FANCJ localization by mismatch repair is vital to maintain genomic integrity after UV irradiation

**Short Title:** FANCJ functions in the UV response

Shawna Guillemette¹, Amy Branagan¹, Min Peng¹, Aashana Dhruva¹, Orlando D. Schärer², and Sharon B. Cantor¹*

1) Department of Cancer Biology, University of Massachusetts Medical School
Women's Cancers Program, UMASS Memorial Cancer Center, Worcester, MA 01605 USA

2) Department of Pharmacological Sciences & Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400

*Address correspondence to: Sharon B. Cantor, Department of Cancer Biology, UMASS Medical School, 364 Plantation St., Worcester, MA 01605, Tel. 508-856-4421; Fax: 508-856-1310; Email: Sharon.Cantor@umassmed.edu

**Key Words:** ultraviolet radiation, FANCJ, mismatch repair, nucleotide excision repair, checkpoint

The authors disclose no potential conflicts of interest.
Abstract:

Nucleotide excision repair (NER) is critical for the repair of DNA lesions induced by ultraviolet (UV) radiation, but its contribution in replicating cells is less clear. Here, we show that dual incision by NER endonucleases including XPF and XPG promotes the S-phase accumulation of the BRCA1 and Fanconi anemia (FA)-associated DNA helicase FANCJ to sites of UV-induced damage. In response to UV damage, FANCJ promotes RPA phosphorylation and the arrest of DNA synthesis following UV irradiation. Interaction defective mutants of FANCJ reveal that BRCA1 binding is not required for FANCJ localization, whereas interaction with the mismatch repair (MMR) protein MLH1 is essential. Correspondingly, we find that FANCJ, its direct interaction with MLH1, and the MMR protein MSH2 function in a common pathway in response to UV irradiation. FANCJ deficient cells are not sensitive to killing by UV irradiation, yet we find that mutations are significantly enhanced. Thus, we considered that FANCJ deficiency could be associated with skin cancer. Along these lines, in melanoma we found several somatic mutations in FANCJ some of which were previously identified in hereditary breast cancer and Fanconi anemia (FA). Given that mutations in XPF can also lead to FA, we propose collaborations between FA, NER and MMR are necessary to initiate checkpoint activation in replicating human cells to limit genomic instability.
Introduction:

Repair of ultraviolet (UV) irradiation-induced DNA damage depends on the nucleotide excision repair (NER) pathway. Underscoring the essential role of NER in repair of UV-induced DNA damage, inherited defects in NER genes result in the skin cancer-prone disease Xeroderma pigmentosum (1). In non-replicating cells NER factors sense UV-induced DNA damage and excise the lesion in a multi-step process. The remaining short single-stranded DNA (ssDNA) region serves as a template for repair synthesis, “gap” repair (2, 3). Lesions escaping NER gap repair stall replication forks and initiate checkpoint responses. Some NER factors interact with the replisome and contribute to the early S phase checkpoint response (4, 5). In post-replication repair lesions are managed largely through DNA-damage tolerance mechanisms (6-8). Among the recent factors found to be involved in this process is the hereditary breast cancer-associated gene product BRCA1, which function independently of NER to suppress mutations (9).

Several lines of evidence indicate that UV-induced damage is also limited by proteins of the DNA mismatch repair (MMR) pathway. MMR factors induce checkpoints, apoptosis, preserve genomic stability, and suppress cancer induced by UV irradiation (10-14). The mechanism by which MMR functions in response to UV irradiation could stem from its general role in genome surveillance and mismatch correction. Canonical MMR begins with the recognition of replication errors (15) where MSH2-MSH6 (MutSα) or MSH2-MSH3 (MutSβ) assemble and recruit the heterodimer MLH1-PMS2 (MutLα). These complexes function in the repair of mismatched bases. As such, loss of MMR confers a mutator phenotype and a predisposition to hereditary nonpolyposis colon cancer (HNPCC) (16). However, it is also well appreciated that MMR proteins respond to DNA damage from exogenous sources, such as to DNA alkylating agents, known to induce mismatches following DNA replication (17). In response to UV irradiation, MMR factors could have an alternative non-canonical role in UV lesion processing given that the MSH2-MSH6 complex directly binds UV lesions (18, 19). Clarifying how MMR contributes to genomic stability in the UV response will be central to understanding the HNPCC variant, Muir-Torre syndrome that is characterized by skin cancers (20-22).

Both the MMR protein MLH1 and BRCA1 bind directly to the DNA helicase FANCJ which has essential functions in activating checkpoints following replication stress, although it has not hitherto
been linked to the UV-induced damage response (23-27). FANCJ is mutated in hereditary breast and ovarian cancer as well as in the rare cancer-prone syndrome Fanconi anemia (FA) (24, 28). Complementation studies using FANCJ-deficient (FA-J) patient cells demonstrated that MLH1 binding is critical for FANCJ function in the repair of DNA interstrand crosslinks (ICLs) (24, 29). Here, we reveal that MLH1 binding to FANCJ is also essential for the response to UV-induced damage, in which FANCJ promotes an S phase checkpoint and limits UV-induced mutations. Because dual incision by NER also promotes FANCJ accumulation at sites of UV induced damage, and the NER endonuclease XPF was recently shown to be an FA gene (30, 31), our analysis suggests that FA, MMR and NER pathways collaborate to process UV lesions in S phase cells to preserve the genome.

**Materials and Methods**

**Cell Culture**

A549, MCF7, and U2OS cells were cultured in DMEM (Gibco, Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. FA-J, 48BR, MEFs, GM04429 XP-A, XP2YO XP-F, and XPCS1RO XP-G cells and their respective complements were cultured in DMEM supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. Patient cell lines XP2YO XP-F, XPCS1RO XP-G and their respective complements were generated by Dr. Orlando Schärer and MSH2-/- or +/- MEFs were a generous gift of Janet Stavnezer.

