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The Fanconi Anemia Protein, FANCG, Binds to the ERCC1-XPF Endonuclease via its Tetratricopeptide Repeats and the Central Domain of ERCC1†

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Abstract

There is evidence that Fanconi anemia (FA) proteins play an important role in the repair of DNA interstrand cross-links (ICLs), but the precise mechanism by which this occurs is not clear. One of the critical steps in the ICL repair process involves unhooking of the cross-link from DNA by incisions on one strand on either side of the ICL and its subsequent removal. The ERCC1-XPF endonuclease is involved in this unhooking step and in the removal of the cross-link. We have previously shown that several of the FA proteins are needed for production of incisions created by ERCC1-XPF at sites of ICLs. In order to more clearly establish a link between FA proteins and the incision step(s) mediated by ERCC1-XPF, yeast two-hybrid analysis was undertaken to determine whether FANCA, FANCC, FANCF, and FANCG directly interact with ERCC1 and XPF and, if so, to determine the sites of interaction. One of these FA proteins, FANCG, was found to have strong affinity for ERCC1 and moderate affinity for XPF. FANCG has been shown to contain seven tetratricopeptide repeat (TPR) motifs, which are motifs that mediate protein-protein interactions. Mapping the sites of interaction of FANCG with ERCC1, using site-directed mutagenesis, demonstrated that TPRs 1, 3, 5, and 6 are needed for binding of FANCG to ERCC1. ERCC1, in turn, was shown to interact with FANCG via its central domain, which is different from the region of ERCC1 that binds to XPF. The present demonstrated binding between FANCG and the ERCC1-XPF endonuclease, combined with our previous studies which show that FANCG is involved in the incision step mediated by ERCC1-XPF, establishes a link between an FA protein and the critical unhooking step of the ICL repair process.

Fanconi anemia (FA)1 is a genetic disorder characterized by genomic instability, bone marrow failure, diverse congenital abnormalities, an increased incidence of cancer and a marked cellular hypersensitivity to DNA interstrand cross-linking agents (1–5). This hypersensitivity correlates with a defect in ability to repair cross-links produced by these agents (5–12). Thirteen FA complementation groups have been identified (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, and -N) (5,13–15). Proteins encoded by eight of the FA genes form a nuclear core complex (FANCA, FANCB, FANCC, FANCE, FANCF,

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SUPPORTING INFORMATION AVAILABLE

A table showing the primers used to amplify cDNA for the domains of ERCC1, a table showing the primers used to amplify the cDNAs for TPR motifs mutated in FANCG, and a table showing the wild-type and mutated sequences of the TPR motifs in FANCG. This material is available free of charge via the Internet at http://pubs.acs.org.

[1]Abbreviations: FA, Fanconi anemia; ICL, interstrand cross-link; NER, nucleotide excision repair; HR, homologous recombination; αIISp, nonerythroid α spectrin; TPR, tetratricopeptide repeat; BD, DNA-binding domain; AD, activation domain; HnH2, helix-hairpin-helix domain; DSB, double strand break; MMC, mitomycin C; SH3, src-homology 3; WD, wild type;

FANCG, FANCL, and FANCM) (4,16,17). There is evidence that the core as well as the other FANC proteins are involved in DNA interstrand cross-link (ICL) repair, but the precise mechanism by which this occurs is not clear (5,10,12,18–22). ICLs represent important blocks to DNA replication and are of particular significance in FA cells where there is a defect in replication-dependent ICL repair (4,12,22–24). Repair of DNA interstrand cross-links at stalled replication forks has been proposed to involve a number of steps which include creation of a double strand break (DSB) at the site of the stalled replication fork, unhooking of the cross-link, translesion synthesis, excision of the monoaduct by nucleotide excision repair (NER) and homologous recombination (HR) (10,12,23–25). FA proteins have been implicated in one or more of these steps (5,10,12,18–22,26).

The initial unhooking of the cross-link is a critical step in the ICL repair process. ERCC1-XPF is a heterodimeric complex which is a structure specific endonuclease that has been shown to create incisions at the site of a DNA ICL (9,11,27). It has been proposed that it plays an important role in the unhooking step in the cross-link repair process (28–32). Potentially it may also be involved in subsequent steps in which the cross-link is excised from the DNA and in the completion of homologous recombination (31,32). Whether there are other proteins that may interact with ERCC1-XPF and are involved in its role in the repair process is not known. We have shown that the structural protein nonerythroid α spectrin (αIISp) is involved in the repair of DNA ICLs (19,20,26,33). αIISp and three FANC proteins which are components of the FA core complex, FANCA, FANCC and FANCG, are important for production of incisions created by ERCC1-XPF at the site of a DNA ICL (11,19,26). Antibodies against αIISp and these FANC proteins inhibit production of these incisions (11). There is a deficiency in these incisions in FA-A, FA-C, and FA-G cells after they are exposed to a DNA interstrand cross-linking agent, which correlates with reduced levels of αIISp in these cells, and this deficiency is corrected when levels of the corresponding FANC protein have been restored by transfection of these cells with the appropriate FA cDNA (11). αIISp is also important in formation of ERCC1-XPF nuclear foci after DNA ICL damage: knockdown of αIISp in normal cells by siRNA leads to loss of formation of ERCC1-XPF foci and in FA-A cells, where there are reduced levels of αIISp, there is decreased formation of these foci (20,33). It is thus possible that the interaction of these FANC proteins with αIISp and ERCC1-XPF is necessary for the incisions produced by ERCC1-XPF in the cross-link repair process.

The present studies were undertaken in order to examine whether there is direct physical interaction between αIISp, several members of the FA core complex and either of the two proteins in the ERCC1-XPF heteroduplex, and, if so, to determine the sites of interaction. Importantly, if a direct interaction could be demonstrated between any of the FA proteins examined and either ERCC1 and/or XPF, this would more clearly establish a link between FA proteins and the incision step(s) in the ICL repair process. The present studies examined the ability of αIISp, FANCA, FANCC, FANCF and FANCG to bind directly to ERCC1 and XPF using yeast two-hybrid analysis. The results demonstrated that one of the FANC proteins, FANCG, had strong affinity for ERCC1 and moderate affinity for XPF. FANCG contains seven tetratricopeptide repeat (TPR) motifs, which have been shown to mediate protein-protein interactions and are thought to be important in assembly of multiprotein complexes (34–39). Mapping the sites of interaction of FANCG with ERCC1 demonstrated that TPR motifs 1, 3, 5, and 6 are needed for binding of FANCG to ERCC1. In contrast, FANCA, FANCC and FANCF, which did not contain these motifs, did not bind directly to ERCC1. αIISp also did not bind directly to ERCC1. ERCC1, in turn, was shown to interact with FANCG via its central domain. This is different from the region of ERCC1 that binds to XPF, which is the C-terminal HhH_2 domain (40–42). These studies thus demonstrate that there is binding between a FA protein, FANCG, and the ERCC1-XPF endonuclease. These

results, combined with our previous studies (11,19), provide evidence that there is a link between at least one of the proteins in the FA core

