

The FANCI/MutL α interaction is required for correction of the cross-link response in FA-J cells

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FANCI also called BACH1/BRIP1 was first linked to hereditary breast cancer through its direct interaction with BRCA1. FANCI was also recently identified as a Fanconi anemia (FA) gene product, establishing FANCI as an essential tumor suppressor. Similar to other FA cells, FANCI-null (FA-J) cells accumulate 4N DNA content in response to DNA interstrand crosslinks (ICLs). This accumulation is corrected by reintroduction of wild-type FANCI. Here, we show that FANCI interacts with the mismatch repair complex MutL α , composed of PMS2 and MLH1. Specifically, FANCI directly interacts with MLH1 independent of BRCA1, through its helicase domain. Genetic studies reveal that FANCI helicase activity and MLH1 binding, but not BRCA1 binding, are essential to correct the FA-J cells' ICL-induced 4N DNA accumulation and sensitivity to ICLs. These results suggest that the FANCI/MutL α interaction, but not FANCI/BRCA1 interaction, is essential for establishment of a normal ICL-induced response. The functional role of the FANCI/MutL α complex demonstrates a novel link between FA and MMR, and predicts a broader role for FANCI in DNA damage signaling independent of BRCA1.

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Introduction

In the absence of DNA repair proteins, cell cycle checkpoints and/or DNA damage repair pathways are not properly activated. This inability to actively respond to DNA damage can lead to massive chromosomal damage and even cell death. In some cases, mutations in DNA repair proteins can contribute to multiple cancer syndromes. Studies on the genetic causes of the cancer-prone syndrome Fanconi anemia (FA) revealed

that genetic mutations associated with hereditary breast cancer were also associated with FA. For example, the hereditary breast cancer gene, *BRCA2* was shown to be the gene defect in the FA-D1 patient complementation group, revealing that *BRCA2* was *FANCD1* (Howlett *et al*, 2002). Likewise, *FANCI* (also called *BACH1/BRIP1*) was identified as the gene defective in the *FANCI*-null (FA-J) patient complementation group (Levitus *et al*, 2005; Levran *et al*, 2005; Litman *et al*, 2005), and was initially linked to hereditary breast cancer. This link was based on its direct binding to *BRCA1* and through the identification of two breast cancer patients with mutations in *FANCI*, which also altered its helicase activity *in vitro* (Cantor *et al*, 2004, 2001). This connection was furthered by the finding that *FANCI* (*BRIP1*) mutations confer a two-fold increase in the risk of developing breast cancer (Seal *et al*, 2006).

While other FA genes have not been linked to breast cancer, the network of at least 13 genes (designated *FANCA* to *FANCN*) is critical for maintaining chromosomal integrity (Thompson, 2005). Although the molecular function of these proteins is not clear, several gene products, including *FANCA*, *B*, *C*, *D*, *E*, *F*, *G*, *L* and *M*, form a nuclear core complex (the FA core complex), that is required for monoubiquitination of *FANCD2*. The FA proteins *BRCA2/FANCD1*, *PALB2/FANCN* and *FANCI* are not required for this event and are considered downstream of *FANCD2* monoubiquitination. Nevertheless, all FA proteins contribute to processing interstrand crosslinks (ICLs) (Thompson, 2005). Consequently, in the absence of FA proteins, ICL treatment leads to reduced cell viability and an accumulation of cells with a 4N DNA content representing cells in either late S or G2/M. This ICL-induced cell cycle progression defect and sensitivity to ICLs is restored upon reintroduction of the missing FA gene (Dutrillaux *et al*, 1982; Kaiser *et al*, 1982; Kupfer and D'Andrea, 1996; Kupfer *et al*, 1997; Heinrich *et al*, 1998; Sala-Trepat *et al*, 2000; Chandra *et al*, 2005). However, the FA-related function or associated partners required for a proper ICL response is not known.

Consistent with other FA cells, FA-J cells have an ICL-induced cell cycle progression defect that can be corrected upon re-introduction of wild-type (WT) *FANCI* cDNA (Litman *et al*, 2005). This cell cycle progression defect has also been described as a prolonged G2/M arrest (Miglierina *et al*, 1991) or 4N DNA content accumulation (Akkari *et al*, 2001). The cause of this ICL response in FA cells is not presently understood, but is thought to involve delayed repair and/or failure to restart replication (Thompson *et al*, 2005). Unlike the majority of FA proteins, *FANCI* has defined domains. Specifically, *FANCI* binds directly to *BRCA1* (Cantor *et al*, 2001) and is a DNA helicase (Cantor *et al*, 2004). Determining the importance of these domains could further our understanding of how FA proteins function in an ICL-induced response. Attempts to define the functions of *FANCI* domains in the ICL response have been limited to chicken DT40 cells, where the *FANCI/BRCA1* interaction is not conserved (Bridge *et al*, 2005). If *FANCI* operates

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independent of BRCA1 for a particular ICL response function, a remaining question will be whether FANCJ forms a complex with other proteins independent of BRCA1 to perform that function.

Here, we investigated whether FANCJ helicase activity or the FANCJ interaction with two distinct proteins was required for restoring FANCJ's ICL response. Specifically, we identified that FANCJ interacts with the MutL α mismatch repair complex, independent of BRCA1. Our findings demonstrate for the first time that the FANCJ/MLH1 interaction is as critical as FANCJ helicase activity for restoring a normal cell cycle progression and resistance of FA-J cells to ICLs. In contrast, the FANCJ/BRCA1 interaction is dispensable for normalizing the response of FA-J cells to ICLs, suggesting that FANCJ functions in distinct complexes to facilitate multifaceted DNA repair functions.

Results

FANCJ functions independently of BRCA1 to correct FA-J cells

We had previously shown that introduction of WT FANCJ cDNA into FA-J cells corrects the ICL-induced cell cycle progression defect (Litman *et al*, 2005). However, it is unclear how FANCJ contributes to the ICL response to restore the FA pathway, especially given that FANCJ's role in the FA pathway appears to be independent of BRCA1, at least in chicken cells (Bridge *et al*, 2005). To verify and extend this finding, we addressed whether FANCJ binding to BRCA1 was required to correct the ICL-induced cell cycle progression defect in FA-J cells. We reconstituted FA-J cells with vector, WT, or the S990A FANCJ construct that is ablated for BRCA1 binding (Yu *et al*, 2003) (Figure 1C). Both WT and S990A versions of FANCJ corrected the ICL-induced cell cycle progression defect observed in FA-J cells compared to vector alone (Figure 1A and B). These data support the finding that FANCJ operates independent of BRCA1 to correct FA-J cells.

FANCJ is physically linked to the MutL α complex

Since FANCJ binding with BRCA1 was not required to correct the ICL-induced cell cycle progression defect in FA-J cells, we set out to identify additional FANCJ interacting partners that may function with FANCJ in this ICL-induced response. A WT double tagged FANCJ construct was used to create a stable line of HeLa S3 cells. Using a two-step immunoaffinity strategy, the double tagged FANCJ was sequentially immunopurified (Nakatani and Ogrzyzko, 2003). Interacting proteins co-purifying with the double tagged WT FANCJ were eluted and visualized by silver stain. FANCJ migrated at the expected ~140 kDa size (Figure 2A). Individual bands were excised from the gel and analyzed by mass spectrometry (LC-MS/MS). As expected, FANCJ copurified with BRCA1 that was identified as the 250 kDa band. Unique partners were identified, including the MMR proteins, MLH1 and PMS2, which form the MutL α heterodimer (Schofield and Hsieh, 2003) (Figure 2A). Western blot analyses using specific antibodies (Abs) confirmed the presence of these proteins (Figure 2B). To determine whether the MutL α complex associated with the native FANCJ protein, MCF7 cell extracts were immunoprecipitated (IP) with FANCJ Abs E67 and E47, and the presence of co-precipitating MLH1, PMS2 and BRCA1 proteins was evaluated by Western blot (Figure 2C). While

FANCJ Ab precipitated the MutL α complex in the MCF7 cells, a MutL α complex was not precipitated with preimmune Abs (PI) or FANCJ Abs, in 293T cells, which lack expression of the MutL α complex (Trojan *et al*, 2002). Moreover, FANCJ was not precipitated with the MLH1 Ab in FA-J cells, which lack expression of FANCJ, unless FANCJ was reintroduced (Figure 2D). In contrast, a FANCJ/MLH1 interaction was readily detected in other FA cell lines, irrespective of gene correction, such as FA-A, FA-D1 and FA-D2 (Supplementary Figure 1). Furthermore, the interaction between FANCJ and the MutL α complex was stable in HeLa cells in the presence or absence of DNA damage (Figure 2E).

