stained for

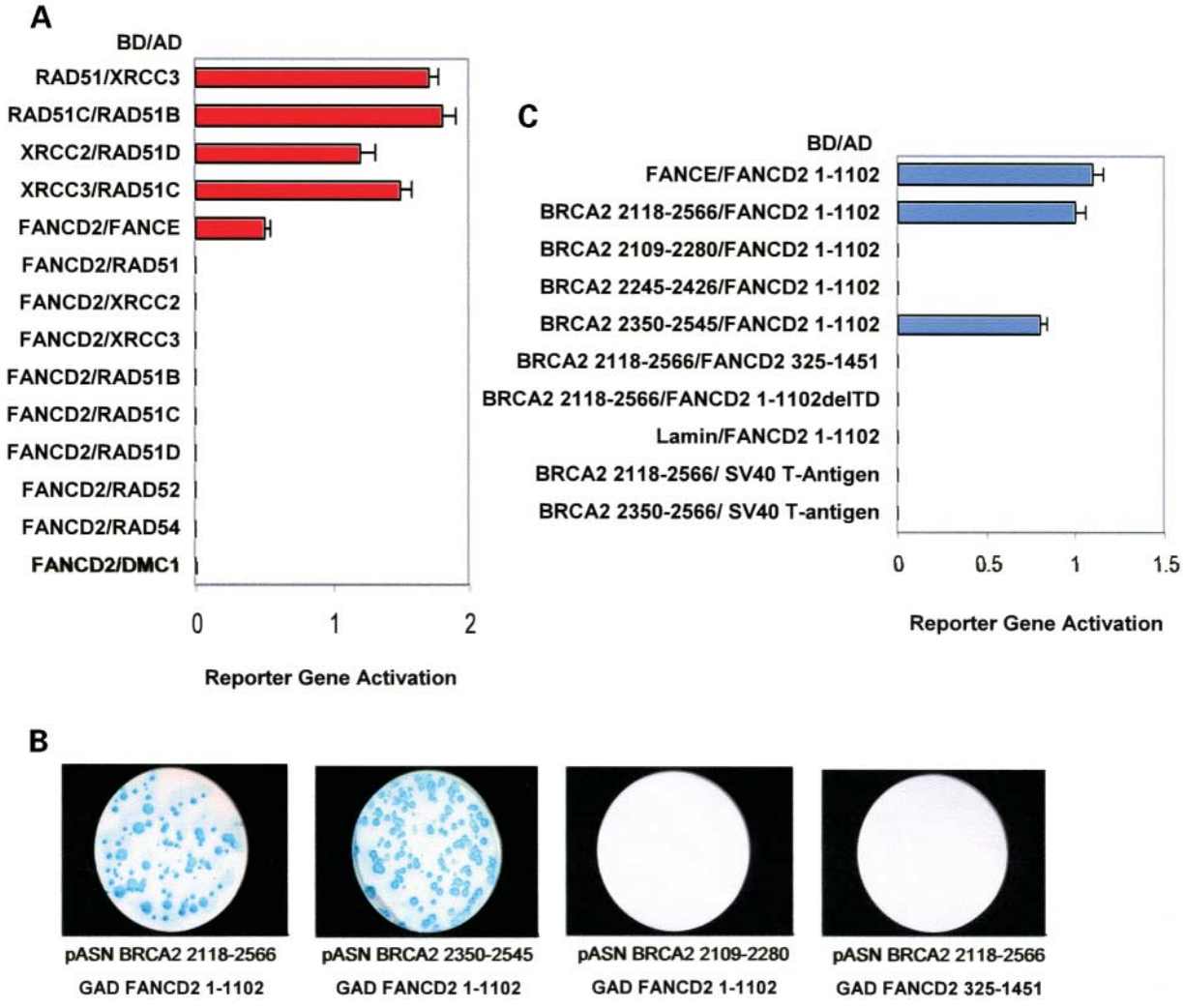

Figure 1. FANCD2 interacts with BRCA2 but not with other DNA repair proteins in yeast two-hybrid analysis. (**A**) Screening of FANCD2 for interaction with homologous recombination repair proteins. The strengths of interactions detected were determined by a semiquantitative method (see Materials and Methods) (15,53). The results are from three independent mating experiments. Error bars show standard deviations from the mean. Positive interactions of RAD51 paralogs and FANCD2/FANCE have been reported previously (16,58–60). (**B**) Filter lifts showing that FANCD2 binds to BRCA2 amino acids 2118–2566, with amino acids 2350–2545 being required for the interaction. The presence of blue colonies on filter lifts represents activation of three independent reporters: *HIS3*, *ADE2* and *LacZ*. (**C**) Semiquantitative analysis of yeast two-hybrid interaction strengths showing that the FANCD2-BRCA2 interaction appears to be comparable in strength to the previously reported FANCD2/FANCE interaction (16,26). The FANCD2 1–1102 delTD construct carries an in-frame deletion of amino acids 248–359. Results are from four to six independent mating experiments.

endogenous FANCD2 and RAD51. Treatment resulted in a large increase in the number of FANCD2 foci per cell and the number of cells containing FANCD2 foci (Fig. 3B and Table 1). As seen with MMC, HU caused a substantial increase in the degree of colocalization of FANCD2 with RAD51 (Fig. 3B and Table 1).

