Crosstalk between BRCA-Fanconi anemia and mismatch repair pathways prevents MSH2-dependent aberrant DNA damage responses

Min Peng1, Jenny Xie2, Anna Ucher2, Janet Stavnezer2 & Sharon B Cantor1,*

Abstract

Several proteins in the BRCA-Fanconi anemia (FA) pathway, such as FANCJ, BRCA1, and FANCD2, interact with mismatch repair (MMR) pathway factors, but the significance of this link remains unknown. Unlike the BRCA-FA pathway, the MMR pathway is not essential for cells to survive toxic DNA interstrand crosslinks (ICLs), although MMR proteins bind ICLs and other DNA structures that form at stalled replication forks. We hypothesized that MMR proteins corrupt ICL repair in cells that lack crosstalk between BRCA-FA and MMR pathways. Here, we show that ICL sensitivity of cells lacking the interaction between FANCJ and the MMR protein MLH1 is suppressed by depletion of the upstream mismatch recognition factor MSH2. MSH2 depletion suppresses an aberrant DNA damage response, restores cell cycle progression, and promotes ICL resistance through a Rad18-dependent mechanism. MSH2 depletion also suppresses ICL sensitivity in cells deficient for BRCA1 or FANCD2, but not FA. Rescue by Msh2 loss was confirmed in Fancd2-null primary mouse cells. Thus, we propose that regulation of MSH2-dependent DNA damage response underlies the importance of interactions between BRCA-FA and MMR pathways.

Keywords Fanconi anemia; FANCJ; mismatch repair; MLH1; replication stress

Subject Categories DNA Replication, Repair & Recombination

Introduction

DNA interstrand crosslinks (ICLs) induce a range of cellular responses, including recruitment of DNA repair proteins to the lesion and/or a stalled replication fork. Subsequent processing of ICLs and restart of replication forks require the coordination of several repair pathways, including homologous recombination (HR) and the error-prone DNA damage tolerance mechanism, translesion synthesis (TLS; Sale, 2012). Cells derived from Fanconi anemia (FA) patients or BRCA1/2-associated tumors that lack the BRCA-FA pathway (BRCA-FA cells) are extremely sensitive to agents such as mitomycin C (MMC) that induce ICLs (Moldovan & D’Andrea, 2009; Muniandy et al., 2010). This inter-strand crosslink (ICL) sensitivity and associated chromosomal aberrations are key determinants to diagnosing genetic deficiency in the BRCA-FA pathway, which has up to 16 components (Sharma & Canman, 2012).

The ICL sensitivity in BRCA-FA cells has been attributed to defects in the repair of intermediates of ICL processing such as DNA double-strand breaks (DSBs; Moldovan & D’Andrea, 2009). In particular, loss of the BRCA-FA proteins BRCA1 and FANCD2 leads to defects in recombination-directed repair. This has been attributed to non-homologous end-joining (NHEJ) proteins that occupy the ends of broken DNA and interfere with DNA end-processing required for HR (Bunting & Nussenzweig, 2010; Aly & Ganesan, 2011). In FANCD2-deficient cells, the NHEJ protein DNA-PKcs is aberrantly phosphorylated (Adamo et al., 2010). Furthermore, in BRCA1-deficient cells, HR is restored by elimination of the NHEJ factor, 53BP1 (Bouwman et al., 2010; Bunting et al., 2010; Aly & Ganesan, 2011). Remarkably, loss of 53BP1 also overcomes early embryonic lethality in BRCA1-nullizigous mice (Cao et al., 2009; Bouwman et al., 2010; Bunting et al., 2012), suggesting that 53BP1 underlies the proliferation defect in BRCA1 mice.

While elimination of NHEJ can normalize growth and HR defects in BRCA-FA cells, ICL repair is not fully restored. For example, in Brca1-null mouse cells, eliminating NHEJ restored HR, but did not fully restore ICL resistance (Bunting et al., 2012). Moreover, in Fancd2-null mouse cells, ICL sensitivity was enhanced by inactivation of NHEJ and mice had more severe development defects (Houghtaling et al., 2005; Bunting et al., 2012). Furthermore, in worms that are mutant for the FANCJ (BACH1/BRIP1) homologue, dog-1, ICL sensitivity was not suppressed by eliminating NHEJ (Adamo et al., 2010). Failure to restore ICL repair in BRCA-FA cells by suppression of NHEJ suggests that the BRCA-FA pathway has additional roles besides suppression of NHEJ. Other functions include protecting replication forks from degradation by nucleases.
(Schlacher et al., 2011, 2012) and orchestrating replication restart through HR, TLS, and other post-replication repair pathways (Kim & D’Andrea, 2012).