The helicase domain of FANCJ binds directly to MLH1 independent of BRCA1

MLH1 was previously reported to be part of a BRCA1 complex (Wang *et al*, 2000; Greenberg *et al*, 2006); therefore, we examined whether BRCA1 mediated the interaction between FANCJ and the MutL α complex. First, we noted that unlike FANCJ, BRCA1 was not readily detected in an MLH1 precipitation (Figure 2C). Next, we addressed whether FANCJ precipitated with the MutL α complex in BRCA1-deficient cells. Expression of BRCA1 was stably suppressed in MCF7 cells by an shRNA vector, as previously demonstrated (Litman *et al*, 2005). In cells expressing both a control shRNA specific to eGFP or an shRNA specific to BRCA1, FANCJ Abs efficiently co-precipitated the two components of the MutL α complex (Figure 3A), suggesting that FANCJ binds the MutL α complex independent of BRCA1. In support of this finding, the helicase domain of FANCJ was required for MLH1 binding, while a C-terminal region of FANCJ was required for BRCA1 binding (Figure 3B). To further assess the nature of the FANCJ/MLH1 interaction, we incubated recombinant FANCJ or BRCA1 with MLH1 that had been translated *in vitro*. MLH1 and FANCJ were precipitated by their corresponding Abs, and their interactions were analyzed by Western blot. FANCJ and MLH1 proteins were co-precipitated with both FANCJ and MLH1 IPs, whereas BRCA1 was robustly precipitated only in the FANCJ IP (Figure 3C). A direct interaction between FANCJ and MLH1 was confirmed by ELISA assay using purified recombinant proteins. FANCJ bound MLH1 in a protein concentration-dependent manner (Figure 3D). Furthermore, the interaction of FANCJ and MLH1 was demonstrated to be DNA independent, as evidenced by the similar colorimetric signal observed for FANCJ/MLH1 interaction in the presence of ethidium bromide (EtBr) or DNaseI (Figure 3E). These results suggest that FANCJ makes direct contacts with MLH1, independent of BRCA1 or PMS2.

PMS2 contributes to the FANCJ/MLH1 interaction in vivo

Given that MLH1 forms a heterodimer with PMS2, we next assessed whether PMS2 binding to MLH1 contributed to the MLH1/FANCJ interaction *in vivo*. To address this possibility, we tested the ability of different MLH1 constructs to precipitate FANCJ in the absence or presence of PMS2. WT full-length MLH1 and several MLH1-myc fusion proteins of varying length were generated and transiently transfected into MutL α -null 293T cells. To determine which of these MLH1 fragments were expressed and/or co-IPed FANCJ, MLH1 was precipitated from cell lysates with either myc or

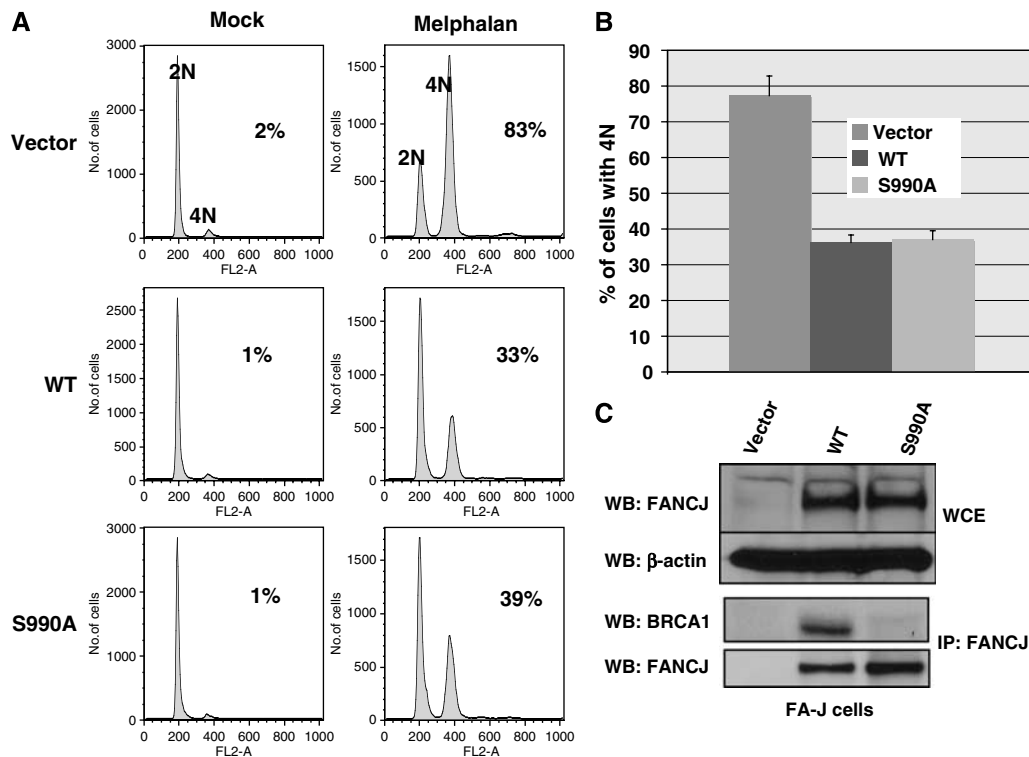


Figure 1 The FANCD2/BRCA1 interaction is dispensable for correction of the 4N DNA accumulation defect in FA-J cells. (A) FA-J cells were reconstituted with vector, WT or S990A, and FANCD2 expression was analyzed in whole-cell extracts (WCE) by Western blot. β -Actin served as a loading control for the WCE samples. (B) Immunoprecipitations with FANCD2 (E67) were analyzed by Western blot with the indicated Abs. (C) FA-J cells reconstituted with vector, WT, or S990A FANCD2 were either left untreated or treated with melphalan, and the percentage of cells with 4N DNA content was analyzed by FACS. The percentage of cells with 4N DNA content after ICL-treatment was averaged for each cell line from four independent experiments, with standard deviation (s.d.) indicated by error bars.

MLH1 Abs. While cotransfecting PMS2 with MLH1 did not alter the expression of MLH1, the ability of FANCD2 to form a complex with MLH1 was enhanced. With the addition of PMS2, FANCD2 precipitated with the MLH1 constructs N2, C2, and C3, which in the absence of PMS2 had failed to precipitate FANCD2 (Figure 4A). Thus, in the presence of PMS2, only one of the two MLH1-FANCD2 interacting domains (478–508) (D1) or (736–744) (D2) was required (see Figure 4C), suggesting that PMS2 facilitates the MLH1/FANCD2 interaction. PMS2 stability is dependent on the MLH1 C-terminus (Mohd *et al*, 2006); not surprisingly, we found that ablation of a C-terminal region of MLH1 (703–725) reduced both PMS2 and FANCD2 binding (Figure 4B).

MutL α functions downstream of FANCD2 monoubiquitination

To appreciate the physiological significance of a FANCD2/MutL α interaction, we next, addressed whether the MutL α complex functioned with FANCD2 in the FA pathway. We had previously shown that in FANCD2-deficient cells, DNA damage-induced FANCD2 monoubiquitination was intact (Litman *et al*, 2005). Similarly, we found that incubation of MutL α -deficient cells (HCT116 and HEC-1A) with hydroxyurea (HU) leads to efficient FANCD2 monoubiquitination (Supplementary Figure 2A), suggesting that similar to FANCD2, MutL α functions downstream of FANCD2.

Given that suppression of MMR proteins has been reported to reduce the survival of cells upon ICL-treatment, (Aquilina *et al*, 1998; Fiumicino *et al*, 2000), we next asked whether

similar to FANCD2 deficiency, MutL α deficiency also sensitizes cells to ICLs. First, we suppressed MutL α using siRNA reagents in MCF7 cells versus a luciferase control. Second, we reconstituted HCT116 cells null for MutL α with vector or MutL α expressing cDNAs. In both experiments, there was no measurable change in ICL sensitivity, in the presence or absence of MutL α expression (Supplementary Figure 2B and data not shown). Given that MMR proteins bind and process ICLs (Duckett *et al*, 1996; Yamada *et al*, 1997; Zhang *et al*, 2002), activate multiple DNA damage-induced checkpoints, such as intra S and G2/M (4N) arrest (Brown *et al*, 2003; Cejka *et al*, 2003), and participate in the repair of ICLs by promoting recombination (Zheng *et al*, 2006), we considered that MutL α suppression could bypass ICL sensitivity through loss of checkpoint (Cejka *et al*, 2003), and/or by activating default non-recombination-based repair pathways, as reported (Zheng *et al*, 2006). Thus, we considered that to unmask function of MutL α in the ICL response with FANCD2, it would be necessary to selectively ablate the MLH1/FANCD2 interaction, while maintaining other MLH1 functional interactions (i.e. PMS2 binding).