EXPERIMENTAL PROCEDURES

Bacterial and Yeast strains

Escherichia coli strain DH5α (Invitrogen) was used in the construction and propagation of all yeast two-hybrid plasmid constructs. *E. coli* was grown in Luria Broth at 37 °C. *Saccharomyces cerevisiae* strain EGY48 (MATα trp1 his3 ura3 leu2:6 LexAop-Leu2) (Origene Technologies) was grown in liquid YPD (Q-Biogene) or YPD agar plates at 30 °C.

Yeast Two-Hybrid Vectors and Expression Constructs

All yeast expression constructs were created in the DupLexA yeast two-hybrid system (OriGene Technologies). Constructs containing the LexA DNA-binding domain (BD) were subcloned into the pEG202 vector and constructs containing the B42 transcriptional activation domain (AD) were subcloned into the pJG4-5 vector as previously described (43).

Four overlapping regions of αIISp, R1(residues 1-887), R2 (residues 864-1239), R3 (residues 1089-1766) and R4 (residues 1661-2477), which had been constructed and cloned into pJG4-5 were utilized in these studies (43). FANCC, FANCF, FANCG cDNAs were created as previously described (43). Constructs of a N-terminal and a C-terminal fragment of FANCG, residues 1-293 ($FANCG_{1-293}$) and residues 261-622 ($FANCG_{261-622}$), were created as previously described (43). The FANCG fragment, residues 261-355 ($FANCG_{261-355}$), was created by introducing a stop codon at residue 355 using $FANCG_{261-622}$ as a template. FANCA cDNA was amplified by PCR from pREP4 (a gift from Dr. Hans Joenje, Vrije Universiteit Medical Center, Amsterdam, The Netherlands) using Accuprime Pfx DNA polymerse supermix (Invitrogen) and *Eco*RI and *Not*I restriction sites were added to the 5′ and 3′ ends of the cDNA. The forward primer used was 5′-GCGCGAATTCATGTCCGACTCGTGGGTCCC-3′ and the reverse primer was 5′-GCGCGCGGCCGCTCAGAAGAGATGAGGCTCCT-3′. Then FANCA cDNA was then inserted into the pEG202 vector. The sequences for all of the these constructs were verified by sequencing (Molecular Resource Facility, New Jersey Medical School, UMDNJ, Newark, NJ).

Full-length human XPF cDNA was excised using the restriction enzymes *Nco*I and *Not*I from a cER4-40 cosmid (a gift from Dr. Michael P. Thelen, Lawrence Livermore National Laboratory) and inserted into the corresponding sites of pEG202. To make XPF in-frame with LexA, this construct was first digested with *BamH*I and then treated with the Klenow fragment of DNA polymerase I (New England Biolab) and circularized by ligation. The final sequence of the in-frame pEG202-XPF construct was confirmed by sequencing. The pJG4-5-XPF construct was created by excising the XPF cDNA from pEG202 and subcloning it in to the pJG4-5 vector.

Four regions of ERCC1 were constructed by PCR amplification of sequences from human full-length ERCC1 cDNA in the pOTB7 vector (American Type Culture Collection) using Accuprime Pfx DNA polymerase supermix. Primers (Operon) used to amplify each of these four regions are listed in Table S1. The regions of ERCC1 amplified were: the N-terminal domain (residues 1-99); the central domain plus the C-terminal tendom helix-hairpin-helix (HhH_2) domain (residues 88-297); the ERCC1 central domain minus the N-terminal residues 120-220; and the ERCC1 C-terminal HhH_2 domain (residues 214-297). The constructs were then subcloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). After sequence verification, the inserts as well as full-length ERCC1 were subcloned into the yeast two-hybrid vectors pEG202 or pJG4-5.

Site-Directed Mutagenesis

In order to evaluate the importance of each of the seven TPR motifs in FANCG to its binding to ERCC1, specific point mutations were separately created in these motifs in full-length FANCG. For these studies, FANCG was subcloned into the pENTR3C vector (Invitrogen). Seven FANCG TPR mutants were created with a PCR mutagenesis strategy based on the protocol used in ExSite PCR-based site-directed mutagenesis Kit (Stratagene): TPR1 (G216Q), TPR2 (G253Q), TPR3 (A351Q), TPR5 (G460Q), TPR6 (G521Q), TPR7a (A567Q) and TPR7b (R563E). The primers used for this are listed in Table S2. After sequence verification, each FANCG mutant cDNA was subcloned into the yeast two-hybrid pJG4-5 vector as previously described (43). FANCG containing a mutation in TPR4 was not examined in these studies due to difficulities in cloning the cDNA of FANCG with a mutation in TPR4 into the yeast two-hybrid vector. However, this TPR may not be as important for FANCG function as the other TRP motifs since FANCG containing a TPR4 mutation has been shown to fully complement the cross-link sensitivity of FANCG deficient cells (38).

Yeast Two-Hybrid Analysis

The DupLEX-A yeast two-hybrid system (OriGene Technologies) was used for yeast two-hybrid analysis. Yeast Strain EGY48 was transformed with the AD vector (pEG202), BD vector (pJG4-5), and reporter vectors using a PEG/ssDNA/lithium acetate procedure as previously described (43). Six colonies from each of these yeast transformations were randomly selected and inoculated in rows onto YNB (X-gal-His-Trp-ura) plates and grown for 2–5 days to screen for activation of the *LacZ* reporter gene. Activation of *LacZ* results in production of β-galactosidase, which cleaves X-gal and produces a blue color. Positive interactions between the AD and BD fusion proteins were documented by the presence of blue colonies on the plates. Each experiment was repeated four to six times.