**DNA constructs**

FA-J cells were infected with pOZ retroviral vectors (32) expressing FANCJ\textsuperscript{WT}, FANCJ\textsuperscript{K141/142A}, FANCJ\textsuperscript{S990A}, or empty vector as described earlier (23, 24). Stable cell lines were generated by sorting with anti-IL-2 magnetic beads (Dyna Beads). The pCDNA-3myc-6xhis vectors were generated with a QuickChange site-directed mutagenesis kit (Stratagene) using published primers for FANCJ\textsuperscript{K52R} (23). GFP-polη was expressed in U2OS cells as described in (28, 33, 34).

**RNA Interference**

The packaging cell line 293TL was used to produce lentiviral particles containing pGIPZ or pLKO.1 vectors and 293TD cells were used to produce retroviral particles containing pStuffer vector. Cells were transfected with 1:1:2 ug of DNA packaging versus insert using Effectene\textsuperscript{®} transfection reagent (Qiagen) 48 h prior to harvesting retroviral or lentiviral supernatants. Supernatants were filtered and
added to recipient cell lines with 1 ug/ml polybrene. Cells infected with shRNA vectors were selected with either puromycin (pGIPZ, pLKO.1) or hygromycin (pStuffer). For shRNA-mediated silencing the mature antisense was used for pLKO.1 shNSC 5’CCGCAGGTATGCACGCGT3’, shMLH1 5’AATACAGAGAAAGAAGACAC3’, shMSH2 5’AAACTGAGAGATTGCCAGG3’, shXP5’AAATCACTGATAACTCTGCGC3’, shFANCJ 5’TATGGATGCTGTTTCTTAGCT3’, for pGIPZ shNSC 5’ATCTCGCTTGGGCGAGAGTAAG3’, shMSH2-1 5’ATTACTTCAGCTTTTAGCT3’, shMSH2-2 5’GCATGTAATAGAGTGCTAA3’, shMSH6-1 TTCAACTCGATTTCTTCTGGC, and shMSH6-2 TTCAACTCGATTTCTTCTGG. The pStuffer vectors were a generous gift of Dr Junjie Chen. The pStuffer shRNA targeting luciferase was 5’GUGCGCUGCUGGUGCCAAC3’, shFANCJ-1 5’GUACAGUACCCACCUCUAU 3’, and shFANCJ-2 5’GAUUUCAGAUCCACAAU3’. RNAi mediated depletion of Luciferase, FANCJ, or RAD18 using siRNA reagents was performed as described previously (28).

**Local UV irradiation and Immunofluorescence**

Local UV irradiation was performed as described (35) using a 254 nm UV lamp (UVP inc., Upland, CA) with a dose of 100 J/m² though 3 or 5 um Isopore polycarbonate membrane filters (Millipore). Cells were fixed for 10 min with either ice cold methanol or 3% paraformaldehyde/2% sucrose in phosphate buffered saline (PBS), permeabilized for 5 min with 0.5% Triton X-100, and treated with 0.08M NaOH for 2 min. only prior using 6-4 PP or CPD Abs. Coverslips were rinsed 3x in 1xPBS prior to each step. For primary and secondary staining, cells were incubated for 40 min. each in a humid chamber, face down on a 100uL meniscus of Abs diluted in 3%BSA in PBS. Primary Abs used were anti-FANCJ (1:500 Sigma, Lot #051M4759, #014K4843), anti-pS4/S8 RPA32 (1:500 Bethyl), anti-MLH1 (1:200 BD Bioscience), anti-MSH2 (1:200 Calbiochem), anti-XPF (1:200 Neomarkers), anti-ERCC1 (1:500 Santa Cruz), anti-XPC (1:500 Abcam), anti-6-4 PP, and anti-CPD (both 1:1000 CosmoBio). Secondary Abs used include Rhodamine Red-X conjugated AffiniPure Goat anti-rabbit or anti-mouse IgG and fluorescein (FITC)-conjugated AffiniPure Goat anti-rabbit IgG (Jackson Immuno-research Laboratories Inc). Coverslips were mounted on slides using Vectashield® mounting media with DAPI (Vecta laboratories, Inc) and analyzed on a fluorescence microscope (Leica DM 5500B) with a Qimaging Retiga 2000R Fast 1394 camera. For each experimental time point ≥400 DAPI-positive cells (≥1200 in triplicate) were analyzed using Q-Capture Pro line intensity profile software with the intensity gated at ≥0.1 for positive LUDs for 6-4 PP or CPD staining. The accumulation of a
protein at an LUD was considered positive if its’ intensity was 10-fold greater than the line drawn over the rest of the nucleus.

**Mutation Frequency Assays**

The HPRT assay was performed in A549 cells as described (36) with the following modifications. After culturing cells for 1 week in media containing hypoxanthine, aminopterin, and thymidine (HAT selection) to eliminate background HPRT mutations, cells were stably depleted of FANCJ with two unique shRNA targets versus a non-silencing control and selected with hygromycin. UV-induced HPRT mutants were obtained by seeding 6 plates at a confluence of 1x10^6 cells/10 cm dish 24 h prior to either mock treatment, 5, or 10 J/m² UV irradiation in a 254 nm Spectrolinker™ XL-1500 (Dot Scientific, Inc). Post-treatment cells were allowed to recover to 6x10^6 cells or with mock cells 6 population doublings and 6x10^6 cells were seeded at a confluence of 1x10^6/10 cm dish in media containing 24 uM 6-Thioguanine (6-TG) to select for HPRT-inactivated colonies. At the same time 200 cells were also seeded in 6-TG-free media to determine colony-forming efficiency. The frequency of inactivating mutations at the HPRT locus was calculated as the [(# of total 6-TG resistant colonies) / (6x10^6 cells seeded)] x the colony-forming efficiency. HPRT inactivation frequency represents the mean of three independent experiments. Individual colonies were picked and grown until enough cells were obtained for RNA isolation using TRIzol® Reagent (Life Technologies) according to the manufacturer’s protocol. The HPRT gene was subjected to RT-PCR using SuperScript® (Invitrogen) followed by sequencing using overlapping primers HPRT1 5′CTTCCCCCTCCTGAGCAGTC3′; HPRT2–5′AAGCAGATGGC-CACAGA ACT3′; HPRT3–5′CCTGGCGTCTGATATTAGTG3′; HPRT4–5′TTTAC-TGGCGATGTCAATAGGA3′; HPRT5–5′GACCAGTCAACAGGGGACAT3′; and HPRT6 5′ATGTCCCCTGGTGACTGGTC3′. Patient-derived FA-J cells were complemented with empty vector or FANCJ^WT and treated as described with A549 cells. As FA-J cells do not make colonies, the % increase in 10uM 6-TG survival was calculated as the % of UV-irradiated cells surviving 6-TG minus the % of untreated cells surviving 6-TG. The % increase in 6-TG survival represents the mean of three independent experiments.