Disruption of the native MLH1/FANCD2 interaction generates ICL sensitivity

To define the domain on FANCD2 required for MLH1 binding, we generated several FANCD2-myc fusion proteins of varying length and expressed them in MCF7 cells (Figure 5A and E). To determine which of these FANCD2 fragments were expressed and/or co-IPed MLH1, FANCD2 was precipitated from

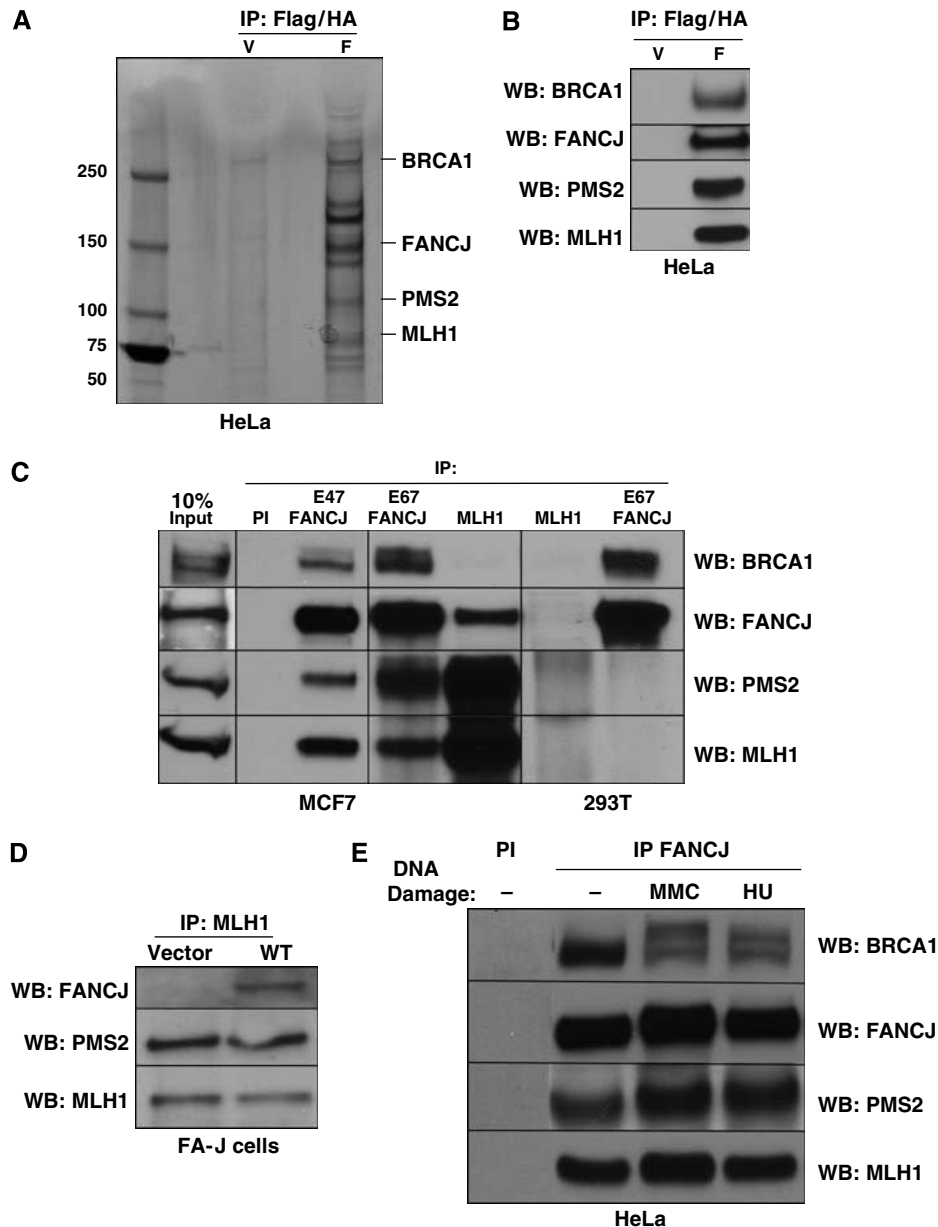


Figure 2 FANCJ interacts with the MMR proteins MLH1 and PMS2. (A) Silver-stained gel of the WT FANCJ (F) compared to vector (V)-purified complexes from HeLa S3 cells by consecutive Flag and HA purification steps (Flag/HA). Identified unique bands are indicated and FANCJ is observed as two species, the 140 kDa band is labeled. (B) Western blot detection of Flag/HA-purified FANCJ complexes. (C) Immunoprecipitations with either FANCJ (E67 or E47) or MLH1 Abs from MCF7 or 293T cells were analyzed by Western blot with the indicated Abs. (D) Western blot shows the presence of the indicated proteins from MLH1 IPs from FA-J cells reconstituted with vector or WT FANCJ. (E) HeLa cells were either left untreated or treated with 1 mM HU for 24 h or 2.4 µg/ml MMC for 1 h. HeLa cell lysates were IPed with PI or FANCJ Abs followed by Western blot analysis with the indicated Abs.

cell lysates with myc Abs. Full-length FANCJ and FANCJ expression constructs including the FANCJ N-terminal amino-acid residues 1–145 precipitated MLH1 (Figure 5A and C). These results suggested that FANCJ N-terminal residues 1–145 were required for binding to MLH1. To assess whether residues in this region were sufficient for MLH1 binding, we inserted FANCJ residues 128–158 within the eGFP gene sequence to create an eGFP-fusion protein. In contrast to eGFP alone, the eGFP-FANCJ fusion protein readily co-precipitated MLH1 (Figure 5B), suggesting that FANCJ 128–158 was sufficient for MLH1 binding. Furthermore, expression of the eGFP-FANCJ fusion protein

in cells perturbed the formation of the native FANCJ/MLH1 interaction, as determined by both FANCJ and MLH1 IP and Western blot experiments (Figure 5D), confirming that this region of FANCJ was essential for mediating the MLH1 interaction.

Next, we addressed whether expression of the 128–158 FANCJ-eGFP fusion protein and the resulting perturbation of the native FANCJ/MLH1 interaction would render cells sensitive to ICLs. MCF7 cells were transfected with vectors expressing either the 128–158 FANCJ-eGFP fusion protein or eGFP alone, plated and treated with increasing concentrations of Mitomycin C (MMC). The overall trend upon expres-