Coimmunoprecipitation of Proteins

Normal human lymphoblastoid cells (GM 3299) (Coriell Institute for Medical Research) were grown in suspension culture in RMPI 1640 medium. Nuclear extracts of the cells were prepared according to the method of Yamashita et al. (44). Coimmunoprecipitation of FANCG and ERCC1 was carried out as previously described (26,45). Briefly, anti-ERCC1 and rabbit IgG, which was used as a control, were bound to protein-G-coated agarose beads (Sigma Aldrich Corp.) and the beads washed in binding buffer [25 mM Tris-Cl, pH 7.3, 150 mM NaCl, 1% Triton X-100 plus protease inhibitor mixture (Roche Molecular Biochemicals)], blocked with 1% BSA (Sigma Aldrich Corp.) and washed in binding buffer as described previously (26,45). The agarose bead-antibody suspension was incubated with the protein extract as described (26,45). For reciprocal IPS, anti-FANCG and pre-immune serum, which served as a control, were bound to protein A-coated agarose beads (Sigma) and the samples processed as described above. The IPs were subjected to SDS-PAGE and the proteins analyzed by Western blot analysis as previously described (19,26,45). For the primary antibodies, an affinity purified antibody against FANCG was generated by Bethyl Laboratories (19) and anti-ERCC1 (FL297, Santa Cruz Biotechnology Inc.) was used. The specificity of this particular antibody for ERCC1 has been validated by Bhagwat et al., (46).

RESULTS

αIISp Does Not Directly Interact with ERCC1 or XPF

Whether αIISp interacted directly with ERCC1 or XPF was examined by yeast two-hybrid analysis. Because of its large size, four overlapping regions of αIISp were constructed. No direct interaction was detected between any of the four regions of αIISp (R1–R4) and either

ERCC1 or XPF (Figure 1). We have previously shown that each of these four regions of αIISp is expressed in the yeast cells, indicating that this lack of interaction was not due to lack of expression of the hybrid proteins in the experimental system (43). We have previously shown that region 2 of αIISp binds to FANCG (43) and this interaction and the known interaction of ERCC1 and XPF (40–42) was used as a positive control. Direct binding between these two groups of proteins was evidenced by the presence of blue colonies, indicating that the yeast two-hybrid system was functional (Figure 1).

FANCG Interacts Directly with ERCC1 and XPF

Yeast two-hybrid analysis was carried out to determine whether the FA core complex proteins, FANCA, FANCC, FANCG and FANCF, interacted directly with either XPF or ERCC1. The interaction of FANCA with FANCG and XPF with ERCC1 was used as a positive control since these proteins have been shown to directly interact with each other (40–42,47). Direct binding between these two groups of proteins was evidenced by the presence of blue colonies (Figure 2A). Very low levels of autoactivation were observed for FANCA, as seen by the presence of pale blue colonies. Interaction between FANCA and either XPF or ERCC1 was not detected, as seen by the presence of white colonies, which indicated that the reporter gene had not been activated and that there was no positive interaction between the fusion proteins (Figure 2A). Direct interaction was also not detected between FANCC and either XPF or ERCC1 (Figure 2B). A very weak interaction was detected between FANCF and ERCC1, but this interaction was not consistently observed, and no interaction was observed between FANCF and XPF (Figure 2B). Analysis of the binding reactions showed, however, that FANCG displayed very strong binding to ERCC1, as evidenced by activation of the reporter gene and the development of an intense blue color in the colonies (Figure 2B). FANCG also bound to XPF, but to a lesser degree (Figure 2B). All of these results are from a single experiment in which six randomly chosen colonies were examined. Each of these binding experiments, as well as those in the subsequent studies, was repeated four to six times with similar results. The results of these protein interactions are summarized in Figure 2C. Immunoblot analysis of protein extracts of the transformed yeast cells showed that FANCA, FANCC and FANCF were expressed in the yeast cells indicating that lack of interaction of these proteins with XPF or ERCC1 was not due to lack of expression of these proteins in these cells (43).

The interaction of FANCG with ERCC1 was examined further in order to determine the region in FANCG that binds to ERCC1. For this, three overlapping fragments of FANCG were constructed and expressed as fusion proteins in yeast two-hybrid experiments: $FANCG_{1-291}$, $FANCG_{261-355}$ and $FANCG_{261-622}$. The $FANCG_{261-622}$ fragment showed some direct binding to ERCC1, while both the $FANCG_{1-291}$ and $FANCG_{261-355}$ fragments failed to show positive interaction with ERCC1 (Figure 2A,B). Autoactivation was not observed with these FANCG fragments. The $FANCG_{261-622}$ fragment contained over half of the FANCG protein from the carboxyl terminal, indicating that this is an important region of binding to ERCC1 (Figure 3B). However, compared to full-length FANCG, $FANCG_{261-622}$ bound to ERCC1 with much less intensity (Figure 3A,B). This suggested that residues outside of this fragment may also be needed for binding to ERCC1 and/or that the overall structure of FANCG is important in this interaction.

The TPR Motifs in FANCG are Essential for its Binding to ERCC1

Since it has been shown that FANCG contains seven TPR motifs, and a number of these have been demonstrated to be important in FANCG's interactions with other proteins (38,39), studies were carried out to determine whether one or more of these TPR motifs could be important in mediating the binding of FANCG to ERCC1 (Figure 4A). Single amino acid substitutions were created at selected residues for each motif by site-directed

mutagenesis. In each of these seven TPR motifs, the 8th amino acid is highly conserved and is usually an alanine or a glycine (38). In the TPR motifs 1, 2, 3, 5 and 6, the 8th amino acid was replaced by the hydrophilic residue glutamine (Table S2 and S3). Since in TPR7, the 8th residue is neither alanine nor glycine, the highly conserved alanine at the 20th position was chosen and replaced with glutamine to create TPR7a (Table S2 and S3). In addition, another mutant TPR7 was created, TPR7b, in which the arginine at position 16, which is also conserved between species (38), was substituted with a glutamic acid (Table S2 and S3). The cDNAs of these FANCG TPR mutants were subcloned into the pJG4-5 vector and western blot analysis showed that all of these FANCG TPR mutants were expressed in yeast cells (Figure 4B).