These observations suggested that FANCD2 could have a role either in the cellular response to stalled replication forks or in the repair of DNA damage, which is encountered following stalled replication. We tested this idea by looking for colocalization of FANCD2 with PCNA foci which can be used to mark sites of DNA replication and repair (37). There was little colocalization of FANCD2 and proliferating cell nuclear antigen (PCNA) foci before HU treatment. However, HU treatment caused a striking increase in the level of colocalization of FANCD2 and PCNA (Fig. 3C and Table 2). Indeed, whereas the level of colocalization between FANCD2 and RAD51 even after MMC and HU treatments was only partial, FANCD2 and PCNA colocalization was in excess of 90% in >90% of cells displaying foci following HU treatment (Fig. 3C and Table 2).

DISCUSSION

In this study we demonstrate a direct interaction between the FANCD2 and BRCA2 proteins. The interaction mapped to part of the highly conserved BLAT domain in BRCA2 which has also been shown to bind FANCG/XRCC9 (25). Very recently an FA-D1 patient-derived missense mutation in

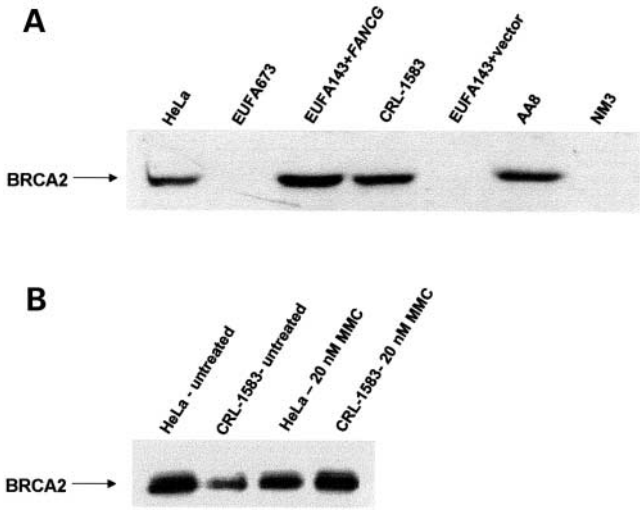

Figure 2. (**A**) Cells were treated with 20 nM MMC for 18 h prior to preparation of cell lysates. Cell lysates were immunoprecipitated with anti-FANCD2 antibody. Samples were loaded onto an SDS–PAGE gel and electrophoresed. Blots were probed with an anti-BRCA2 C-terminal (amino acids 3245–3418) antibody. The FANCD2/BRCA2 interaction could be detected in the wild-type human (HeLa and CRL-1583) and hamster (AA8) cell lines but was not present in the three cell lines mutated in *FANCG* (EUFA143, EUFA673 and NM3). The FANCD2/BRCA2 interaction was restored in EUFA143 transduced with *FANCG* cDNA. (**B**) Coimmunoprecipitation of FANCD2 and BRCA2 was also observed in untreated human cells. Immunoprecipitation and blotting were performed as in (A).

BRCA2 (L2510P) has also been identified in this region (38). The fact that FANCD2 can interact with BRCA2 when expressed in the yeast two-hybrid system indicates that mono-ubiquitination of FANCD2 is not required for binding of the two proteins. However, recruitment of FANCD2 into nuclear foci has been shown to be dependent on its ubiquitination (18) and this is consistent with the proposed role of mono-ubiquitination in protein trafficking (39). Our observation that FANCD2 and BRCA2 fail to coimmunoprecipitate in several FA-G cell lines (Fig. 2) that do not express monoubiquitinated FANCD2 (18,27) suggests that it may be the ubiquitinated form that interacts with BRCA2 *in vivo*. However, it is also possible that FANCG, which itself interacts directly with BRCA2, may facilitate the FANCD2/BRCA2 interaction. BRCA2 does not appear to be required for the recruitment of FANCD2 in nuclear foci, since this process is undisturbed in CAPAN-1 cells, but it does require BRCA1 (18,40).

In this study we also show that FANCD2 colocalizes with nuclear foci of RAD51 in response to various forms of DNA damage agents such as MMC and the replication blocking agent HU. DNA damage-induced RAD51 foci have been proposed to be sites where DSB repair is occurring by homologous recombination (41). It has been suggested previously that the repair of ICLs may occur via the formation of a DSB intermediate during replication (42,43), and HU-induced replication fork blockage may also result in the formation of a DSB (36). It is therefore possible that FANCD2 localizes to sites of DSBs, irrespective of the type of primary DNA lesion, to co-operate with BRCA2 and RAD51 in their repair.