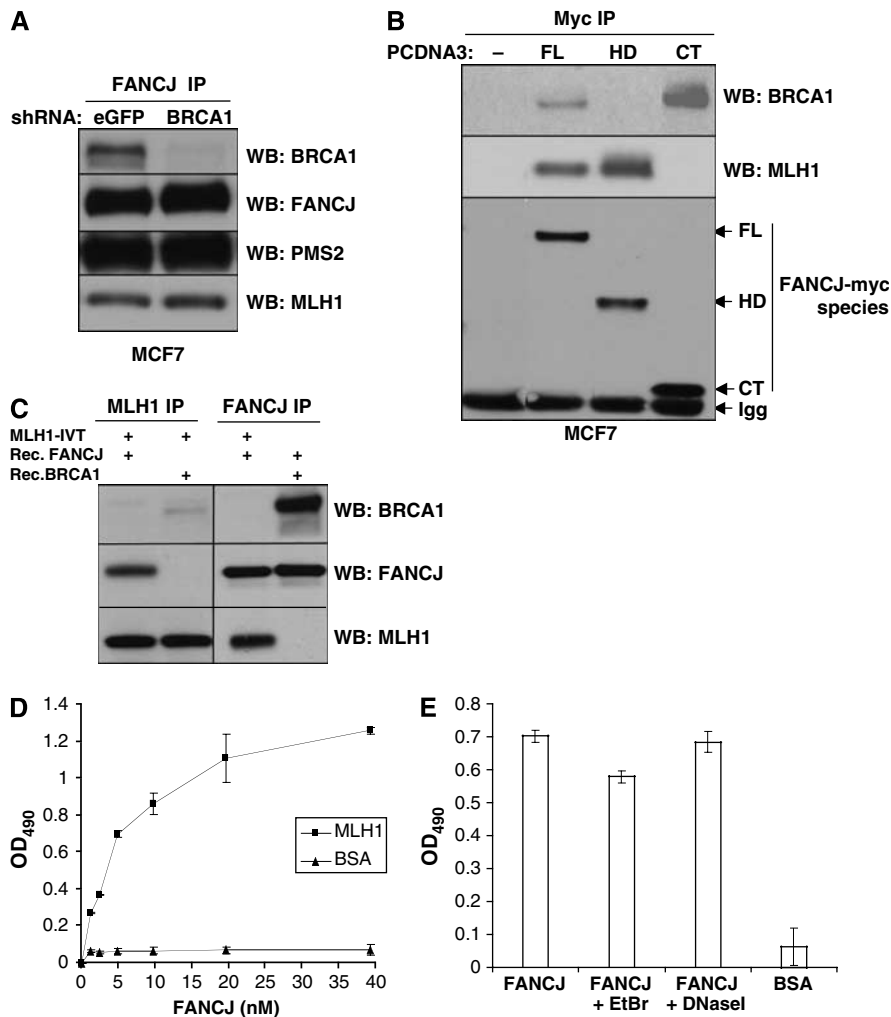


Figure 3 FANCF helicase domain associates with the MutL α complex independent of BRCA1 and through a direct interaction with MLH1. (A) MCF7 cells were stably infected with a lentivirus encoding shRNA for either eGFP or BRCA1. FANCF IP was performed followed by Western blot for the indicated proteins. (B) MCF7 cells were transiently transfected with pCDNA3 vectors containing no insert (-), full-length FANCF (FL), helicase domain including amino-acid residues 1–882 (HD) or C-terminus including residues 882–1249 (CT) of FANCF, and then IPed with the Myc Ab (9E10). Arrows indicate the respective FANCF myc-tagged species. Immunoglobulin (IgG) is shown. (C) Western blot of the indicated IP experiments in which *in vitro* translated MLH1 was incubated with recombinant FANCF or BRCA1 proteins. (D) Purified recombinant MLH1 or BSA was coated onto ELISA plates. Following blocking with 3% BSA, the wells were incubated with increasing concentrations of purified recombinant FANCF (0–40 nM) for 1 h at 30°C, and bound FANCF was detected by ELISA using a rabbit polyclonal Ab against FANCF, followed by incubation with secondary horseradish peroxidase (HRP)-labeled Abs and OPD substrate. Data points are the mean of three independent experiments performed in duplicate, with s.d. indicated by error bars. (E) ELISA was performed as described in panel D using 4.9 nM FANCF alone or in the presence of EtBr (50 μ g/ml) or DNaseI (2 μ g/ml). BSA (3%) was used as a control instead of MLH1 during the coating step.

sion of the 128–158 FANCF-eGFP fusion protein was reduced cellular survival compared to expression of the eGFP control, despite some variability between experiments (Figure 5E). While the enhanced sensitivity was consistent with the possibility that a FANCF/MLH1 interaction was required for ICL repair, we considered that binding of the fusion protein to MLH1 might have altered additional MLH1 functions not specific to FANCF. Thus, we sought to identify a method to ablate the FANCF/MLH1 interaction without altering native MLH1 protein or being reliant on transfection efficiency to disrupt the native FANCF/MLH1 interaction.

Lysines 141 and 142 of FANCF are required for the FANCF/MLH1 interaction

Given that mutational analysis revealed that FANCF co-precipitated with MLH1, except when FANCF residues

140–145 were absent (Figure 5A and C), we assessed the importance of these residues for binding MLH1 within the context of the full-length FANCF protein. Thus, we generated three independent FANCF mutant constructs that converted lysine 141 and 142 to alanine (K141/142A), glutamine 143 to a glutamic acid (Q143E), or serine 145 to an alanine (S145A). While the WT FANCF and all three mutant versions were expressed and efficiently co-precipitated BRCA1, the K141/142A version demonstrated a dramatic reduction in the co-precipitation of MLH1 (Figure 6A), suggesting that these two lysines were required for MLH1 binding.

We considered that the K141/142A mutation in FANCF could have not only disrupted MLH1 binding, but also FANCF helicase activity. Thus, we generated recombinant versions of WT (Cantor *et al*, 2004) and the K141/142A FANCF proteins to assess whether this mutant version was

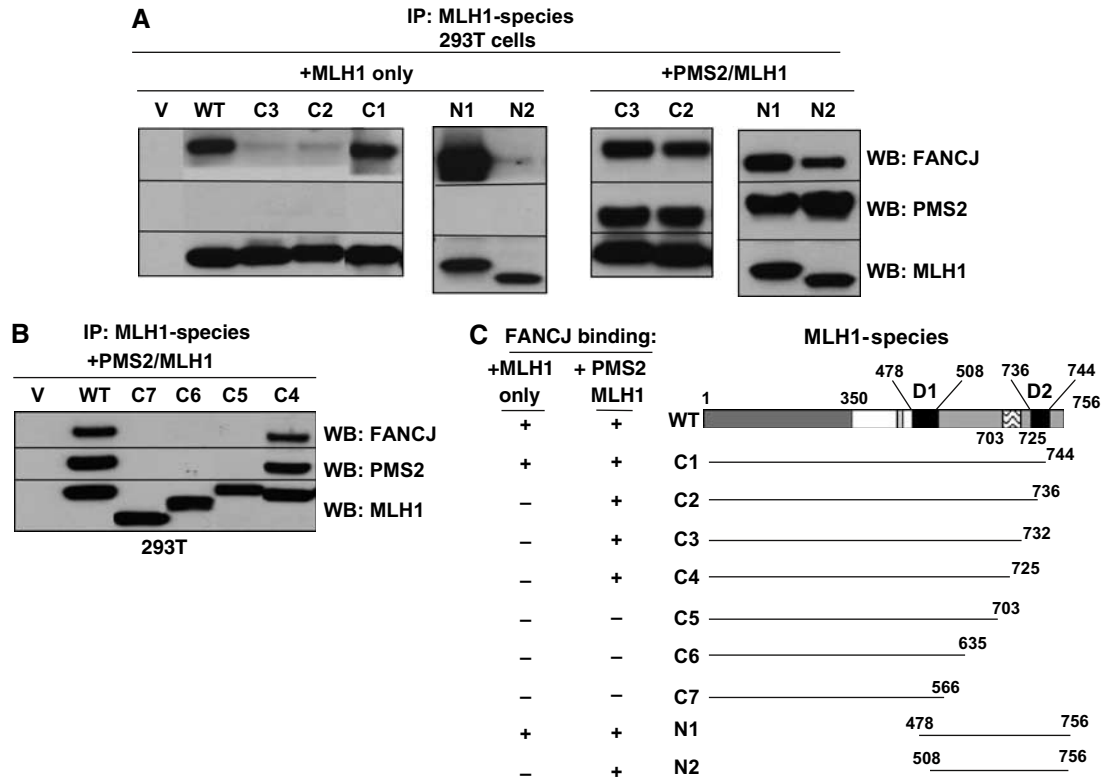


Figure 4 PMS2 facilitates the FANCF interaction with the MLH1 C-terminus. (A) MLH1 or Myc (9E10) IP experiments were performed from 293T cells that were transfected with vector alone (V), full-length MLH1 (WT) or MLH1 species, alone or in combination with PMS2 (C1–C3, N1, N2). IP products were analyzed by Western blot with FANCF, PMS2, and MLH1 Abs. (B) MLH1 IP experiments were performed from 293T cells that were transfected with vector alone (V), full-length MLH1 (WT) or MLH1 species, in combination with PMS2 (C4–C7). IP products were analyzed by Western blot with FANCF, PMS2, and MLH1 Abs. (C) Schematic representation of the MLH1/FANCF dimer domains (D1, D2), and the region between 703–725 is highlighted as an essential element for maintaining the MLH1/PMS2/FANCF complex.

enzymatically active. The recombinant K141/142 FANCF protein was detected as a single Coomassie-stained band analyzed by SDS-PAGE, that co-migrated with the WT FANCF recombinant protein (Supplementary Figure 3A and D). The DNA unwinding activity of K141/142A FANCF on a forked duplex DNA substrate was compared to unwinding activity of WT FANCF. Both K141/142A FANCF and WT FANCF were found to be proficient in unwinding, whereas K52R FANCF, as previously demonstrated, failed to unwind the forked duplex substrate (Gupta *et al*, 2005) (Supplementary Figure 3B). Furthermore, K141/142A FANCF and WT FANCF unwound the forked duplex substrate in a protein concentration-dependent manner, achieving 90% of unwound substrate at the highest helicase concentration tested (Supplementary Figure 3C). Thus, the K141/142 mutant only disrupts MLH1 binding, but not FANCF helicase activity.