**EdU Labeling**

EdU incorporation was performed as described previously (Limsiriachaikul et al 2009), except cells were seeded on coverslips and left untreated or UV-irradiated through 5 micron filters prior to 3 h
incubation in 10uM EdU diluted in serum free media. When using global UV-irradiation, cells were left untreated and pulsed 45 min in 10 uM EdU or UV-irradiated and pulsed 16 h later for 45 min with 10 uM EdU. Cells were processed by Click-iT® EdU imaging kit (Invitrogen) using the manufacturer’s instructions immediately followed by the above immunofluorescence protocol.

**Western Blot**

Cells were harvested and lysed in 150 mM NETN lysis buffer (20mM Tris (pH 8.0), 150 mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail) for 30 minutes on ice. Cell extracts were clarified by centrifugation at 14,000 rpm, protein was quantified by Bradford assay, and lysates were boiled in SDS loading buffer. Chromatin extracts were prepared as described (37). For CPD immune-precipitation cells were lysed in 150 nM NETN buffer, spun down, and the insoluble pellet was re-suspended in RIPA buffer and sonicated. The RIPA fraction was spun down and chromatin lysate was quantified by Bradford assay. Lysates were then pre-cleared with Protein A beads and immuno-precipitated overnight with CPD Abs. Proteins were separated by SDS-PAGE on 4-12% bis Tris or 3-8% Tris Acetate gels (Novex, Life Technologies) and electrotransferred onto nitrocellulose membranes. Membranes were blocked in 5% milk diluted in PBS. Antibodies used for Western blot analysis included anti-FANCJ (1:1000 Sigma, 1:1000 E67 (previously described (38), anti-Bactin (1:5000 Sigma), anti-MLH1 (1:500 BD Bioscience), anti-MSH2 (1:500 Calbiochem), anti-MSH2 (mouse specific, Santa Cruz), anti-XPF (1:1000 Neomarkers), anti-ERCC1 (1:500 Santa Cruz), anti-XPC (1:1000 Abcam), anti-CHK1 (1:500 Bethyl), anti-p317 CHK1 (1:500 Bethyl), anti-RPA32 (1:500 Bethyl), and anti-pS4/S8 RPA32 (1:500 Bethyl). Membranes were washed and incubated with horseradish peroxidase-linked secondary antibodies (Amersham 1:5000), and detected by chemiluminescence (Ambersham). The ratio of phospho-protein to total protein was measured and quantified using Image J software.

**FACS Analysis**

FA-J cells cultured to ~ 80% confluency were left untreated or globally irradiated with 5 J/m² before collecting and fixing in 70% ethanol 4 h post UV irradiation. For antibody labeling, cells were rinsed with1x PBS, pearmabilized with 0.5% Triton-X 100 in PBS 20 minutes at room temperature, and then washed with 1% BSA/0.25% Tween-20 in PBS (PBS-TB) before re-suspending 1 h in PBS-TB with pS4/S8 RPA 32 antibody (1:250 Bethyl). Cells were then collected and washed 2x in PBS-TB prior to
1 h incubation in PBS-TB containing FITC-conjugated AffiniPure Goat Anti-Rabbit IgG (1:200 Jackson Immuno-research Laboratories, Inc.). After washing with PBS, cells were re-suspended in RNAse A solution (100 μg/mL in PBS) for 20 min at room temperature and again washed with PBS prior to FACS analysis. Cells were labeled with propidium iodide prior to analysis on a FACSCalibur flow cytometer (Becton-Dickinson) performed at the University of Massachusetts Medical School flow cytometry core facility using Cellquest software. The fluorescence intensity of pS4/S8 RPA 32 positive cells was gated as FITC-positive cell populations compared to no antibody control.

Results:

FANCJ accumulation at sites of UV induced damage is dependent on NER dual incision

We examined the response of FANCJ to UV irradiation by assessing whether FANCJ accumulated at sites of UV-induced damage. Following UV-irradiation through 3 or 5 micron filters to generate sites of localized UV damage (LUDs) (35, 39), we found that FANCJ co-localized with UV-induced 6-4 pyrimidine-pyrimidones (6-4 PPs), cyclobutane pyrimidine dimers (CPDs), and the NER endonuclease XPF in the breast cancer cell line, MCF7. FANCJ localization to 6-4 PP- or XPF-positive LUDs peaked ~3 h after UV irradiation and diminished by ~12 h (Figure 1 A-B).

To address the relationship of FANCJ to NER, we used XP fibroblast cell lines and their functionally complemented counterparts. We found that the accumulation of FANCJ at LUDs was reduced ~2- to 3-fold in NER-deficient XP-A, XP-F and XP-G cells when compared to the wild-type complemented cells (Figure 1C-F). Contributing to FANCJ localization was XPF and XPG endonuclease activity as complementation with XPF or XPG nuclease-defective mutant species, XPF<sup>D676A</sup> or XPG<sup>E791A</sup> (40) failed to restore robust FANCJ accumulation at LUDs between ~1-5h post UV-induced damage (Figure 1E-F). Following global UV irradiation, FANCJ foci were also more prominent in XP-F cells complemented with wild type XPF endonuclease (Figure S1A-B). By contrast, FANCJ depletion did not affect the localization dynamics of NER factors (Figure S1C-D). Collectively, these data indicate that NER incision events potentiate the accumulation of FANCJ to sites of UV-induced damage.