Our observation that FANCD2 colocalizes very tightly with PCNA following treatment with HU is consistent with a role for FANCD2 either in the cellular response to stalled replication forks or in the repair of DSBs that arise subsequently. The involvement of BRCA2 in replication-associated DNA repair has also been suggested by the fact that it is expressed mainly in the S-phase (44), and colocalizes with PCNA foci following HU treatment (45). Recently, it has been proposed that BRCA2 stabilizes DNA structures at stalled forks, and that the chromosomal instability associated with a deficiency of BRCA2 is a consequence of an inability to respond to stalled replication (46). The direct interaction of FANCD2 with BRCA2 and its tight colocalization with PCNA following HU treatment suggests that it may also be a part of this process. The interaction does not require exogenous DNA damage, since we found that FANCD2 and BRCA2 could be coimmunoprecipitated in untreated cells. This is consistent with our observation of substantial colocalization of FANCD2 with RAD51 even in untreated cells, which are likely to be in the S-phase (35). It is possible that endogenous compounds such as reactive oxygen species may induce DNA damage which generates stalled replication forks (47), thereby promoting the interaction of FANCD2 with the BRCA2/RAD51 complex. Also, stalling of replication forks occurs commonly in normally dividing cells (48).

The observation that FANCG and FANCD2 bind to the same site in BRCA2 suggests that these three proteins may co-operate in the repair process. Chicken DT40 cells that are deficient in FANCG have a 9-fold reduction in HR compared with wild-type cells (49), which implies a direct role for FANCG in at least some types of HR-mediated DSB repair. BRCA2 is thought to bind inactive RAD51 and mediate its loading onto single-stranded DNA (ssDNA) where active recombination complexes assemble (reviewed in 24). The interactions of FANCG and FANCD2 with BRCA2 may lead to conformational changes important for RAD51–ssDNA interactions. For example, a structure has recently been proposed for the BRCA2/RAD51 interaction in which the BRC repeats of BRCA2 wrap around RAD51 ring structures, which brings N- and C-terminal regions of BRCA2 into close apposition (50). It is proposed that the complex is recruited to a DSB, where the BRC repeats disassemble the ring, and RAD51 monomers are loaded onto ssDNA. Since we have shown previously that FANCG binds to both the N- and C-terminal regions of BRCA2, it is possible that FANCG binds both these regions of BRCA2 simultaneously, thus stabilizing the structure until the correct signal is received at the site of a DSB for unloading of RAD51. FANCD2 could be a part of this signal by replacing FANCG at the same C-terminal region of BRCA2. Although we currently lack an understanding of the precise function of the FA pathway, these studies indicate that at least some of the FA proteins are directly associated with key elements of homologous recombination repair, such as the BRCA2/RAD51 complex.

MATERIALS AND METHODS

Plasmid constructs

Full length cDNAs for human RAD51, XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, RAD52, RAD54 and DMC1 were cloned into yeast two-hybrid vectors to give in-frame