FANCF function depends on MLH1 binding to correct FA-J cells

Next, we tested the ability of K141/142A FANCF cDNA to correct the cell cycle progression defect in FA-J cells. We used retroviral infection to stably infect FA-J cells with cDNA encoding the vector, WT, K141/142A or K52R Flag/HA-tagged FANCF constructs, which expressed similarly (Figure 6A). Moreover, an MLH1 IP demonstrated that the MLH1/PMS2 complex was intact in FA-J cells and was able to precipitate the reconstituted FANCF (Figure 2D). As in MCF7 cells, in

FA-J cells MLH1 co-IPed with WT FANCF, but was dramatically reduced with the K141/142A mutant FANCF (Figure 6A). FA-J cells containing vector, WT, K52R, or K141/142A FANCF were treated with melphalan to induce ICLs, as described (Litman *et al*, 2005). The proportion of vector containing FA-J cells with 4N DNA content increased after melphalan treatment, similar to previous experiments. As before, the proportion of WT FANCF containing FA-J cells with 4N DNA content (~30%) was about half that of vector containing cells (~70%). We found that cells containing the catalytically inactive FANCF helicase (K52R) failed to correct the 4N accumulation defect (~66%). Likewise, the proportion of K141/142A FANCF containing FA-J cells with 4N DNA content (~68%) resembled that of vector containing FA-J cells, suggesting that introduction of K141/142A FANCF did not correct the cell cycle progression defect in FA-J cells (Figure 6B). These data suggest that the FANCF/MLH1 interaction is essential for restoration of the FA pathway in FA-J cells.

To further confirm and examine the importance of FANCF binding to MLH1 for the ICL-induced response, we assessed whether FANCF binding to MLH1 was required for FANCF to correct the ICL-induced sensitivity of FA-J cells. However, the ability of WT FANCF to correct the ICL sensitivity of FA-J cells had not been previously reported. Thus, we first tested and confirmed that reintroduction of WT FANCF corrected the ICL sensitivity of FA-J cells treated with MMC. The vector-reconstituted FA-J cells were

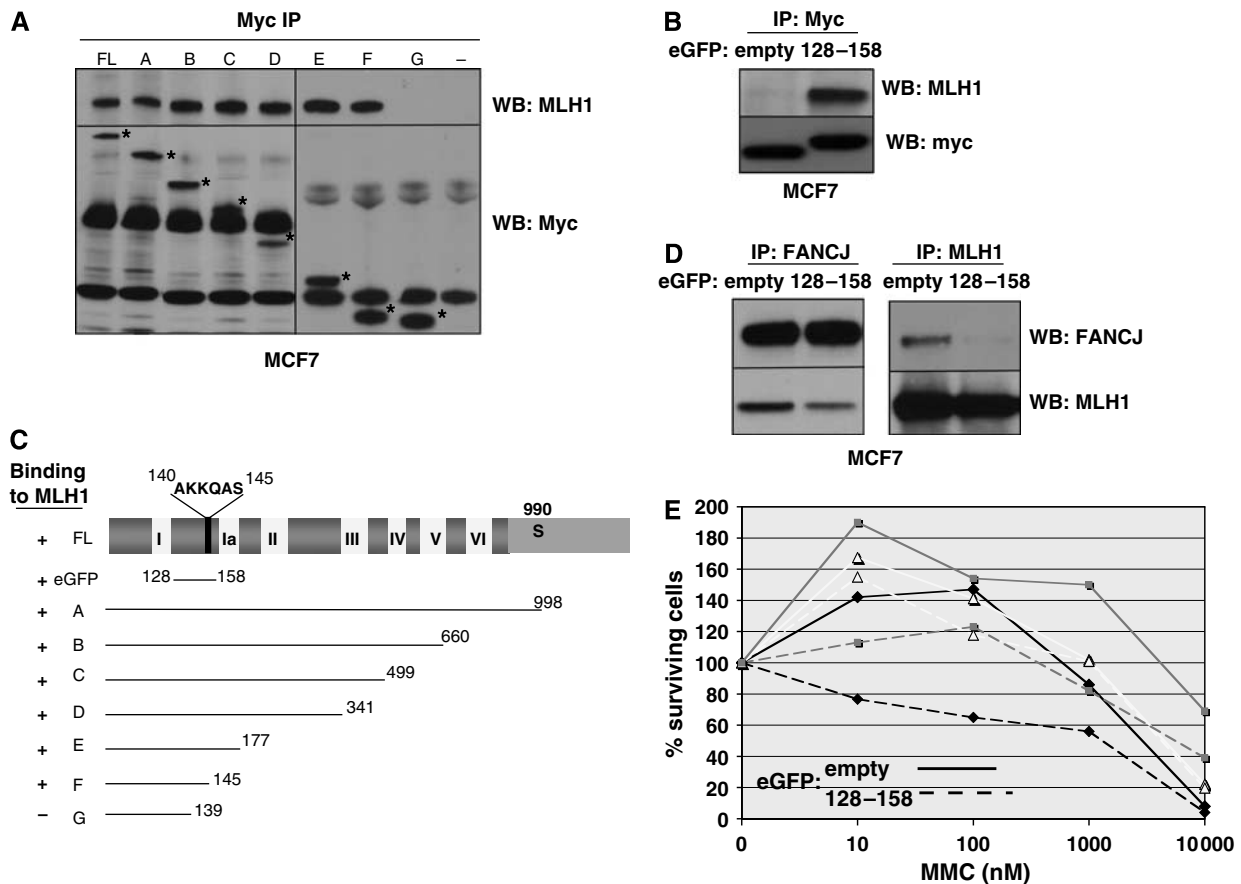


Figure 5 Expression of FANCF residues 128–158 disrupts the FANCF/MLH1 interaction to generate ICL sensitivity. (A) Myc (9E10) IP experiments were performed from MCF7 cells that were transfected with vector alone (–), full-length FANCF (FL) and the different FANCF constructs (A–G) shown in panel C, followed by Western blot with MLH1, and Myc Abs. The asterisk denotes the migration of the different myc-tagged FANCF species. (B, D) Myc IP experiments were performed from MCF7 cells that were transfected with either eGFP empty vector or the 128–158 FANCF-eGFP constructs, followed by Western blot with the indicated Abs. (C) The different FANCF constructs are indicated with a positive (+) or negative (–) to indicate binding to MLH1. (E) MCF7 cells transfected with vector alone or the 128–158 FANCF-eGFP construct, treated with increasing concentrations of MMC and incubated for 4–5 days. Cell growth was measured by ATP content. Three independent representative experiments are shown and depicted by lines with squares, triangles, and diamonds. Solid lines represent cells transfected with empty-eGFP vector and hatched lines represent cells transfected with 128–158-eGFP vector (for color figure see online version).

more sensitive to MMC than the WT FANCF-reconstituted cells with an IC_{50} of 250 and ~900 mM MMC, respectively (Figure 6C). In contrast to WT, both the K141/142A- and K52R FANCF-reconstituted FA-J cells were sensitive to ICLs with an IC_{50} of less than 250 mM MMC. Moreover, the K141/142A FANCF-reconstituted FA-J cells were more sensitive than the vector or K52R FANCF-reconstituted FA-J cells ($P < 0.01$). As before, the S990A FANCF corrected the ICL response similar to WT. Although the correction of the ICL sensitivity was greater with the S990A FANCF than with the WT FANCF, at 250 mM MMC the values were not significantly different (with P -values between 0.0027 and 0.22) (Figure 6C). These findings clearly demonstrate that both FANCF helicase activity and FANCF binding to MLH1 are required for FANCF to functionally correct the ICL sensitivity of FA-J cells.

Discussion

In this study, we addressed whether FANCF helicase activity or different FANCF complexes are essential for FANCF's

function in the ICL response. Specifically, we have shown that FANCF forms a complex with the MutL α heterodimer, which is composed of the mismatch repair proteins MLH1 and PMS2. FANCF directly interacts with MLH1 independent of BRCA1, and this DNA-independent interaction is within the FANCF helicase domain, C-terminal to nucleotide binding box 1, and includes lysines 141 and 142. This is the first report that demonstrates that a direct interaction between FANCF and MLH1 is as essential for the ICL-induced response as the FANCF helicase activity. Furthermore, our data suggest that formation of a FANCF/BRCA1 complex is not required for normalization of the ICL-induced response in FA-J cells.