NER promotes the accumulation of FANCJ at UV induced damage in S phase
Next, we investigated whether NER contributed to FANCJ accumulation at LUDs in a specific cell cycle phase. Cells within S-phase and non-S phase can be easily distinguished after local UV irradiation by staining for 5-ethynyl-2’-deoxyuridine (EdU) incorporation into genomic DNA (41). In S-phase cells, EdU staining is bright and pan nuclear. In non-S phase cells, EdU staining is restricted to sites of LUDs, representing sites of unscheduled DNA synthesis that occurs during gap repair in NER (42). Following localized UV irradiation, cells were incubated in media with EdU for 3 h and immunostained with FANCJ antibodies. Consistent with a role for XPF in NER dependent gap filling, EdU positive LUDs in non-S phase cells were only present in XPFWT cells (Figure 1G-H). FANCJ recruitment to LUDs was not significantly improved in non-S phase XPFWT cells, however it was significantly enhanced in S-phase XPFWT cells indicating that XPF potentiates FANCJ accumulation in cells undergoing DNA synthesis (Figure 1G-H).

**FANCJ localization to sites of UV induced damage is MMR dependent**

FANCJ directly binds BRCA1, which functions in the response to UV irradiation selectively in S/G2 phase cells (9, 23). FANCJ also directly binds MLH1 (24), which along with other MMR factors function in the response to UV irradiation and preserve genomic integrity (43). Because both BRCA1 and MLH1 contribute to FANCJ localization and function in the DNA damage response (24, 28, 29, 44), we investigated whether BRCA1 or MLH1 interactions were required for FANCJ localization to LUDs. We analyzed FANCJ recruitment in FANCJ deficient FA-J patient cells complemented with empty vector, FANCJWT, the BRCA1-interaction defective mutant (FANCJS990A) (45), or the MLH1-interaction defective mutant (FANCJK141/142A) (24). While the FANCJ species expressed at similar levels, we found that FANCJK141/142A localization was dramatically reduced as compared to FANCJS990A, which localized to LUDs just as efficiently as FANCJWT (Figure 2A-C). Importantly, FANCJ positive LUDs were not detected in FANCJ-null FA-J cells unless complemented with wild-type FANCJ confirming the specificity of our FANCJ antibody (Figure 2A-C). Further validating that FANCJ localization to LUDs requires functional MMR, we found that as compared to a non-silencing control (NSC), FANCJ recruitment to LUDs was severely reduced in U2OS cells depleted of MLH1, MSH2, or MSH6 (Figure 2D-I, S2A-D). In contrast, XPC and ERCC1 recruitment to LUDs was not affected by MSH2 depletion (Figure S2E-F), indicating that MMR is required for accumulation of FANCJ at LUDs, but not XPC or ERCC1. Likewise, MSH2 recruitment to LUDs was similar in vector
and XPF\textsuperscript{WT} complemented \textit{XP-F} cells while as expected ERCC1 was only present in the XPF\textsuperscript{WT} complemented \textit{XP-F} cells suggesting that MMR and NER accumulation at LUDs is not interdependent (Figures S2G-H). We also noted that the residual accumulation of FANCJ found at LUDs in \textit{XP-F} cells was eliminated by depletion of MSH2 (Figure 2J-L), suggesting that NER and MMR operate in a parallel manner to support FANCJ localization. In the \textit{XP-F} cells, however MSH2 depletion did not perturb FANCJ nuclear or chromatin localization suggesting MMR and NER contribute to FANCJ localization to LUDs as opposed to nuclear import (Figure S2I).

We expected that both NER and MMR would also be present in S phase cells given that they contribute to the S phase localization of FANCJ. However, NER proteins are best known for their UV repair function in non-S phase cells (3) and from the literature it was not clear if MMR proteins had a cell cycle dependent localization to LUDs. We used primary immortalized 48BR fibroblasts that have been used to characterize NER proteins in gap repair by means of EdU incorporation (42). While XPF was clearly present in non-S phase cells at sites of gap filling, as expected, we also detected XPF in nearly all LUDs in S phase cells, \~95\% (Figure S3A-C). MLH1 and MSH2 were also present at LUDs with a similar percent in both non-S and S phase cells. Instead, FANCJ was primarily at LUDs in S phase cells, \~86\% and only in \~19\% of non-S phase cells (Figure S3A-C). Collectively, these studies show that MMR and NER proteins localize to LUDs in both non-S and S phase cells whereas FANCJ localizes primarily in S phase cells.