The interaction of FANCG with ERCC1 was severely disrupted by mutations in TPR 1, 3, 5 and 6 in FANCG, as seen by the absence of reporter gene activation (Figure 4C). However, the mutations in TPR7a and TPR7b had no significant effect on the interaction of FANCG with ERCC1. Both of these FANCG mutants maintained their ability to interact with ERCC1 as demonstrated by a level of reporter gene activation which was as strong as that of wild-type FANCG (Figure 4C). The mutation in TPR2 mutant resulted in a moderate decrease in interaction of FANCG with ERCC1 (Figure 4C). Each of these binding experiments was repeated four to six times with similar results and the results of these protein interactions are summarized in Figure 4D. This indicates that TPR1, 3, 5 and 6 are critical for the binding of FANCG to ERCC1. TPR2 may have some importance as well. However, TPR7 does not appear to be needed for the interaction. Figure 4A shows the localization of each of the TPR motifs in the three FANCG regions examined for binding to ERCC1 (Figure 4C and 4D). It can be seen that the $FANCG_{261\text{-}622}$ fragment, which showed some binding to ERCC1, contains TPR motifs 3–7. Based on the present findings, this binding could be contributed by TPR motifs 3, 5 and 6. The presence of TPR1, along with TPR2, is not sufficient, however, for binding of the $FANCG_{1\text{-}191}$ fragment to ERCC1 (Figure 4C,D). However, TPR1 and possibly 2 are probably contributing to the binding of FANCG to ERCC1 since full length FANCG had much greater binding to ERCC1 than did any of the FANCG fragments. These studies demonstrate the importance of the TPR motifs 1, 3, 5 and 6 in mediating FANCG's interaction with ERCC1. The possibility also exists that the overall TPR structure, rather than a subset of these motifs, is required for efficient ERCC1 binding.

The Central Domain of ERCC1 Binds to FANCG

Since strong binding was observed between FANCG and ERCC1, the domains in ERCC1 that interacted with FANCG were examined. ERCC1 contains three domains (Figure 5A): the N-terminal domain; the central domain, which contains the sites of interaction with XPA; and the C-terminal HhH_2 domain, which is the domain that binds to XPF (40–42,48–51). For these studies, ERCC1 was divided into four overlapping regions: the first region contained the N-terminal domain (residues 1-99); the second region contained the central and C-terminal HhH_2 domains (residues 88-297); the third region contained the central domain minus its N-terminal residues (residues 120-220); and the fourth region contained the C-terminal (HhH_2) domain (residues 214-297) (Figure 5A). The cDNAs corresponding to these regions were subcloned into the yeast two-hybrid vectors.

The regions of ERCC1 which contained the central plus the HhH_2 domains, $ERCC1_{88\text{-}297}$, and the central domain minus its N-terminus, $ERCC1_{120\text{-}220}$, showed strong binding to FANCG (Figure 5B). The N-terminal domain of ERCC1, $ERCC1_{1\text{-}96}$, and the HhH_2 domain, $ERCC1_{214\text{-}297}$, did not bind to FANCG (Figure 5B). The results of 4–5 experiments are summarized in Figure 5C. These results indicate that ERCC1 interacts with FANCG through its central domain, which is different than the domain that binds to XPF.

FANCG Coimmunoprecipitates with ERCC1 in Human Cells

The interaction of FANCG and ERCC1 was further assessed by carrying out coimmunoprecipitation experiments. Immunoprecipiation of normal human cell extracts with anti-ERCC1 and immunoblotting with either anti-ERCC1 or anti-FANCG showed that FANCG coimmunoprecipitated with ERCC1 (Figure 6A). Similarly, when coimmunoprecipiation was carried out using anti-FANCG, immunoblotting with anti-FANCG or anti-ERCC1 showed that ERCC1 coimmunoprecipitated with FANCG (Figure 6B). These studies indicate that FANCG and ERCC1 interact with each other in human cells.

DISCUSSION

Though there is evidence that FA proteins are involved in repair of DNA interstrand cross-links (ICLs), the exact role they play is not yet clear (5,10–12,18–21). Our laboratory has shown that several of the FA proteins are important in the incision step(s) of the repair process in which ERCC1-XPF incises DNA at the site of a ICL: FANCA colocalizes with XPF and αIISp at cross-linked induced nuclear foci (20); antibodies against FANCA, FANCC and FANCG inhibit incisions produced by ERCC1-XPF at the site of a psoralen interstrand cross-link (11); in FA-A, FA-C, and FA-G cells there is a deficiency in production of incisions by ERCC1-XPF at the site of a DNA interstrand cross-link (9,11); and in corrected FA-A, FA-C, and FA-G cells, which express the FANCA, FANCC, and FANCG proteins, respectively, levels of incisions produced by ERCC1-XPF at sites of cross-links are restored to normal (9,11). ERCC1-XPF, which is known to be involved in nucleotide excision repair (NER) (40,41,52–54), has also been shown to play a role in repair of DNA interstrand cross-links and is believed to be important in repair of these cross-links at stalled replication forks (9,11,27–31). In FA cells, there is a defect in DNA repair that occurs at DNA replication forks stalled at sites of interstrand cross-links (4,12,21–23). Of the proposed steps in the repair of DNA interstrand cross-links at stalled replication forks, one of the most critical involves the incisions that are produced at the site of the cross-link and result in its unhooking. In order for the cross-link to become unhooked from one strand of DNA, incisions must be made on the 3′ and 5′ sides of the lesion. The structure-specific nuclease, Mus81-Em1, is thought to facilitate formation of the 3′ incision producing a double strand break (DSB) (10,12,18,55,56). ERCC1-XPF is then proposed to incise the DNA on the 5′ side of the cross-link leading to its unhooking (12,18,29). Though our studies indicate that several of the FA proteins are important in this step, the precise role that FA proteins play in this process is not known. In order to elucidate this, the present study examined whether there is a direct association between several of the FANC core complex proteins, in particular, FANCA, FANCC, FANCF or FANCG, with proteins involved in the unhooking step of the repair process, specifically with either ERCC1 or XPF.

The results showed, using yeast two-hybrid analysis, that there is a strong interaction between one of these FA proteins, FANCG, and ERCC1. Full length FANCG was needed for this interaction. Overlapping N- and C-terminal fragments of FANCG showed no or only weak binding to ERCC1 compared to the binding of full length FANCG. This indicates that sites in both fragments are critical for this interaction or that the overall structure of FANCG is important. There was also interaction between FANCG and XPF, however, it was not as strong. That the interaction between FANCG and ERCC1 occurs *in vivo* was confirmed by studies which showed that FANCG and ERCC1 coimmunoprecipitated from human cell extracts.

FANCG contains seven TPR motifs, which are 34-amino acid helix-turn-helix motifs that are involved in mediating protein-protein interactions (34–39). These TRP motifs have been shown to be important in the interaction of FANCG with several different proteins (39).

TPR5 and TPR6 are required for the interaction of FANCG with XRCC3, FANCA, and the N-terminus of BRCA2; TPR1 and TPR2 are involved in the interactions FANCG with the C-terminus of BRCA2; and TPR1, TPR2, TPR5 and TPR6 are important in binding of FANCG to FANCF (39).