The question remains as to how FANCF or mismatch repair proteins function in the ICL response. Following exposure to ICLs, ATR is activated and initiates a signal cascade through the phosphorylation of downstream substrates, ultimately leading to checkpoint activation, DNA damage repair and/or apoptosis. Intriguingly, MMR proteins were recently proposed to act as direct sensors of DNA methylation and initiate the intra S-phase checkpoint, by helping to recruit ATR-ATRIP

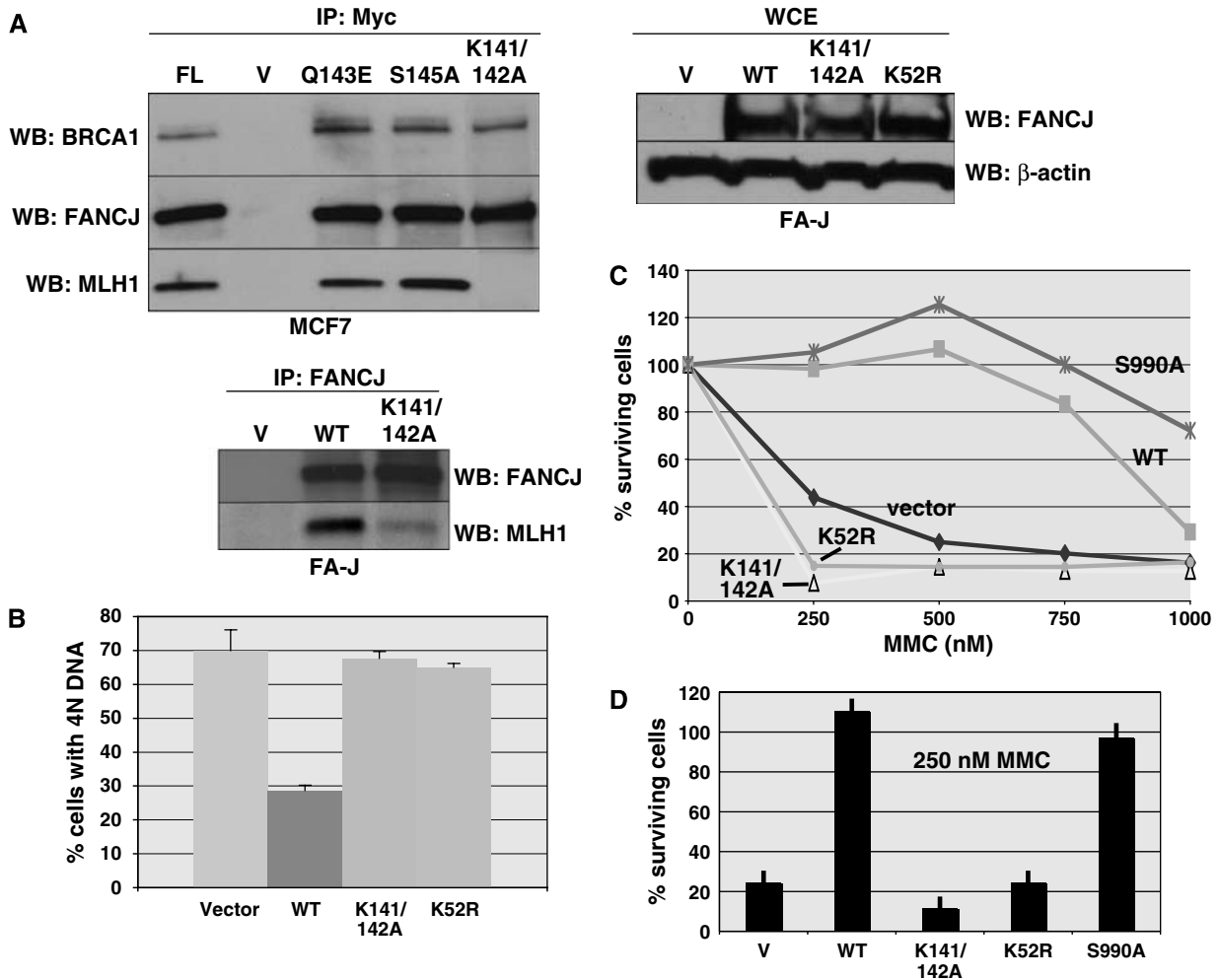


Figure 6 MLH1 binding to FANCJ is essential to correct FA-J cells. (A) Myc IP experiments were performed from MCF7 cells that were transfected with vector alone (–), FL, V, Q143E, S145A, and K141/142A FANCJ constructs, followed by Western blot with the indicated Abs. FA-J cells were reconstituted with empty vector, WT, K141/142A, or K52R FANCJ vectors, and FANCJ expression was analyzed by whole-cell extracts; β -actin served as a loading control for the WCE samples. Western blot shows the presence of the indicated proteins from FANCJ IPs from FA-J cells reconstituted with vector, WT, or K141/142A FANCJ. (B) FA-J cell lines reconstituted with empty vector, WT, K141/142A, or K52R FANCJ were either left untreated or treated with melphalan. The percentage of cells with 4N DNA content after ICL treatment was averaged for each cell line from four independent experiments, with standard deviation (s.d.) indicated by error bars. (C) FA-J cells reconstituted with vector, WT, K141/142A, K52R, or S990A FANCJ were seeded on 24-well plates and incubated overnight under normal growth conditions. The cells were then treated with the indicated doses of MMC and incubated for 8 days. On the final day, the cells were counted and the percentage of live cells was calculated. Experiments were performed in triplicate and a representative plot is shown. (D) The IC_{50} dose for the FA-J vector (250 nM) was compared for all mutants, and error bars represent the standard deviation.

to sites of DNA damage (Yoshioka *et al*, 2006). Furthermore, mismatch repair proteins have been implicated in sensing and processing ICLs. In particular, the MutS β complex was shown to bind to intrastrand crosslinks produced by cisplatin (Duckett *et al*, 1996; Yamada *et al*, 1997), and the MutS β complex was shown to be involved with the removal of ICLs produced by psoralen (Zhang *et al*, 2002). Furthermore, in the absence of MMR signaling, ICL repair proceeded in an alternate pathway promoting a non-recombination-dependent mechanism (Zheng *et al*, 2006). Our study now suggests that MLH1 binding to FANCJ is functionally important for the ICL-induced response, as disruption of the native FANCJ/MLH1 interaction reduced cell survival following ICL treatment. Moreover, FANCJ K141/142A mutant, defective in MLH1 binding, fails to correct FA-J cells. The finding that MLH1 deficiency did not generate gross changes in the

cellular survival following ICLs may stem from the loss of both MMR DNA damage repair and signaling. In fact, the loss of DNA damage signaling and the subsequent repair is essential in establishing the resistance of MMR-deficient cells to DNA methylation (O'Brien and Brown, 2006). The multiplicity of MMR functions in the DNA damage response has been recently uncovered through separation-of-function mutations (O'Brien and Brown, 2006).

Here, we attempted a similar approach to dissect the role of MLH1 and FANCJ in the ICL response. Three approaches were attempted to selectively ablate the FANCJ/MLH1 interaction *in vivo*. In one of these approaches, we attempted to generate an MLH1 mutant that lacked FANCJ binding. While MLH1 and FANCJ bind directly *in vitro*, the interaction is facilitated by PMS2 *in vivo*. In the absence of PMS2, FANCJ binding to MLH1 requires two MLH1 C-terminal domains D1