**FANCJ promotes the UV induced arrest of DNA synthesis and the induction of RPA phosphorylation**

UV irradiation activates checkpoint responses and inhibits DNA replication in S phase cells (46). Given the role of FANCJ in checkpoint responses (25-27) and the accumulation of FANCJ at LUDs during S-phase, we tested if FANCJ contributed to the UV-induced checkpoint response. By pulsing cells with EdU, we found that \textit{FA-J} cells expressing FANCJ\textsuperscript{WT} underwent a 10.5-fold reduction in S-phase cells when examined 16 h after UV irradiation. By comparison, \textit{FA-J} cells expressing vector underwent a 2.0-fold reduction (Figure 3A-B), indicating that FANCJ contributes to the arrest of DNA synthesis in response to global UV irradiation.
The UV induced arrest of DNA synthesis is also associated with changes in phosphorylation of the single-stranded DNA-binding protein RPA (47). Following UV-irradiation, the 32 kDa subunit of RPA is phosphorylated on several serine residues in the N-terminal of the protein in a cell cycle dependent manner by DNA-PK and cyclin dependent kinases (48, 49). By examining phosphorylation of serines4/8 on RPA32 with a specific antibody, we found that in response to global UV irradiation FANCJ complementation was sufficient to enhance RPA serines4/8 phosphorylation in FA-J cells by fluorescent activated cell sorting (FACS) (Figure 3C) and immunoblot (Figure S3D). By FACS analysis basal phospho-S4/8 RPA32 was ~1-2% in both untreated vector and wild-type FANCJ complemented FA-J cells. Following UV-irradiation, phospho-S4/8 RPA32 was induced to ~22% in FANCJ<sup>WT</sup> FA-J cells as compared to only ~7% in vector FA-J cells (Figure 3C). Likewise, using phospho-S4/8 RPA32 immunostaining in conjunction with EdU pulse, we uncovered that phospho-S4/8 RPA32 staining was detected only in S phase cells (Figure 3D-E). Furthermore, we found that FA-J patient cells complemented with FANCJ<sup>WT</sup> or the BRCA1-interaction defective mutant (FANCJ<sup>S990A</sup>) had significantly greater EdU positive S-phase cells with phospho-S4/8 RPA32 positive LUDs as compared to the FA-J cells complemented with empty vector or the MLH1-interaction defective mutant (FANCJ<sup>K141/142A</sup>) (Figure 3D-E). This finding further suggested that FANCJ and the FANCJ-MLH1 interaction, but not the BRCA1-interaction contributes to checkpoint responses in S phase cells.

Immunoblotting in FANCJ depleted MCF7 cells also revealed that phospho-S4/8 RPA32 as well as the soluble checkpoint factor phospho-317 CHK1 was reduced compared to non-silencing control (NSC) while total CHK1 and RPA levels were unchanged (Figure 3F-G). Moreover, co-immunostaining with phospho-S4/8 RPA32 and 6-4 PP antibody was used to visually mark UV induced LUDs and revealed that phospho-S4/8 RPA32 was significantly reduced in FANCJ depleted cells as compared to NSC (Figure 3H-J). Interestingly, by 12h post-UV damage, 6-4 PP LUDs persisted in FANCJ depleted cells (Figure 3H-J). FANCJ or MSH2 depletion also consistently enhanced the persistence of 6-4 PP positive LUDs in the male lung cancer cell line, A549 in which the formation of phospho-S4/8 RPA32-positive LUDs was also significantly reduced (Figure S4A-C). Furthermore, the combination of FANCJ and MSH2 depletion was not additive (Figure S4A-C), suggesting that FANCJ and MSH2 function in a common pathway that is not cell type specific.
Recently NER factors were shown to promote the S phase checkpoint response, including RPA phosphorylation in response to UV irradiation (4, 5, 50). Given that the mechanism by which NER promotes the S phase checkpoint is unclear, we considered whether the NER-dependent accumulation of FANCJ at LUDs in S phase was required. As before, XP-F patient cells were segregated into non-S and S phase cells by labeling with EdU and phospho-S4/8 RPA32 staining was detected only in S phase cells (Figure 4A-B). We found that phospho-S4/8 RPA32 induction was greatest in XP-F cells complemented with XPF WT (Figure 4A-C). Strikingly, depletion of FANCJ or MSH2 profoundly reduced the phospho-S4/8 RPA32 induction of XPF WT complemented XP-F cells (Figure 4A-E). Notably, the residual phospho-S4/8 RPA32 positive LUDs found in vector complemented XP-F cells were also reduced by depletion of FANCJ or MSH2 (Figure 4A-E). Thus, FANCJ promotes S phase checkpoint responses in not only cancer cell lines, but also in non-transformed fibroblasts. Together, these data suggest that MSH2 and FANCJ contribute to NER-dependent and independent UV-induced phospho-S4/8 RPA32 induction at LUDs in S phase cells. In contrast, when either FANCJ or MSH2 were depleted, we found gap filling was proficient in the XP-F cells complemented with XPF WT (Figure 4A-B,E-H). Gap filling was also proficient in 48BR cells depleted of FANCJ, MLH1, or MSH2, but reduced in cells depleted of XPF (Figure S4D). Msh2 -/- and Msh2+/+ mouse embryonic fibroblasts also had similar levels of gap filling (Figure S4E), suggesting that FANCJ and MMR factors are not required for NER-dependent gap filling.

Collectively, our data indicate that FANCJ contributes to the UV induced arrest of DNA synthesis by potentiating checkpoint induction pathways. While FANCJ does not contribute to NER-dependent gap repair, it influences the clearance of UV induced lesions in a common pathway with MSH2.