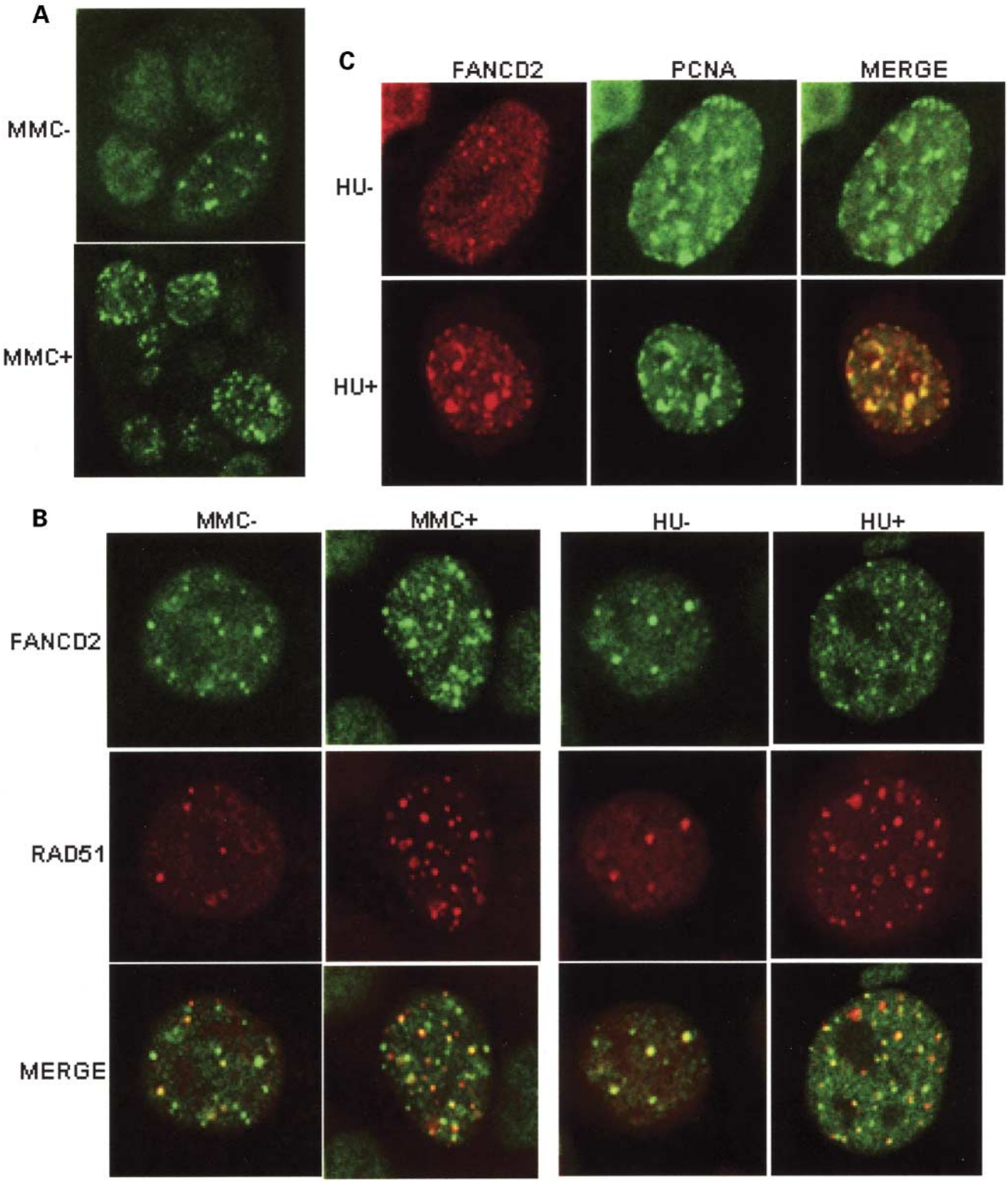

Figure 3. Localization of FANCD2 in nuclear foci. (**A**) BRCA2 is not required for the formation of DNA damage-induced FANCD2 nuclear foci. CAPAN-1 cells were treated with MMC and stained for endogenous FANCD2 using an affinity-purified rabbit polyclonal FANCD2 antibody. The formation of FANCD2 foci following MMC treatment was normal, suggesting that the process is BRCA2-independent. (**B**) FANCD2 colocalizes with RAD51 in response to DNA damage. HeLa cells were treated with MMC and HU and stained for endogenous FANCD2 and RAD51 using affinity-purified antibodies. Although there was some degree of colocalization in untreated cells, both MMC and HU caused a substantial increase in the degree of colocalization. (**C**) FANCD2 colocalizes with PCNA following HU treatment. HeLa cells were treated with HU and stained for endogenous FANCD2 and PCNA.

Table 1. Colocalization of FANCD2 with RAD51

	Cells displaying more than five FANCD2 nuclear foci (%)	Cells displaying more than five RAD51 nuclear foci (%)	Cells showing more than 50% colocalization of FANCD2 and RAD51 nuclear foci (%)[a]	Mean no. of FANCD2/RAD51 colocalizing foci per cell[a]
MMC−	16	14	54	3.6
MMC+	39	42	82	13.2
HU−	13	16	48	4.7
HU+	42	40	90	13.7

[a]Only cells displaying more than five FANCD2 or RAD51 foci were scored. At least 100 cells were scored for each count.

Table 2. Colocalization of FANCD2 with PCNA

	Cells displaying more than five FANCD2 foci (%)	Cells displaying more than five PCNA foci (%)	Cells showing more than 90% colocalization of FANCD2 and PCNA foci (%)[a]	Mean no. of FANCD2/PCNA colocalizing foci per cell[a]
HU−	12	41	12	3.2
HU+	38	39	93	16.4

[a]Only cells displaying more than five FANCD2 or PCNA foci were scored. At least 100 cells were scored for each count.

fusions with either the GAL4 binding domain (BD) or the GAL4 activation domain (AD) and were verified by sequencing. FANCD2 and BRCA2 constructs have been described elsewhere (51,52). Constructs contained full length cDNAs unless otherwise stated.

Yeast two-hybrid analysis

The MATCHMAKER Two Hybrid System 3 (Clontech) was used for yeast two-hybrid analysis according to the manufacturer's instructions and as described previously (15,53). Briefly, GAL4 AD and GAL4 BD constructs were either sequentially transformed into AH109 yeast cells and subjected to selection on (−trp−leu−his−ade) medium, or transformed separately into AH109 and Y187 yeast strains and mating cultures plated onto selection medium. Transformations were performed using a PEG/ssDNA/lithium acetate procedure. Colonies were then transferred onto filters and tested for β-galactosidase expression with X-gal. Each experiment was performed at least in triplicate, and constructs were tested for autoactivation and against a series of control plasmids. Five mM 3-AT was added to the media to suppress slight autoactivation of some constructs. A semiquantitative test of interaction strength based on the extent of reporter gene activation was carried out as described previously, where reporter gene activation $= (n \times C/t \times \ln T)$, where $n =$ number of blue colonies, $C =$ intensity of blue colour, $t =$ time of colony growth prior to *LacZ* expression testing and $T =$ time taken for colour development.