Investigation in the present study as to whether any of the TPR motifs were responsible for the binding between FANCG and ERCC1 showed that mutations in consensus position 8 of TPR1, TPR3, TPR5 and TPR6 in FANCG resulted in loss of binding of FANCG to ERCC1. This demonstrated that these TPR motifs are critical for this interaction. Of interest, TPR 3, 5 and 6 are in the C-terminal fragment of FANCG which showed some binding to ERCC1. TPR 1 is in the N-terminal fragment of FANCG which did not bind to ERCC1. However, since TPR1 is needed for interaction of FANCG with ERCC1, this indicates that the combined action of TPR1 with TPR 3, 5 and 6 is needed for binding of FANCG to ERCC1. Missense mutations at position 8 in TPR1, TPR5 and TPR6 in FANCG have been shown to cause failure of these mutant proteins to correct the cellular sensitivity of FA-A cells to a DNA interstrand cross-linking agent, mitomycin C (MMC) (38). These findings, combined with our present studies, which show that TPR1, 3, 5 and 6 are involved in the binding of FANCG to ERCC1, and with our previous finding that FANCG is needed for the incisions produced by ERCC1-XPF at the site of a cross-link (11), indicate that binding of FANCG to ERCC1 is critical for the repair response after exposure to a cross-linking agent.

Studies on which of the three domains in ERCC1 bind to FANCG demonstrated that the central domain of ERCC1, minus residues (96-119), binds to FANCG. It has been shown that residues 96-119 are necessary for interaction of ERCC1 with XPA (47,57,58). Recent studies indicate that residue Asn110 within this region is required for NER and that residue Tyr145 is also important for interaction of ERCC1 with XPA and for NER (51). However, though these sites of interaction of ERCC1 with XPA have been shown to be important for NER, they do not appear to be involved in ICL repair (51). Since the present results combined with our previous studies show that residues 120-220 but not residues 96-119 in ERCC1 are needed for binding of FANCG to ERCC1, and that FANCG is necessary for production of incisions by ERCC1-XPF at sites of DNA interstrand cross-links (11), this suggests that the sites on ERCC1 that are involved in NER are different from those involved in ICL repair. The present studies thus indicate that ERCC1, in addition to sites for binding of XPA in its central domain and of XPF in its C-terminal HhH2 domain, contains a region in its central domain for binding of FANCG (Figure 5A).

Recent studies indicate that ERCC1-XPF is involved in the unhooking of the cross-link at the site of a stalled replication fork and that this process is not required for FA pathway activation but is required for the localization of monoubiquitinated FANCD2 on chromatin (30). Since we have shown that association of ERCC1 with XPF is necessary for the ability of XPF to incise DNA at sites of cross-links (59) and that FANCG is also needed for these incisions (11), it is possible that the binding of FANCG to ERCC1 is of key importance for this unhooking step.

In addition to being a component of the FA core complex, studies indicate that FANCG can have a role independent of the this complex (60). It forms a complex with FANCD1/BRCA2, FANCD2 and XRCC3 and is proposed to play a role in homologous recombination repair that occurs during DNA interstrand cross-link repair (60). The present studies, combined with our previous findings, indicate that FANCG may form another complex or interact directly with proteins involved in the unhooking step of the cross-link repair process, ERCC1-XPF and αIISp. We have shown that FANCG, in addition to binding to ERCC1 via TPR 1, 3, 5 and 6, interacts with the Src-homology 3 (SH3) domain of αIISp via a motif that binds to SH3 domains (43). This motif, residues 380-388 in FANCG, is located

between TPR 3 and 4. SH3 domains are modular domains that play a role in protein-protein interactions and formation of protein networks (61–64). Thus FANCG has two different types of motifs that have been shown to be important in protein-protein interactions and assembly of complex protein networks. It has TPR motifs, several of which interact with ERCC1, and it has a SH3 binding motif which interacts with αIISp. We have shown that this interaction of FANCG with αIISp is important in the ERCC1-XPF mediated unhooking step in the repair of DNA interstrand cross-links. Based on the present studies as well as previous work in our laboratory (19,20,26,33), we have proposed a model for the involvement of αIISp in repair of DNA interstrand cross-links (Figure 7). At a replication fork stalled at the site of a cross-link, a nuclease such as Mus81-Eme1 cleaves the DNA on the 3′ side of the ICL which leads to the production of a DSB (12,18,30,65). αIISp binds at the site of the DNA interstrand cross-link and acts as a scaffold to aid in the recruitment of repair proteins to the site of damage. FANCG is either recruited to bind to αIISp or is bound to the SH3 domain of αIISp at the time it binds to the cross-linked DNA. Since the present studies indicate that the interaction of αIISp with ERCC1 or XPF is indirect, we further propose that αIISp, which we have shown binds directly to DNA containing an interstrand cross-link (19) and to FANCG (43), recruits ERCC1-XPF to sites of damage via FANCG. Thus the interaction of FANCG with ERCC1-XPF would be critical in the recruitment of this protein complex to the site of the cross-link. This is analogous to the recruitment of ERCC1-XPF to sites of damage in NER by XPA (53,54). ERCC1-XPF then incises the DNA on the 5′ side of the cross-link and unhooking of the cross-link takes place. Subsequent steps in the repair process would then occur which involve localization of monoubiquinitated FANCD2 and additional FA proteins to chromatin, translesion synthesis, NER to excise the monoadduct, HR and repair of the DSB. Thus the demonstrated binding of FANCG to ERCC1 establishes a link between FA proteins and the critical unhooking step of the cross-link repair process.