**FANCJ suppresses UV-induced mutations**

Given that FANCJ is dispensable for survival after UV exposure (28), we sought to examine if FANCJ preserves the integrity of the genome, as has been found for MMR (14). A549 cells are useful for analyzing mutations at the endogenous hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus (36). Similar to other cell lines examined, in A549 cells FANCJ localized to sites of UV induced
damage as demonstrated by co-precipitation of FANCJ with CPD and modified PCNA following UV-induced damage (Figure S5A). Using RNAi-mediated FANCJ silencing, we confirmed that FANCJ was not essential for survival following UV irradiation, but was essential for survival following exposure to the DNA cross-linking agent cisplatin (Figure 5A-C, Figure S6A-B). As compared to NSC, we found that FANCJ depletion enhanced UV-induced HPRT inactivating mutations determined by clonal selection in 6-thioguanine (36). FANCJ depletion did not affect spontaneous HPRT mutations, but the frequency of inactivating HPRT mutations after 10J/m² UV irradiation was enhanced ~10-fold in A549 cells (Figure 5D). Sequencing of clones arising from HPRT inactivation indicated no gross deletions or rearrangements as a consequence of FANCJ deficiency in response to global UV irradiation. Instead, HPRT inactivation was predominated by ~8-fold more C to T transitions in both the transcribed strand (TS) and non-transcribed strand (NTS) of HPRT in FANCJ depleted cells (Figure 5E, F, Supplemental Table 1). These findings suggest that FANCJ is involved in a specific process that suppresses the formation of point mutations in response to UV irradiation. Further supporting that FANCJ suppresses inactivating mutations at the HPRT locus, FANCJWT-complementation in FANCJ-deficient FA-J patient cells was sufficient to reduce survival in 6-thioguanine (6-TG) after UV irradiation, indicating that it averted the occurrence of mutations (Figure S6C). Furthermore, complementation with FANCJWT enhanced resistance to DNA cross-linking agent, mitomycin C (MMC), while in accordance with the results from Figure 4C, the resistance to UV irradiation was unchanged (Figure S6D,E). Together, these results suggest that FANCJ, similar to MMR (10-14), contributes to the prevention of mutations in response to UV irradiation, without affecting long-term survival following this treatment. Thus, we propose that in collaboration with NER, the MMR-FANCJ pathway is important for the response to UV irradiation in S phase to ensure checkpoint responses, genome stability and limit tumorigenesis (Figure 5G).

Discussion

Here, we show that both NER and MMR proteins promote the localization of the FANCJ DNA helicase to sites of UV-induced lesions to ensure a robust S phase checkpoint response. MMR proteins initially recruit FANCJ and its further accumulation requires dual incision by the NER endonucleases XPF and XPG (Figures 1C-H; 2D-L). Although FANCJ-deficiency does not cause UV-induced sensitivity, our analysis revealed an important role for FANCJ in promoting an S phase checkpoint response, lesion repair, and suppressing UV-induced mutations (Figures 3A-J; S3D; 4A-
E; S4A-C; 5A-F; S6C). Consistent with FANCJ and MMR functioning in a common pathway, we found that FANCJ or MMR deficiency alone or in combination generated similar defects (Figure S4A-C). Correspondingly, the direct interaction between MLH1 and FANCJ is essential for both FANCJ localization and function at sites of UV-induced damage, whereas the BRCA1 interaction is not required (Figures 2A-C; 3E-F). Similar to NER, MMR proteins localize to LUDs in both non-S and S phase cells whereas FANCJ is predominantly found at sites of UV-damage in S phase cells (Figure S3A-C). Together, our work demonstrates that distinct pathways merge in S phase cells to ensure a robust UV-induced DNA damage response.

These findings are important in light of the fact that defects in MMR have been associated with skin cancers found in the HNPCC variant Muir-Torre syndrome. Furthermore, we searched for both FANCJ and MMR mutations within sequenced melanoma genomes using cBioPortal (51, 52) and the Catalogue of Somatic Mutations in Cancer (CoSMiC) database (53). We identified mutations in FANCJ, MSH2, MSH6, MLH1, and PMS2 (Figure S7A-F). The majority of FANCJ mutations target the helicase domain including domains important for enzyme function, such as the Fe-S domain and helicase boxes III, IV, and V (54) (Figure S7A). In addition, some of the mutations have been detected previously. The FANCJP47 residue was targeted in breast cancer and was shown to be ATPase and helicase inactive in vitro (38). The splice mutant FANCJR831 is an allele in FA and eliminates conserved helicase boxes required for enzyme function (38, 54, 55). To determine if loss of FANCJ ATPase/helicase/translocase activity disrupts the UV response, we attempted to express the catalytic inactive FANCJK52R mutant in FA-J cells. Because FANCJ deficient FA-J cells are defective in the UV response and the FANCJK52R mutant has weak expression compared to FANCJWT, it was unclear if the mutant was defective in complementing FA-J cells or mediating the UV response. Thus, we overexpressed the FANCJK52R mutant in U2OS cells. Here, we found significant defects in lesion clearance at 16 h following UV damage, but no significant affect on RPA phosphorylation at this time point (Figure S8A-C). Thus, loss of FANCJ expression could disrupt checkpoint activation, whereas expression of an enzyme inactive mutant could dominantly disrupt repair.

Further supporting that multiple pathways contribute to high fidelity repair after UV irradiation, similar to skin tumors from XP patients (56), MMR deficient and FANCJ-deficient cells display an elevated frequency of UV-induced C>T point mutations (Supplemental Table 1) (14). Similar to
FANCJ deficiency, MMR deficiency also has modest affects on UV sensitivity despite reduced checkpoint and apoptotic responses (12, 57). Thus, we propose that FANCJ intersects MMR and NER dependent repair pathways to promote efficient checkpoint activation, lesion clearance, and suppress UV-induced mutations (Figure 5G). Conceivably, in the absence of FANCJ and its checkpoint function error-prone polymerases induce mutations at sites of UV induced lesions. Indeed, the high-fidelity TLS polymerase, polη has reduced foci formation in response to global UV irradiation in FANCJ deficient cells (Figure S9A-C). Correspondingly, MSH2 deficient cells have defective UV-induced PCNA mono-ubiquitination and TLS foci formation (58).

The NER factor XPA contributes to the S phase checkpoint following UV-induced irradiation. However, not all NER factors are required suggesting that this checkpoint function is distinct from NER repair in G1 phase (4). Our findings further suggest that XPF promotes RPA phosphorylation in S phase cells (Figure 4A-C). Interestingly, XPF is the FA gene, FANCQ (30). Given that XPF promotes FANCJ accumulation in S phase cells, our data also suggest that FANCJ functions downstream of this FA factor to promote RPA phosphorylation throughout S phase. NER dependent incision may provide a better substrate or change the DNA structure, enabling distribution of FANCJ at the lesion site (Figure 5G). Here, FANCJ could facilitate repair of lesions ahead of the replication fork through checkpoint induction and the arrest of DNA synthesis to limit mutation induction. Conceivably this function is shared by FANCJ partners, such as BLM or the FA pathway explaining its link to the UV response and checkpoints that limit genomic instability (59-62). It has been long proposed that ATR-BLM and FA pathway interactions maintain genomic stability by restoring productive replication following replication stress (63-65).