Coimmunoprecipitation and immunoblotting

Wild-type human cell lines HeLa and CRL-1583 and hamster cell lines AA8 and NM3 were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) as previously described (54). CRL-1583 is a human SV40-transformed placental cell line (55). AA8 is a Chinese hamster ovary cell line and NM3 is a *FANCG* mutant derived from AA8 that has a frameshift mutation in exon 3, which causes premature truncation of *FANCG* (27,56). FA-G cell lines EUFA143 and EUFA673 were grown in RPMI-1640 medium with 15% FCS. EUFA143 cells transduced with either human *FANCG* cDNA in the pMMP vector or empty vector were kindly provided by Gary Kupfer (57). FANCD2 antibody (ab2187) was obtained from Abcam Limited (Cambridge, UK) and BRCA2 antibody (Ab-2) was obtained from Oncogene Research Products (Merck, UK). Coimmunoprecipitation was performed using the EZview red protein affinity gel system provided by Sigma, essentially using their recommended conditions. Total cell extracts from 1 to 2×10^7 cells, untreated or treated with 20 nM MMC for 18 h, were prepared by lysis in 1 ml of ice-cold RIPA buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 1% sodium dodecyl sulfate (SDS) with protease inhibitor cocktail]. After centrifugation at 4°C, undiluted anti-FANCD2 was added to the supernatant and mixed gently for 2 h at 4°C. Antibody-antigen complexes were then purified using the recommended procedure and the resulting 25 μl sample loaded onto an SDS–PAGE gel. Electrophoresis and transfer of proteins onto nitrocellulose membrane was as previously described (27). The membrane was blocked for 1 h in 5% dried skimmed milk in Tris-buffered saline (TBS, pH 8.8), washed three times in TBS and incubated with primary antibody (α-BRCA2 in TBS) overnight at 4°C. After washing three times with TBS, the membrane was incubated with anti-rabbit horseradish peroxidase-linked secondary antibody, washed three times with TBS and chemiluminescence used for detection (Amersham, UK).

Immunofluorescence and microscopy

HeLa cells used for all immunofluorescence experiments were cultured at 37°C at 5% CO_2 in DMEM supplemented with 10% FCS. CAPAN-1 cells were cultured in RPMI-1640 plus

10% FCS. Exponentially growing cells on four-well chamber slides (Becton Dickinson) were incubated with 50 ng/ml MMC for 1 h in normal growth media. After washing cells extensively in serum-free media, fresh growth media was added and the cells were returned to incubation for 3 h. Alternatively, cells were treated with 3 mM HU for 90 min. For FANCD2/RAD51 staining, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized with 2% Triton X-100 in PBS for 10 min. For FANCD2/PCNA staining, cells were fixed and permeabilized in 70% methanol/30% acetone for 5 min at room temperature. Blocking buffer (5% FCS, 0.2% fish skin gelatine, 0.2% Tween-20 in PBS) was then added to the cells for 30 min. Cells were then incubated with an affinity-purified rabbit polyclonal anti-FANCD2 antibody at 1:200 dilution and/or affinity-purified mouse monoclonal anti-RAD51 antibody (Abcam ab213) at 1:100 dilution and/or fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-PCNA antibody (Santa Cruz PC10) at 1:200 dilution in blocking buffer for 2 h at room temperature. After washing five times with PBS + 0.2% Tween-20, cells were then incubated in blocking buffer with either TRITC-conjugated anti-mouse antibody or TRITC- or FITC-conjugated anti-rabbit antibody (Jackson Immunoresearch) at 1:300 dilution as appropriate for 1 h at room temperature. After five more washes in PBS + 0.2% Tween-20, chamber walls were removed and slides were mounted in vectashield (Vector Laboratories). All imaging was performed using a confocal laser scanning microscope (Zeiss LSM 510). At least 100 nuclei were scored for each type of count, and a threshold of five foci per cell was set to reduce the likelihood of scoring artefacts.