This direct interaction of FANCG with ERCC1/XPF may also be important in any other function attributed to either of these proteins, such as the recombination step of the interstrand cross-link repair process or the second set of incisions to release the cross-link from the DNA during the proposed NER step of cross-link repair. Further studies will be needed to examine these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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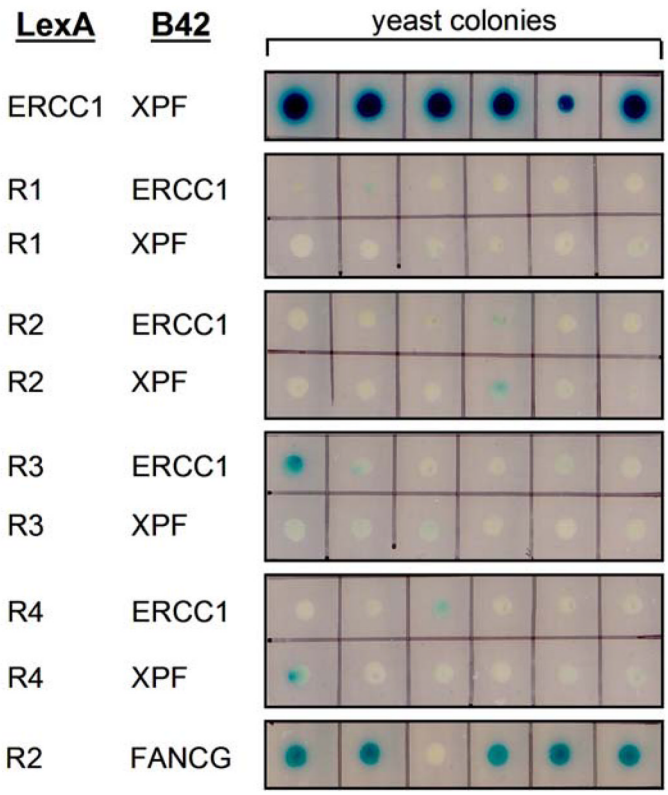
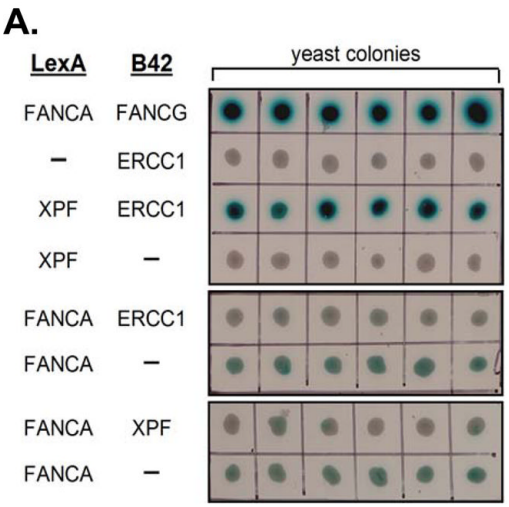
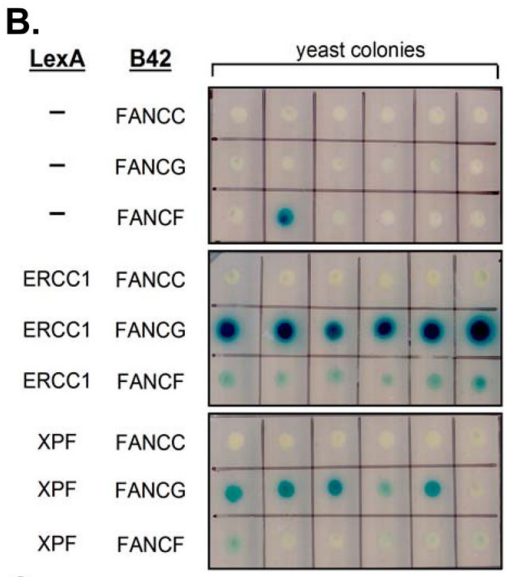


FIGURE 1.

No direct interaction was detected between any of the four regions of αIISp and either ERCC1 or XPF. αIISp was divided into four overlapping regions: R1 (residues 1-887), R2 (residues 864-1239), R3 (residues 1089-1766), and R4 (residues 1661-2477), which were subcloned into yeast two-hybrid vectors. The LexA fusion proteins were co-expressed in yeast with the B42-XPF or B42-ERCC1 constructs. Six randomly selected colonies from each of these yeast transformations were replica plated in a row to plates containing X-gal to test for activity of the reporter gene, β-galactosidase. Positive interaction between fusion proteins is seen by the presence of blue colonies. White colonies indicate no positive protein interactions. The interaction between ERCC1 and XPF and between R2 of αIISp and FANCG were used as positive controls.





C.

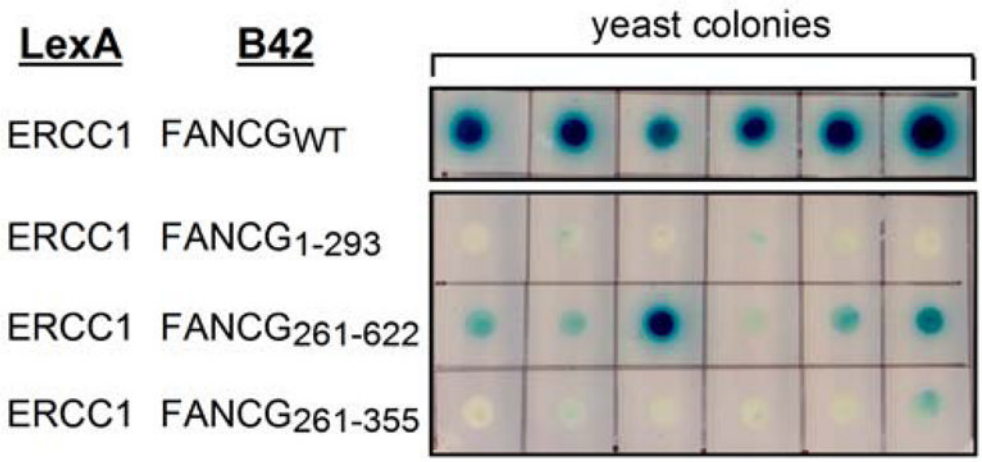
	ERCC1	XPF
FANCA	-	-
FANCC	-	-
FANCF	-	-
FANCG	++	+

FIGURE 2.

Yeast two-hybrid analysis of interaction ofERCC1 and XPF with FA proteins. (A) The LexA-FANCA fusion protein was co-expressed in yeast with an empty vector (−) or the B42-XPF or B42-ERCC1 constructs. Six randomly selected colonies from each of these yeast transformations were replica plated in a row to plates containing X-gal to test for activity of the reporter gene, β-galactosidase. Positive interaction between fusion proteins is seen by the presence of blue colonies, which indicates that the reporter gene has been activated. White colonies indicate no positive protein interactions. The interaction between FANCA and FANCG and between XPF and ERCC1 were used as positive controls. (B) The LexA-ERCC1 or LexA-XPF fusion proteins were co-expressed in yeast with the B42-

FANCC, B42-FANCG and B42-FANCF constructs. Protein interaction was assessed as above. (C) Summarization of the results of yeast two-hybrid analysis of the interaction of ERCC1 and XPF with FANCA, FANCC, FANCF and FANCG: (++) very good binding; (+) binding; (−) no binding.

A.