The two-step mobilization of FANCJ to UV-induced lesions, localization by MMR and further accumulation after NER dependent post-incision could ensure pathway coordination. Indeed, the combined loss of NER and MMR enhances UV-induced mutagenesis (10). While MMR and NER proteins have been shown to have overlapping substrates (66), it remains to be determined whether they bind the same or a distinct type of UV lesion. FANCJ loading by MLH1 would be reminiscent of the requirement of the bacterial MutL, homologous to the MutLα complex, for loading helicase II (UvrD) onto DNA (67). Helicase II functions with DNA polymerase I to release oligonucleotide fragments containing UV photoproducts (68). In contrast to Helicase II, our data do not support a role...
for FANCJ or MMR in gap repair (Figures 4A-B,F-H; S4D-E). However, these findings do not exclude the possibility that MMR and FANCJ contribute to the fidelity of NER-dependent gap filling. Alternatively, loading of FANCJ by MMR factors could unwind and disrupt secondary DNA structures that impede NER processing. Indeed, MMR factors bind secondary structures such as G-4 quadruplex DNA that FANCJ unwinds (69, 70). We also reported that FANCJ depends on MLH1 for localization to sites of DNA interstrand crosslinks (29).

Collectively, the data presented in this manuscript provide a framework for understanding the contributions of distinct DNA repair pathways to the DNA damage response to UV irradiation in human cells. The identification of a novel function for MMR in localizing FANCJ to sites of UV induced damage could be useful for several reasons. First, it could help in the discrimination between missense and pathogenic MMR variants. Loss of FANCJ localization and function could be uniquely disrupted by MMR gene mutations as found in tumors in which canonical MMR is intact. Second, the MMR-FANCJ pathway could represent a unique tumor suppression pathway that provides opportunities for selective therapy in effected tumors. In melanoma, loss of FANCJ function or expression could be a consequence of not only FANCJ mutations (Figure 7A), but also MMR mutations. Indeed, ~5.7% of tumors are affected by FANCJ mutations, which did not co-segregate with MMR gene mutations (51, 52). Associated skin tumors may be selectively sensitive to ICL-inducing agents, which is a hallmark of FA-J patient cells. In light of the recent finding that XPF is the FA gene, FANCQ (30), it will be important to determine if the FA pathway has a more fundamental role in the response to UV irradiation and/or in reducing the emergence of disease.

Figure Legends:

Figure 1. FANCJ recruitment to sites of local UV-induced damage (LUDs) is dependent on NER dual incision and is predominantly in S phase (A) MCF7 cells were UV irradiated through 5 um micropore filters to generate LUDs and co-immunostained with the indicated Abs. Representative images are shown 1 h after UV irradiation. (B) Quantification of MCF7 cells positive for FANCJ, XPF, or 6-4 PP LUDs. (C) XP-A cells complemented with empty vector or XPAWT were UV irradiated through 3 um micropore filters to generate LUDs and co-immunostained with the indicated Abs. (D) Quantification of XP-A cells with FANCJ-positive LUDs. (E) XP-F cells complemented with empty vector, XPFWT, or XPF<sup>D676A</sup> were treated as in C and FANCJ-positive LUDs were quantified (F) XP-G cells complemented with empty vector, XPGWT, or XPG<sup>E791A</sup> were treated as in C and FANCJ-positive LUDs were quantified. (G) XP-F cells complemented with empty vector or XPFWT were UV irradiated through 5 um micropore filters, incubated with EdU, and co-immunostained with the indicated Abs.
(H) Quantification of XP-F cells with FANCJ-positive LUDs. Where shown, error bars represent the standard deviation of the mean of three independent experiments, asterisks denote significance from student’s two-tailed, unpaired t-test; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005.

**Figure 2. FANCJ recruitment to sites of local UV-induced damage (LUDs) is MMR dependent.**

(A) FA-J cells were complemented with empty vector, FANCJ<sup>WT</sup>, FANCJ<sup>S990A</sup>, or FANCJ<sup>K141/142A</sup> and analyzed by immunoblot. (B) FA-J cells were UV irradiated through 5 µm micropore membrane filters, co-immunostained with the indicated Abs, and quantified for FA-J cells with (C) FANCJ- and 6-4 PP-positive LUDs. (D) U2OS cells containing shRNA vectors targeting MLH1 or NSC were analyzed by immunoblot and (E) UV irradiated through micropore filters and (F) quantified for cells with FANCJ- or 6-4 PP-positive LUDs. (J) XP-F cells complemented with empty vector were stably depleted of MSH2 vs. NSC and analyzed by immunoblot. (K) Cells were treated as in E, and processed for EdU incorporation and co-immunostained with the indicated Abs and (L) quantified for cells with FANCJ-positive LUDs. Error bars represent the standard deviation of the mean of three independent experiments.

**Figure 3. FANCJ contributes to the UV-induced checkpoint response.**

(A) FA-J cells complemented with empty vector or FANCJ<sup>WT</sup> were left untreated and pulsed for 45 minutes with 10 µM EdU or globally UV irradiated and pulsed for 45 minutes with 10 µM EdU 16 h later. Cells were processed for EdU incorporation and co-stained with DAPI. (B) Quantification of EdU incorporation/total number of DAPI (+) cells. ≥1000 DAPI cells were quantified for each experiment in triplicate. (C) FA-J cells complemented with empty vector or FANCJ<sup>WT</sup> were left untreated or UV-irradiated and analyzed by FACS sorting for pS4/8 RPA32 positive cells, representative plots are shown. (D) FA-J cells were UV irradiated through 5 um micropore filters, incubated with 10uM EdU for 3 h, and co-immunostained with phospho-S4/8 RPA32 Ab. (E) Quantification of phospho-S4/8 RPA32 positive LUDs in S-phase cells. (F) MCF7 cells containing shRNA vectors targeting FANCJ or NSC were analyzed by immunoblot and (G) UV irradiated through micropore filters and (H) quantified for cells with FANCJ- or 6-4 PP-positive LUDs. (J) XP-F cells complemented with empty vector were stably depleted of MSH2 vs. NSC and analyzed by immunoblot. (K) Cells were treated as in E, and processed for EdU incorporation and co-immunostained with the indicated Abs and (L) quantified for cells with FANCJ-positive LUDs. Error bars represent the standard deviation of the mean of three independent experiments.