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REFERENCES

1. Joenje, H., Oostra, A.B., Wijker, M., di Summa, F.M., van Berkel, C.G., Rooimans, M.A., Ebell, W., van Weel, M., Pronk, J.C., Buchwald, M. *et al.* (1997) Evidence for at least eight Fanconi anemia genes. *Am. J. Hum. Genet.*, **61**, 940–944.
2. Levitus, M., Rooimans, M.A., Steltenpool, J., Cool, N.F.C., Oostra, A.B., Mathew, C.G., Hoatlin, M.E., Waisfisz, Q., Arwert, F., de Winter, J.P. *et al.* (2004) Heterogeneity in Fanconi anemia: evidence for two new genetic subtypes. *Blood*, **103**, 2498–2503.
3. Strathdee, C.A., Gavish, H., Shannon, W.R. and Buchwald, M. (1992) Cloning of cDNAs for Fanconi's anaemia by functional complementation. *Nature*, **358**, 763–767 (correction: *Nature*, **358**, 434).
4. Lo Ten Foe, J.R., Rooimans, M.A., Bosnoyan-Collins, L., Alon, N., Wijker, M., Parker, L., Lightfoot, J., Carreau, M., Callen, D.F., Savoia, A. *et al.* (1996) Expression cloning of a cDNA for the major Fanconi anaemia gene, FAA. *Nat. Genet.*, **14**, 320–323.
5. Fanconi Anaemia/Breast Cancer Consortium (1996) Positional cloning of the Fanconi anemia group A gene. *Nat. Genet.*, **14**, 324–328.
6. de Winter, J.P., Waisfisz, Q., Rooimans, M.A., Van Berkel, C.G.M., Bosnoyan-Collins, L., Alon, N., Carreau, M., Bender, O., Demuth, I., Schindler, D. *et al.* (1998) The Fanconi anaemia group G gene *FANCG* is identical with *XRCC9*. *Nat. Genet.*, **20**, 281–283.
7. de Winter, J.P., Leveille, F., van Berkel, C.G., Rooimans, M.A., van Der Weel, L., Steltenpool, J., Demuth, I., Morgan, N.V., Alon, N., Bosnoyan-Collins, L. *et al.* (2000) Isolation of a cDNA representing the Fanconi Anemia complementation group E gene. *Am. J Hum. Genet.*, **67**, 1306–1308.
8. de Winter, J.P., Rooimans, M.A., van Der Weel, L., van Berkel, C.G., Alon, N., Bosnoyan-Collins, L., de Groot, J., Zhi, Y., Waisfisz, Q., Pronk, J.C. *et al.* (2000) The Fanconi anaemia gene *FANCF* encodes a novel protein with homology to ROM. *Nat. Genet.*, **24**, 15–16.
9. Timmers, C., Taniguchi, T., Hejna, J., Reifsteck, C., Lucas, L., Bruun, D., Thayer, M., Cox, B., Olson, S., D'Andrea, A.D. *et al.* (2001) Positional cloning of a novel Fanconi anemia gene, *FANCD2*. *Mol. Cell*, **7**, 241–248.
10. Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., de Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G. *et al.* (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*, **297**, 606–609.
11. Meetei, A.R., de Winter, J.P., Medhurst, A.L., Wallisch, M., Waisfisz, Q., van de Vrugt, H.J., Oostra, A.B., Yan, Z., Ling, C., Bishop, C.E. *et al.* (2003) A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat. Genet.*, **35**, 165–170.
12. Kupfer, G.M., Näf, D., Suliman, A., Pulsipher, M. and D'Andrea, A.D. (1997) The Fanconi anaemia proteins, FAA and FAC interact to form a nuclear complex. *Nat. Genet.*, **17**, 487–490.
13. Garcia-Higuera, I., Kuang, Y., Näf, D., Wasik, J. and D'Andrea, A.D. (1999) Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. *Mol. Cell. Biol.*, **19**, 4866–4873.
14. de Winter, J.P., van Der Weel, L., de Groot, J., Stone, S., Waisfisz, Q., Arwert, F., Scheper, R.J., Kruyt, F.A., Hoatlin, M.E. and Joenje, H. (2000) The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. *Hum. Mol. Genet.*, **9**, 2665–2674.
15. Medhurst, A.L., Huber, P.A., Waisfisz, Q., de Winter, J.P. and Mathew, C.G. (2001) Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway. *Hum. Mol. Genet.*, **10**, 423–429.
16. Pace, P., Johnson, M., Tan, W.M., Mosedale, G., Sng, C., Hoatlin, M.E., de Winter, J.P., Gergely, F. and Patel, K.J. (2002) FANCE: the link between Fanconi anaemia complex assembly and activity. *EMBO J.*, **21**, 3414–3423.
17. Meetei, A.R., Sechi, S., Wallisch, M., Yang, D., Young, M.K., Joenje, H., Hoatlin, M.E. and Wang, W. (2003) A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. *Mol. Cell. Biol.*, **23**, 3417–3426.
18. Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A.D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell*, **7**, 249–262.
19. D'Andrea, A.D. and Grompe, M. (2003) The Fanconi anemia/BRCA pathway. *Nat. Rev. Can.*, **3**, 23–34.
20. Nakanishi, K., Taniguchi, T., Ranganathan, V., New, H.V., Mathew, C.G., Kastan, M.B., Weaver, D.T. and D'Andrea, A.D. (2002) Interaction of FANCD2 and NBS1 in the DNA crosslink response. *Nat. Cell Biol.*, **4**, 913–920.
21. Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P.R., Gregory, R.C., Kim, S.T., Lane, W.S., Kastan, M.B. and D'Andrea, A.D. (2002) Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. *Cell*, **109**, 459–472.
22. Houghtaling, S., Timmers, C., Noll, M., Finegold, M.J., Jones, S.N., Meyn, M.S. and Grompe, M. (2003) Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev.*, **17**, 2021–2035.
23. Rothfuss, A. and Grompe, M. (2004) Repair kinetics of genomic interstrand DNA cross-links: evidence for DNA double-strand break-dependent activation of the Fanconi anemia/BRCA pathway. *Mol. Cell Biol.*, **24**, 123–134.
24. West, S.C. (2003) Molecular views of recombination proteins and their control. *Nat. Rev. Mol. Cell Biol.*, **4**, 435–445.