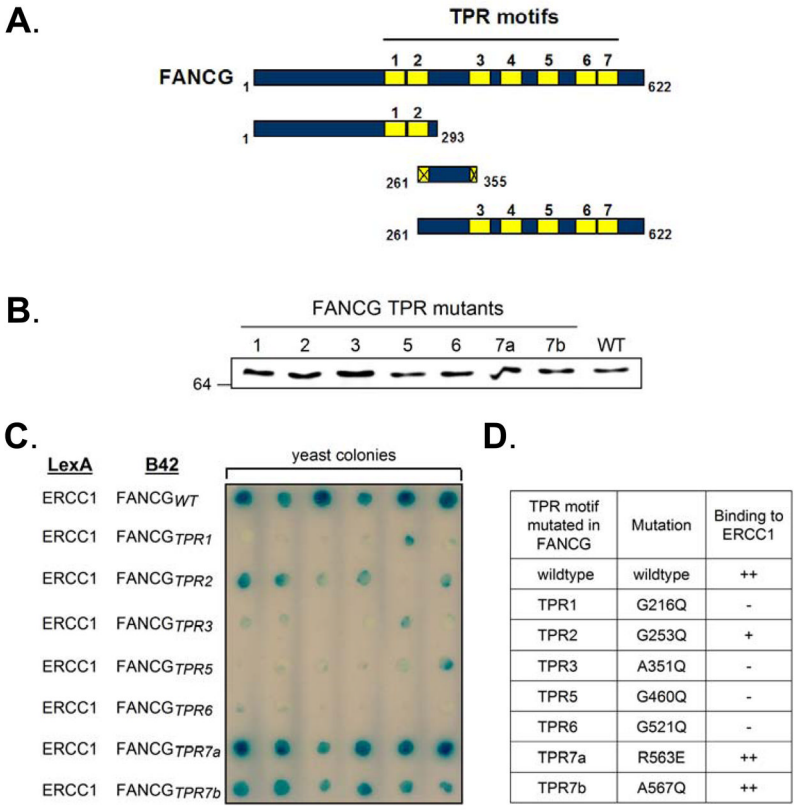


B.

FANCG constructs (residues)	Binding to ERCC1
full-length	++
1-291	-
261-355	-
261-622	+

FIGURE 3.

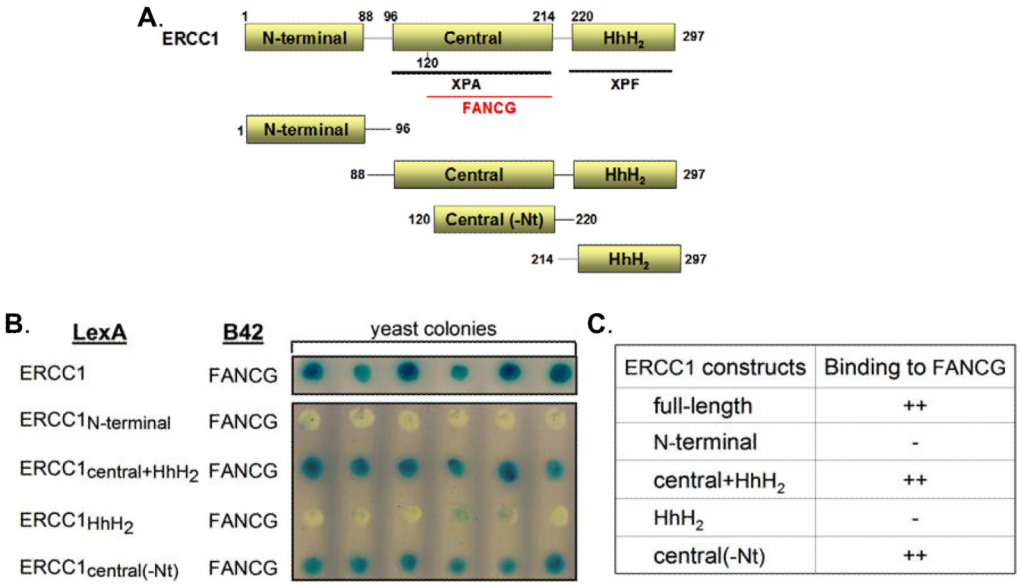
Full-length but not fragments of FANCG bind to ERCC1. (A) Full length and deletion constructs of FANCG (residues 1-293, residues 261-622, and residues 261-355) were created and subcloned into yeast two-hybrid vectors. LexA-ERCC1 fusion proteins were co-expressed in yeast with the B42-FANCG full-length or deletion constructs. Six colonies were selected and replica plated in rows as in Figure 1. Blue colonies indicate positive interaction between fusion proteins. White colonies indicate no positive protein interactions. (B) Summarization of the results of yeast two-hybrid analysis of the interaction of ERCC1 with full-length FANCG and deletion constructs of FANCG: (++) very good binding; (+) binding; (−) no binding.



TPR motif mutated in FANCG	Mutation	Binding to ERCC1
wildtype	wildtype	++
TPR1	G216Q	-
TPR2	G253Q	+
TPR3	A351Q	-
TPR5	G460Q	-
TPR6	G521Q	-
TPR7a	R563E	++
TPR7b	A567Q	++

FIGURE 4.

Specific TPR motifs in FANCG are essential for its binding to ERCC1. (A) Diagram showing the TPR motifs in full-length FANCG and the FANCG fragments. (B) Mutations were created in TPR motifs 1, 2, 3, 5, 6 and 7 in FANCG. Yeast strain EGY48 was transfected with the pJG4-5 vectors containing the TPR mutants of FANCG and wild type (WT) FANCG. The fusion proteins were detected by anti-HA antibody in western blot analysis of yeast cell extracts. (C) Yeast two-hybrid analysis of the interaction of wild type FANCG and FANCG containing mutant TPR motifs with ERCC1. LexA-ERCC1 fusion proteins were co-expressed in yeast with the B42-FANCG WT or B42-FANCG TPR mutants. Six colonies were selected and replica plated in rows as in Figure 1. Blue colonies indicate positive interaction between fusion proteins. White colonies indicate no positive protein interactions. (D) Summarization of the results of yeast two-hybrid analysis of the interaction of wild type FANCG and FANCG containing TPR mutations with ERCC1: (++) very good binding; (+) binding; (−) no binding.



ERCC1 constructs	Binding to FANCG
full-length	++
N-terminal	-
central+HhH_2	++
HhH_2	-
central(-Nt)	++

FIGURE 5.