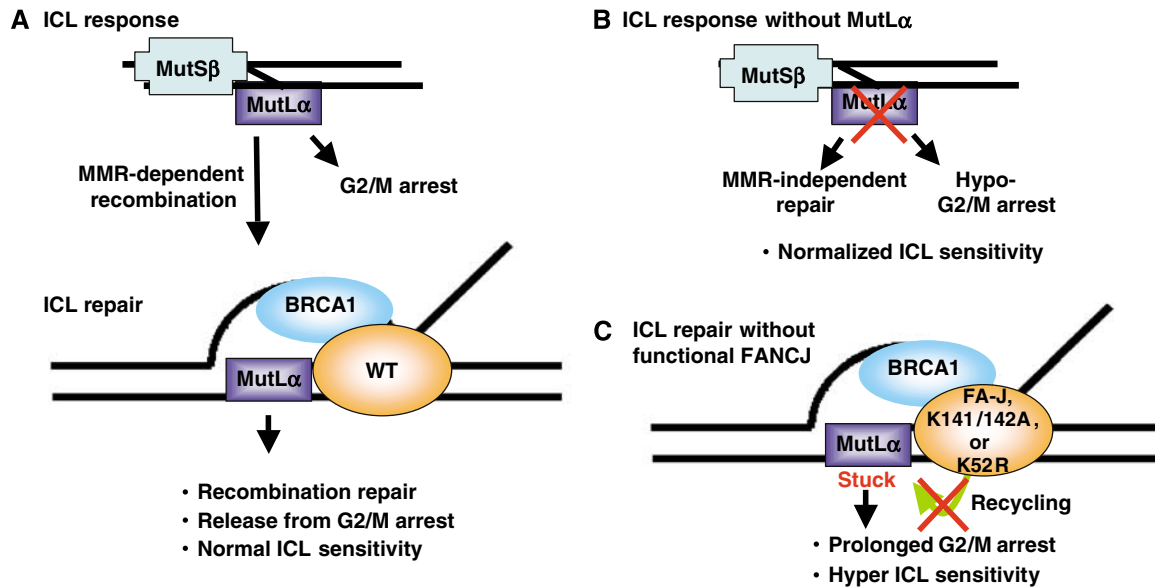


Figure 7 Model depicting how FANCI and MutL α proteins function to mediate the ICL response. (A) The normal ICL response is proposed to include MMR proteins. This is supported by the findings that the MMR machinery has been shown to specifically bind crosslinked DNA adducts (Duckett *et al*, 1996; Yamada *et al*, 1997; Zhang *et al*, 2002), mediate ICL recombination repair (Zheng *et al*, 2006) and induce a G2/M arrest (Cejka *et al*, 2003). Furthermore, MMR proteins including the MutL α complex, similar to BRCA1 and FANCI are essential for recombination processes (de Wind *et al*, 1995; Jasin, 2002; Mohindra *et al*, 2002; Litman *et al*, 2005). (B) The ICL response without MutL α is predicted to lead to a MMR-independent non-recombination-based mechanism with a minimal G2/M arrest, so that ICL sensitivity is normalized. (C) ICL repair without functional FANCI is predicted to be directed to recombination as in panel A, but generates a prolonged G2/M arrest, due to absent or dysfunctional FANCI protein. In the absence of FANCI protein, helicase activity or MLH1 binding, FANCI is unable to displace MutL α from recombination intermediates, and consequently, the MutL α complex remains stuck or tethered to DNA for a longer time period, delaying the exit from the G2/M arrest and enhancing ICL sensitivity.

(478–508) and D2 (736–744). However, in the presence of PMS2, only one of these domains was required. Moreover, both the regions on MLH1 (703–725) required for FANCI binding in the presence of PMS2 are also essential for PMS2 stabilization. The complexity of the MLH1/PMS2/FANCI interaction confounded attempts to selectively ablate the FANCI/MLH1 interaction through MLH1 mutagenesis. Fortunately, the binding of MLH1 to FANCI was less complex and therefore, the FANCI/MLH1 interaction could be selectively disrupted by both mutagenesis and peptide disruption. We found that disruption of this complex caused defects in the ICL response, consistent with the MLH1/FANCI interaction being required for ICL repair.

Conceivably, for ICL repair, mismatch repair complexes including MutL α , mobilize or regulate FANCI helicase activity to unwind DNA in the vicinity of the DNA damage to facilitate repair processes. Thus, disruption of FANCI helicase activity or MLH1 binding could interfere with ICL response. In support of this possibility, the MLH1 homologue in *Escherichia coli*, MutL binds the DNA helicase UvrD gene product Helicase II (Hall *et al*, 1998; Spampinato and Modrich, 2000) and stimulates its helicase activity (Dao and Modrich, 1998; Yamaguchi *et al*, 1998). However, we did not detect an effect of the MutL α complex, inhibitory or stimulatory, on FANCI catalyzed unwinding of a forked duplex, 5' flap, or Holliday Junction substrate (data not shown). It is possible that regulation of FANCI helicase activity by MLH1 may require additional MMR proteins, and/or that the physical interaction between FANCI and MLH1 serves a non-catalytic role in mediating the ICL response.

Alternatively, the catalytic activity of FANCI may serve an entirely different purpose (see Figure 7). For example, the FANCI helicase activity could serve to displace MutL α from DNA. This type of model has also been proposed for other helicases. For example, the Srs2 helicase is proposed to displace a checkpoint protein to facilitate checkpoint exit (Vaze *et al*, 2002). If true, in the absence of FANCI (FA-J cells), or in FA-J cells with a catalytically inactive FANCI and/or a FANCI mutant that lacks MLH1 binding, the MutL α complex would fail to be displaced from DNA. As such, the MutL α complex would be stuck or take longer to be displaced from DNA, leading to a prolonged G2/M arrest and/or delay in the completion of repair. In support of this model, ICL treatment of FA-J cells reconstituted with vector, K52R or K141/142A FANCI constructs demonstrates both hyper-G2/M arrest and sensitivity to ICLs. Furthermore, peptide perturbation of the MLH1/FANCI interaction in MCF7 cells lead to enhanced ICL sensitivity.

This proposed role for FANCI in displacing MutL α is also not at odds with the finding that suppression of the MutL α complex in MCF7 cells did not lead to MMC sensitivity (Supplementary Figure 2). It has been reported that in the absence of MMR signaling there is a reduced G2/M arrest following DNA damage (O'Brien and Brown, 2006), and compensating non-recombination repair pathways are engaged (Zheng *et al*, 2006). The expectation from these findings would be that MMR-deficient cells would have a normalized sensitivity to ICLs, due to the defective checkpoint and compensation by alternative mechanisms of repair (see model in Figure 7). Ultimately, it will be critical to establish whether a FANCI/MLH1 complex facilitates ICL

repair by promoting homologous recombination or other repair functions. While ablation of BRCA1 binding to FANCI may not affect the ability of FANCI to correct the defective response of FA-J cells, the timing or mechanism of repair may be altered. Thus, it is important to consider that the FA-J cells lacking the FANCI/BRCA1 interaction resist the ICL-induced 4N arrest and sensitivity through an alternative mechanism, perhaps by checkpoint avoidance. Experiments are currently underway to investigate this possibility.

Although MMR proteins are essential to elicit a G2/M arrest, it is presently unclear as to how this arrest is overcome. In cells lacking the active FANCI helicase and/or a FANCI/MLH1 interaction, diffusion of the MutL α complex from DNA may lead to an eventual exit from this G2/M arrest. In contrast, this exit may not be achieved in FA-J cells expressing the K52R FANCI, if the MutL α complex is locked on the DNA by the inactive helicase. It follows that a complete failure to re-enter the cell cycle, as apposed to a slower entry, would be more toxic to cells. Consistent with this, we found that the FA-J K52R expressing cells are short-lived in tissue culture and forced expression of K52R FANCI in other cell lines is not stable (data not shown).

Given that the FANCI/MutL α interaction is intact in other FA cells, including FA-A, FA-D2 and FA-D1 (Supplementary Figure 1), loss of this complex is not a general feature of FA cells. However, it remains to be determined whether additional FA-MMR interactions are altered. Moreover, similar to FANCI deficiency, MLH1 deficiency does not affect the ATR-mediated FANCD2 monoubiquitination (Supplementary Figure 2) (Andreassen *et al*, 2004; Levitus *et al*, 2004; Bridge *et al*, 2005; Litman *et al*, 2005), suggesting that the FANCI/MLH1 interaction is not essential for FA-pathway activation. Interestingly, we find that deletions in the MLH1 C-terminus (703–725), which are important for maintaining the stability of PMS2 (Mohd *et al*, 2006), also disrupted the FANCI/MLH1 interaction. This finding has implications for MLH1 clinical mutations identified in colon cancer patients, potentially linking MLH1 function not only to PMS2 but also to FANCI.

In conclusion, these studies have provided the first evidence for a role of the FANCI/MutL α complex in the ICL-induced response. This work extends the already implicated role of MMR proteins in the ICL response. Further study of the role of FANCI, BRCA1 and MMR proteins in this process should advance the understanding of how ICL-induced responses are regulated to preserve genomic integrity.

Materials and methods

Cell lines

HeLa, MCF7, 293T, and HeLa S3 cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml each). FA-J (EUFA30-F) cells were cultured as previously described (Litman *et al*, 2005). HCT116 cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml each). Hi5 insect cell were grown in Grace's Insect Media supplemented with 10% fetal bovine serum and 1% genetemycin at 28°C without CO₂. FA-J cells were infected with the pOZ retroviral vector (Nakatani and Ogryzko, 2003) containing no insert, WT, K141/142A, or K52R FANCI inserts, or with the lentiviral vector pLentiV5 (Invitrogen) vector, containing no insert, WT or S990A FANCI inserts. Stable FA-J pOZ cell lines were generated by sorting pOZ-infected cells with anti-IL-2 magnetic beads (Dyna Beads) and expanding IL-2-positive

cells. Stable FA-J pLenti cell lines were generated by blasticidin selection (7 μ g/ml).

Purification of a FANCI complex

A FANCI complex was purified from nuclear extracts (NE) derived from $\sim 8 \times 10^9$ HeLa cells stably expressing the double tagged FANCI, by two-step immunoaffinity chromatography, according to the standard method (Nakatani and Ogryzko, 2003). Flag-HA double purified material was electrophoresed in 3–8% Tris-acetate gel (Invitrogen). Individual Silver-stained bands were excised and subsequently analyzed by mass spectrometry (Genomine Inc., South Korea).