25. Hussain, S., Witt, E., Huber, P.A.J., Ashworth, A. and Mathew, C.G. (2003) Direct interaction of the Fanconi anaemia protein FANCG with BRCA2/FANCD1. *Hum. Mol. Genet.*, **12**, 2503–2510.
26. Gordon, S.M. and Buchwald, M. (2003) Fanconi anemia protein complex: mapping protein interactions in the yeast 2- and 3-hybrid systems. *Blood*, **102**, 136–141.
27. Wilson, J.B., Johnson, M.A., Stuckert, A.P., Trueman, K.L., May, S., Bryant, P.E., Meyn, R.E., D'Andrea, A.D. and Jones, N.J. (2001) The Chinese hamster *FANCG/XRCC9* mutant NM3 fails to express the monoubiquitinated form of the FANCD2 protein, is hypersensitive to a range of DNA damaging agents and exhibits a normal level of spontaneous sister chromatid exchange. *Carcinogenesis*, **22**, 1939–1946.
28. Goggins, M., Schutte, M., Lu, J., Moskaluk, C.A., Weinstein, C.L., Petersen, G.M., Yeo, C.J., Jackson, C.E., Lynch, H.T., Hruban, R.H. *et al.* (1996) Germline *BRCA2* gene mutations in patients with apparently sporadic pancreatic carcinomas. *Can. Res.*, **56**, 5360–5364.
29. Davies, A.A., Masson, J.Y., McIlwraith, M.J., Stasiak, A.Z., Stasiak, A., Venkitaraman, A.R. and West, S.C. (2001) Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol. Cell*, **7**, 273–282.
30. Pellegrini, L., Yu, D.S., Lo, T., Anand, S., Lee, M., Blundell, T.L. and Venkitaraman, A.R. (2002) Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nat. Cell Biol.*, **420**, 287–293.
31. Yu, D.S., Sonoda, E., Takeda, S., Huang, C.L.H., Pellegrini, L., Blundell, T.L. and Venkitaraman, A.R. (2003) Dynamic control of RAD51 recombinase by self-association and interaction with BRCA2. *Mol. Cell*, **12**, 1029–1041.
32. Wong, A.K., Pero, R., Ormonde, P.A., Tavtigian, S.V. and Bartel, P.L. (1997) RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene *BRCA2. J. Biol. Chem.*, **272**, 31941–31944.
33. Chen, P.L., Chen, C.F., Chen, Y., Xiao, J., Sharp, Z.D. and Lee, W.H. (1998) The BRC repeats in BRCA2 are critical for Rad51 binding and resistance to methyl methanesulfonate treatment. *Proc. Natl Acad. Sci. USA*, **95**, 5287–5292.
34. Yuan, S.S., Lee, S.Y., Chen, G., Song, M., Tomlinson, G.E. and Lee, E.Y. (1999) BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex *in vivo. Can. Res.*, **59**, 3547–3551.
35. Taniguchi, T., Garcia-Higuera, I., Andreassen, P.R., Gregory, R.C., Grompe, M. and D'Andrea, A.D. (2002) S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood*, **100**, 2414–2420.
36. Michel, B., Ehrlich, S.D. and Uzest, M. (1997) DNA double-strand breaks caused by replication arrest. *EMBO J.*, **16**, 430–438.
37. Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D.M. (1997) Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell*, **90**, 425–435.
38. Hirsch, B., Shimamura, A., Moreau, L., Baldinger, S., Hag-alshiekh, M., Bostrom, B., Sencer, S. and D'Andrea, A.D. (2004) Biallelic BRCA2/FANCD1 mutations: association with spontaneous chromosomal instability and solid tumors of childhood. *Blood*, **103**, 2554–2559.
39. Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R. and Gu, W. (2003) Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science*, **302**, 1972–1975.
40. Vandenberg, C.J., Gergely, F., Ong, C.Y., Pace, P., Mallery, D.L., Hiom, K. and Patel, K.J. (2003) BRCA1-independent ubiquitination of FANCD2. *Mol. Cell*, **12**, 247–254.
41. Raderschall, E., Golub, E.I. and Haaf, T. (1999) Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proc. Natl Acad. Sci. USA*, **96**, 1921–1926.
42. Dronkert, M.L. and Kanaar, R. (2001) Repair of DNA interstrand cross-links. *Mutat. Res.*, **486**, 217–247.