**Figure 4. FANCJ and MSH2 are required for NER dependent and independent induction of RPA phosphorylation in S phase, but not for gap repair**

(A) XP-F cells complemented with empty vector or XPFWT were stably depleted of FANCJ, MSH2, or NSC using shRNA vectors and analyzed by immunoblot. (B) Cells were UV irradiated through 5 um micropore filters, incubated with EdU, and co-immunostained with the indicated Abs 3 h post treatment (C) Quantification of phospho-S4/8 RPA32 positive LUDs in S phase cells expressing shNSC (D) shFANCJ and (E) shMSH2. (F) Quantification of NER dependent gap filling in non-S phase cells expressing shNSC (G) shFANCJ and (H) shMSH2. Where shown, error bars represent the standard deviation of the mean of three independent experiments.
Figure 5. FANCJ suppresses UV-induced mutations. (A) A549 cells expressing individual shRNA vectors targeting FANCJ or NSC were analyzed by immunoblot and (B) left untreated or globally UV irradiated and analyzed for colony survival. (C) Quantification of surviving colonies. (D) Quantification of 6-thioguanine (6-TG)-resistant HPRT mutant colonies from mutagenesis assay. (E) Quantification of the distribution of HPRT-inactivating mutations in A549 cells expressing shRNA to NSC and (F) or shRNAs to FANCJ (combined). (G) Model of FANCJ function in response to UV-irradiation. NER and MMR factors are recruited to sites of local UV induced damage in non-S phase cells where NER, but not MMR, is required for gap filling. In S-phase cells, both NER and MMR factors contribute to the accumulation of FANCJ. MMR through MLH1 binding localizes FANCJ to sites of UV induced damage. NER incision enhances the accumulation of FANCJ at the lesion site. Collectively, these events ensure a robust checkpoint response to limit the replication of damaged DNA, induction of mutations, and cancer. Where shown, error bars represent the standard deviation of the mean of three independent experiments.

Funding: This work was supported by the National Institutes of Health [RO1 CA129514-01A1] and from charitable contributions from Mr. and Mrs. Edward T Vitone Jr.

Acknowledgements: We thank Dr. Chris Heinen (University of Connecticut Health Center) for comments on the manuscript. Special thanks to Dr. John Hays (Oregon State University) for helpful discussions. We also thank Benjamin Morehouse, Caroline Brown, and Nimisha Patil for technical assistance and quantification of experiments.

Author Contribution: Shawna Guillemette, Amy Branagan, Min Peng, and Aashana Dhruva performed experiments, Sharon Cantor directed experiments and prepared manuscript, and Orlando Schärer provided reagents and prepared manuscript.

References:

5. Gilljam KM, Muller R, Liabakk NB, Otterlei M. Nucleotide excision repair is associated with the replisome and its efficiency depends on a direct interaction between XPA and PCNA. PloS one 2012;7: e49199.


Figure 1

A. MCF7 Cells

B. % Cells with (+) LUD

C. XP-A + Vector vs. XP-A + XPAWT

D. % Cells with FANCJ (+) LUD

E. XP-F cells

F. XP-G cells

G. XP-F cells EdU/DAPI

H. % Cells with FANCJ (+) LUD (3h)
Figure 5

A

\[ \text{shNSC} \quad \text{shFANCJ-1} \quad \text{shFANCJ-2} \]

\[
\begin{array}{c}
\text{FANCJ} \\
\beta\text{ACTIN}
\end{array}
\]

A549 cells

B

\[ \text{A549 cells} \]

C

\[ \%\text{Colony Survival} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \quad 120 \]

\[ 0 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \quad 9 \quad 10 \quad 11 \quad 12 \]

\[ \text{shNSC} \quad \text{shFANCJ-1} \quad \text{shFANCJ-2} \]

D

\[ \text{A549 cells} \]

E

\[ \text{shNSC} \]

\[ \begin{array}{c}
\text{C>T NTS} \\
\text{C>T TS} \\
\text{T>C NTS} \\
\text{T>A NTS} \\
\text{T>A TS} \\
\text{C>G NTS}
\end{array} \]

\[ \begin{array}{c}
18.2\% \\
54.5\% \\
18.2\% \\
9.1\% \\
12.9\% \\
2.4\%
\end{array} \]

F

\[ \text{shFANCJ} \]

\[ \begin{array}{c}
\text{C>T NTS} \\
\text{C>T TS} \\
\text{T>C NTS} \\
\text{T>A NTS} \\
\text{T>A TS} \\
\text{C>G NTS}
\end{array} \]

\[ \begin{array}{c}
24.7\% \\
52.9\% \\
12.9\% \\
2.4\% \\
4.7\% \\
2.4\%
\end{array} \]

G

Cell Cycle

NER

MMR

NER

ERCC1

XPD

MMR

FANCI

FANCA

FANCG

FANCJ

FANCN

XPA

XPG

MLH1

MLH3

Checkpoint

And Repair

Mutations

Replication fork

G1

S

UVC
FANCI localization by mismatch repair is vital to maintain genomic integrity after UV irradiation

Sharon B. Cantor, Shawna Guillemette, Amy Branagan, et al.

Cancer Res Published OnlineFirst December 18, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-2474

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/12/18/0008-5472.CAN-13-2474.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.