Immunoprecipitations, immunoblotting and Abs

Cells were harvested and lysed in 150 mM NETN lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin) for 30 min on ice. Cell extracts were clarified by centrifugation. The cell lysates were boiled in SDS loading buffer. For immunoprecipitation assays, cells lysates were incubated with protein-A beads, and either FANCI (E67 or E47), MLH1 (BD Bioscience) or Myc (9E10) Abs at 4°C for 2 h. Beads were subsequently washed and boiled in SDS loading buffer. Proteins were separated using SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked in 5% milk phosphate-buffered saline (PBS)/Tween and incubated with primary Ab for 1 h. Abs for Western blot analysis included anti-MLH1 (BD Bioscience, 1:500), anti-PMS2 (BD Biosciences, 1:200), anti-BRCA1 (ms110, hybridoma cell, 1:3), anti-FANCI (Monoclonal pool 2G7, 2C10, 1B4), anti-FANCD2 (Fanconi Anemia Research Foundation) and Myc (9E10, hybridoma cell, 1:3). Membranes were washed, and incubated with horseradish peroxidase-linked secondary Abs (Amersham, 1:5000), and detected by chemiluminescence (Amersham).

ICL-induced 4N accumulation and sensitivity assays

FA-J cells reconstituted with either vector, WT FANCI, K141/142A FANCI, K52R FANCI, or S990A FANCI were either treated with 0.5 μ g/ml of melphalan (Sigma) or left untreated, and incubated for 65 h. Cells were fixed with 90% methanol in PBS and were then incubated 10 min with PBS containing 30 μ l/ml DNase-free RNase A and 50 μ g/ml propidium iodide. A total of 1×10^4 cells was analyzed using a FACs Calibur instrument (Becton-Dickinson, San Jose, CA). Aggregates were gated out and the percentage of cells with 4N DNA content was calculated using Modfit software.

The FA-J cells reconstituted with either vector, WT FANCI, K141/142A FANCI, K52R FANCI, or S990A FANCI were seeded on 24-well plates 1000 cells per well and incubated overnight. The cells were either left untreated or treated with increasing doses of MMC for 1 h and incubated for 8 days. Finally, the cells were collected by trypsinization and counted using a hemacytometer. The percentage of live cells at each concentration was calculated using the untreated controls as the baseline growth.

MCF7 cells transfected with either empty vector or the 128–158 FANCI eGFP construct were seeded at 500 cells per well in 96-well plates, and incubated overnight. The cells were either left untreated or treated with increasing concentrations of MMC for 1 h and incubated 4–5 days. The percentage of live cells was calculated by ATP content, as previously described (Litman *et al*, 2005).

Plasmid construction and in vitro translation

The WT and S990A FANCI pLentiviral vectors were a gift from J Chen (Yu *et al*, 2003). The pCDNA3-myc.his vector (Invitrogen) was digested by *Not1/Apa1*, and different FANCI fragments generated by PCR and digested *Not1* and *Apa1* were inserted. Primers are available upon request. The WT FANCI pOZ-FH vector was generated by PCR cloning. Specifically, 5' *Xho1* and a 3' *Not1* restriction sites were added by using primers: 5'–3' CGCTCGAGGC CACCATGTCTTCAATGTGGTCTGAATATCAATT and 5'–3' CAGCGG CCGCCTTAAACCAGGAAACATGCCTTTATT. The PCR product was digested *Xho1* and *Not1* and subcloned into the pOZ-FH vector. The K52R, S990A and K141/142A pOZ vectors were generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) by using the FANCI-pOZ as a template and the following primers: (K52R) 5'–3' CCCACAGGAAGTGAAGGAGCTTAGCCTT AGCC and 5'–3' GGCTAAGCTAAGCTCCTTCC-ACTTCTGTGGG; (S990A) 5'–3' TCCAGATCCACAGCCCCAACTTCAAC and 5'–3'

GTTGAAAGTTGGGGCTGTGGATCTGGA; (K141/142A) 5'-3' GCAAA GTTATCTGCT GCGGCACAGGCATCCATATAC and 5'-3' GTATATG GATGCTGTGCCGAGCAGATAACTTTGTC. The same set of primers was used to generate the K141/142 A-pCDNA3 and K141/142 A-pVLL132, by using the WT FANCI pCDNA3myc.his- (Cantor *et al*, 2001) and pVLL132Flag-tagged (Cantor *et al*, 2004) constructs, respectively. The Q143E and S145A pCDNA3 were generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) by using the following primers: (Q143E) 5'-3' GCTGCAAAGTTATCTGCTAAGAAAGAGGCATCCATATACAG and 5'-3' CTGTATATGGATGCCTCTTTCTTAGCAGATAACTTTGTCAGC; or (S145A) 5'-3' TCTGCTAAGAAACAGGCAGCCATATACAGAGAT GAA and 5'-3' TTCATCTCTGTATATGGCTGCCTGTTTCTTAGCAGA. All DNA constructs were confirmed by DNA sequencing. MLH1 protein was synthesized *in vitro* by coupled transcription and translation using the T7 Quick-coupled TnT kit (Pormega) and MLH1 pCDNA3 vector as a template (Plotz *et al*, 2003) gift from Guido Plotz (Homburg/Saar, Germany). The WT-MLH1 pCDNA3 vector (Invitrogen) was digested *NotI/ApaI* and different MLH1 fragments were generated by PCR and digested *NotI* and *ApaI* products were inserted. Primers are available upon request.

The FANCI fragment (amino acids (aa) 128–158) was generated by PCR. An eGFP expression vector was generated by subcloning PCR fragments of eGFP into pCDNA3.1 (Invitrogen, Carlsbad, California) (gift from Dr Andrew Kung) to create new unique restriction sites within the active loop of eGFP. The annealed FANCI fragment and the empty eGFP pCDNA3 vector were digested with *BamHI* and *EcoRI* restriction enzymes for 1 h at 37°. The FANCI fragment and eGFP pCDNA3 vector were ligated using Quick ligase (NEB) for 1 h at room temperature. Primers covered FANCI sequence from 128–158 aa.

ELISA studies

Purified recombinant MLH1 protein was diluted to a concentration of 1 ng/μl in Carbonate buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃, pH 9.6) and added to appropriate wells of a 96-well microtiter plate (50 μl/well), which was incubated at 4°C. 3% bovine serum albumin (BSA) was used in the coating step for control reactions. The samples were aspirated, and the wells were blocked for 2 h at 30°C with blocking buffer (PBS, 0.5% Tween 20 and 3% BSA). The procedure was repeated. Purified recombinant FANCI protein was diluted in blocking buffer, and the indicated concentrations were added to the appropriate wells of the ELISA plate (50 μl/well), which was incubated for 1 h at 30°C. For EtBr or DNaseI treatment, 50 μg/ml EtBr or DNaseI (2 μg/ml) was included in the incubation with FANCI during the binding step in the corresponding wells. The samples were aspirated, and the wells were washed five times before addition of rabbit polyclonal anti-FANCI Ab (Sigma, B-1310) that was diluted 1:5000 in Blocking buffer. Wells were then incubated at 30°C for 1 h. Following three washings, horseradish

peroxidase-conjugated anti-rabbit secondary antibody (1:5000) was added to the wells, and the samples were incubated for 30 min at 30°C. After washing five times, any FANCI bound to the immobilized MLH1 was detected using OPD substrate (Sigma). The reaction was terminated after 3 min with 3 N H₂SO₄, and absorbance readings were taken at 490 nm.

Recombinant protein and helicase assays

Hi5 cells were infected with pVLL132 K141/142A FANCI and incubated for 72 h. Cells were collected, lysed in Insect Lysis Buffer (Roche) containing protease inhibitors (Roche) for 30 min at 4°C, and subsequently cleared by centrifugation. The WT, K52R, and K141/142A FANCI proteins were purified as previously described (Cantor *et al*, 2004). Briefly, K141/142A FANCI-Flag was IPed with 50 μl of FlagM2-conjugated beads for 2 h at 4°C. Beads were washed three times in 500 mM NETN (500 mM NaCl, 0.5% NP-40, 1 mM EDTA and 20 mM Tris-HCl (pH8.0)), followed by a final wash with 150 mM NETN, and K141/142A FANCI protein was eluted twice using 3 × Flag peptide. Elutions were pooled and dialyzed overnight in storage buffer. Helicase assay reaction mixtures (20 μl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 2% glycerol, 100 ng/μl BSA, 2 mM ATP, 10 fmol of the specified duplex DNA substrate (0.5 nM DNA substrate concentration), and the indicated concentrations of FANCI helicase. Helicase reactions were initiated by the addition of FANCI, and then incubated at 30°C for 15 min. Reactions were quenched in the presence of a 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand, to prevent reannealing, and products were resolved on non-denaturing 12% (19:1 acrylamide:bisacrylamide) polyacrylamide gels, and quantitated as previously described (Gupta *et al*, 2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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