43. McHugh, P.J., Spanswick, V.J. and Hartley, J.A. (2001) Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol.*, **2**, 483–490.
44. Vaughn, J.P., Cirisano, F., Huper, G., Bergchuk, A., Futreal, P.A., Marks, J.R. and Iglehart, J.D. (1996) Cell cycle control of BRCA2. *Can. Res.*, **56**, 4590–4594.
45. Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G.E., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M. *et al.* (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol. Cell*, **2**, 317–328.
46. Lomonosov, M., Anand, S., Sangrithi, M., Davies, R. and Venkitaraman, A.R. (2003) Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. *Genes Dev.*, **17**, 3017–3022.
47. Bjelland, S. and Seeberg, E. (2003) Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutat. Res.*, **531**, 37–80.
48. Cox, M.M., Goodman, M.F., Kreuzer, K.N., Sherratt, D.J., Sandler, S.J. and Marians, K.J. (2000) The importance of repairing stalled replication forks. *Nature*, **404**, 37–41.
49. Yamamoto, K., Ishiai, M., Matsushita, N., Arakawa, H., Lamerdin, J.E., Buerstedde, J.M., Tanimoto, M., Harada, M., Thompson, L.H. and Takata, M. (2003) Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. *Mol. Cell. Biol.*, **23**, 5421–5430.
50. Shin, D.S., Pellegrini, L., Daniels, D.S., Yelent, B., Craig, L., Yu, D.S., Shivji, M.K., Hitmi, C., Arvai, A.S., Volkmann, N. *et al.* (2003) Full-length archaeal Rad51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2. *EMBO J.*, **22**, 4566–4576.
51. Folias, A., Matkovic, M., Bruun, D., Reid, S., Hejna, J., Grompe, M., D'Andrea, A.D. and Moses, R. (2002) BRCA1 interacts directly with the Fanconi anemia protein FANCA. *Hum. Mol. Genet.*, **11**, 2591–2597.
52. Marston, N.J., Richards, W.J., Hughes, D., Bertwistle, D., Marshall, C.J. and Ashworth, A. (1999) Interaction between the product of the breast cancer susceptibility gene BRCA2 and DSS1, a protein functionally conserved from yeast to mammals. *Mol. Cell. Biol.*, **19**, 4633–4642.
53. Huber, P.A., Medhurst, A.L., Youssoufian, H. and Mathew, C.G. (2000) Investigation of Fanconi anemia protein interactions by yeast two-hybrid analysis. *Biochem Biophys. Res. Comm.*, **268**, 73–77.
54. Johnson, M.A. and Jones, N.J. (1999) The isolation and genetic analysis of V79-derived etoposide sensitive Chinese hamster cell mutants; two new complementation groups of etoposide sensitive mutants. *Mutat. Res./ DNA Rep.*, **435**, 227–238.
55. Warriar, N., Page, N. and Govindan, M.V. (1996) Expression of human glucocorticoid receptor gene and interaction of nuclear proteins with the transcriptional control element. *J. Biol. Chem.*, **271**, 18662–18671.
56. Lamerdin, J.E., Yamada, N.A., George, J.W., Souza, B., Christian, A.T., Jones, N.J. and Thompson, L.H. (2004) Characterization of the hamster *FancG/Xrcc9* gene and mutations in CHO UV40 and NM3. *Mutagenesis*, **19**, 237–244.
57. Qiao, F., Moss, A. and Kupfer, G.M. (2001) Fanconi anemia proteins localize to chromatin and the nuclear matrix in a DNA damage- and cell cycle-regulated manner. *J. Biol. Chem.*, **276**, 23391–23396.
58. Schild, D., Lio, Y., Collins, D.W., Tsomondo, T. and Chen, D.J. (2000) Evidence for simultaneous protein interactions between human Rad51 paralogs. *J. Biol. Chem.*, **275**, 16443–16449.
59. Masson, J.Y., Tarsounas, M.C., Stasiak, A.Z., Stasiak, A., Shah, R., McIlwraith, M.J., Benson, F.E. and West, S.C. (2001) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev.*, **15**, 3296–3307.
60. Liu, N., Lamerdin, J.E., Tebbs, R.S., Schild, D., Tucker, J.D., Shen, M.R., Brookman, K.W., Siciliano, M.J., Walter, C.A., Fan, W. *et al.* (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol. Cell*, **1**, 783–793.