The central domain of ERCC1 binds to FANCG. (A) Diagram of the three domains of ERCC1 and the binding of XPA, FANCG and XPF to specific domains of ERCC1. ERCC1 was divided into four overlapping regions: a region containing the N-terminal domain, a region containing the central and HhH_2 domains, a region containing the central domain minus its N-terminal (−Nt), and a region containing the HhH_2 domain. (B) Yeast two-hybrid analysis of the interaction of full length ERCC1 and the four regions of ERCC1 with FANCG. LexA-ERCC1 fusion protein and LexA-fusion proteins of the four regions of ERCC1 were co-expressed in yeast with the B42-FANCG. Six colonies were selected and replica plated in rows as in Figure 1. Blue colonies indicate positive interaction between fusion proteins. White colonies indicate no positive protein interactions. (C) Summarization of the results of yeast two-hybrid analysis of the interaction of full-length ERCC1 and the four regions of ERCC1 with FANCG: (++) very good binding; (+) binding; (−) no binding.

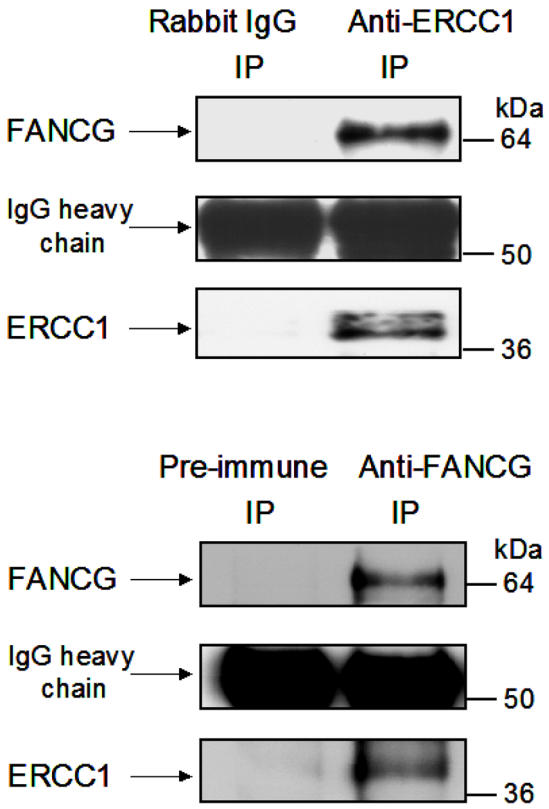


FIGURE 6.

FANCG coimmunoprecipitates with ERCC1. (A) Normal human cell extracts were coimmunoprecipated with anti-ERCC1. Interaction of FANCG with ERCC1 was examined by western blot analysis using anti-FANCG and anti-ERCC1. IgG heavy chain was used as a loading control. (B) Normal human cell extracts were coimmunoprecipated with anti-FANCG. Interaction of FANCG with ERCC1 was examined by western blot analysis using anti-FANCG and anti-ERCC1.

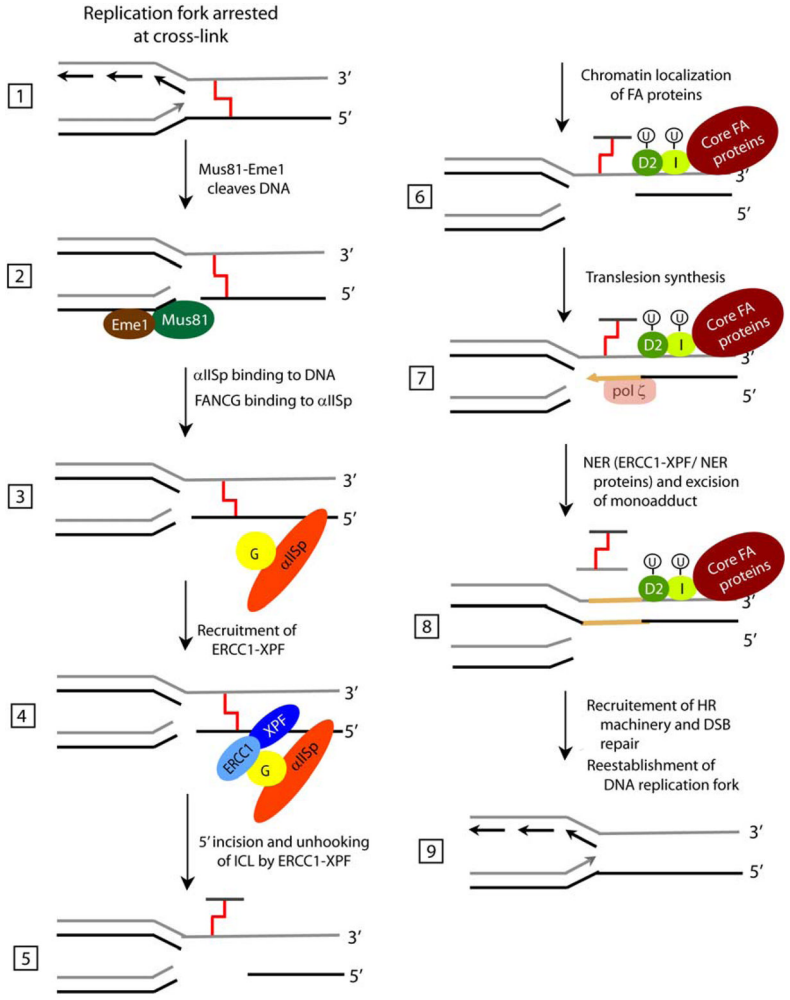


FIGURE 7.

Model showing the interaction of αIISp, FANCG, and ERCC1-XPF in the repair of a DNA interstrand cross-link at an arrested replication fork. [1] A DNA replication fork is arrested at the site of an ICL; [2] Mus81-Eme1 cleaves DNA at the stalled replication fork on the 3′-side of the ICL producing a DSB; [3] αIISp binds to the DNA at the site of the ICL, FANCG binds to the SH3 domain of αIISp; [4] ERCC1-XPF is recruited to the ICL by FANCG, which is bound to αIISp; [5] ERCC1-XPF incises the DNA on the 5′ side of the ICL, thus unhooking the cross-link; [6] monoubiquitinated FANCD2 localizes to the chromatin with other FA proteins; [7] translesion synthesis with a polymerase such as pol ζ fills in the gap resulting from the ICL unhooking; [8] NER with ERCC1-XPF and other NER proteins leads to excision of the monoadduct; [9] the homologous recombination machinery is recruited and the DSB is repaired; the DNA replication fork is reestablished. This is based on our results and on models presented by Thompson and Hinz (12) and Bhagwat et al., (31).