The functional relevance is not fully understood; however, several reports have linked the BRCA-FA pathway with proteins of the mismatch repair (MMR) pathway. In particular, BRCA1, FANC2, SLX4/FANCp, and FANCJ interactions with MMR proteins have been reported (Wang et al., 2000; Svendsen et al., 2009; Kratz et al., 2010; Liu et al., 2010; O’Donnell & Durocher, 2010; Shereda et al., 2010; Smogorzewska et al., 2010; Yoshikiyo et al., 2010; Huang et al., 2011; Williams et al., 2011; Ward et al., 2012; Peng et al., 2007). Moreover, we found that FANCJ binding to the MMR protein MLH1 is essential for ICL repair (Peng et al., 2007; Cantor & Xie, 2010; Xie et al., 2010). Further suggesting a functional connection between these pathways, MMR proteins activate the BRCA-FA pathway, including the promotion of FANC2D monoubiquitination (Huang et al., 2011; Williams et al., 2011) and also localize FANCJ to sites of ICLs and DNA crosslinks induced by ultraviolet light (Suhasesini et al., 2013; Guillemette et al., 2014).

Proteins of the MMR pathway bind DNA lesions/perturbations through either the heterodimer MutSβ (composed of MSH2 and MSH3) or MutSα (composed of MSH2 and MSH6), which subsequently recruit the MutLα complex (composed of MLH1 and PMS2; Duckett et al., 1996; Brown et al., 1997; Yamada et al., 1997; Zhang et al., 2002; Wu & Vasquez, 2008). When processing certain DNA lesions, such as ICLs, MMR complexes from bacteria to human cells have been associated with break induction and promoting apoptosis (Fram et al., 1985; Nowosielska & Marinus, 2005; Zhang et al., 2007; Fink et al., 1996; Nehme et al., 1997). This DNA damage response could result from MMR proteins binding ICLs or other DNA structures that form at stalled DNA replication forks. MMR proteins also have genome surveillance functions that counteract error-prone bypass pathways essential for ICL processing (Jiricny, 2006; Sharma & Canman, 2012).

Here, we considered the hypothesis that MMR-dependent responses in the absence of coordination with the BRCA-FA pathway are detrimental and contribute to defects in FA cells. To interrogate the contribution of MMR to ICL processing defects, we eliminated MMR. We found defects were suppressed by loss of MSH2. MSH2 depletion does not appear to enhance DNA repair, but rather attenuates the DNA damage response that is abnormally increased in FA cells. Reduction of these responses through MSH2 depletion also correlates with the restart of DNA replication. These findings have important clinical implications for BRCA-FA mutation carriers, as MSH2 inactivation might propel tumor formation or reduce the efficacy of platinum therapies used to treat BRCA-FA pathway-associated cancers.

Results

Loss of MSH2, but not MLH1 or NHEJ proteins, reduces the MMC sensitivity of cells deficient in FANCJ

The diversity of MMR functions in ICL processing, including activation of the BRCA-FA pathway and converting ICLs into breaks (Fram et al., 1985; Nowosielska & Marinus, 2005; Zhang et al., 2007; Huang et al., 2011; Williams et al., 2011), suggests that pathway coordination is essential. To test the idea that MMR is toxic in the absence of coordination with the BRCA-FA pathway, we tested whether the ICL sensitivity of FANCJ-deficient cells is due to MMR factors. We used siRNA reagents to disrupt upstream and downstream MMR complexes, through MSH2 or MLH1 depletion, respectively. When siRNAs to FANCJ and MLH1 or MSH2 were used in combination or alone, knockdown was achieved (Fig 1A). The reduction in FANCJ expression resulted in the expected sensitivity to MMC as compared to control (Fig 1B). Furthermore, MMR depletion was sufficient to promote hyper-resistance to the DNA methylating agent methylnitrosourea (MNU; Brown et al., 1997; Supplementary Figure S1A) indicating the siRNAs decreased the function of these proteins. Strikingly, as compared to FANCJ depletion, FANCJ and MSH2 co-depletion enhanced MMC resistance, whereas FANCJ and MLH1 co-depletion did not (Fig 1B and Supplementary Figure S1B). Substantiating these findings, a similar result was obtained in A549 cells with siRNA reagents targeting a distinct MSH2 site (Supplementary Figure S1C–E), indicating that these results were not likely to be cell type specific or the result of off-target effects.

To further validate these findings, we examined a pair of cell lines derived from a colon cancer patient, HEC59 (MSH2-deficient) and HEC59+chr2 (MSH2-proficient). HEC59 cells do not express MSH2 unless chromosome 2 is re-introduced (Fig 1C; Umar et al., 1997). We found that treatment of HEC59+chr2 cells with either of two individual FANCJ siRNAs (a or b) resulted in sensitivity to MMC as compared to treatment with control siRNA (Fig 1D). In contrast, MSH2-deficient HEC59 cells depleted of FANCJ exhibited the same levels of survival after MMC treatment comparable to controls. Altogether, these findings indicate that MSH2 contributes to the ICL sensitivity of FANCJ-deficient cells.

In light of recent studies in which elimination of the NHEJ factors, DNA-PKcs or 53BP1 reduced ICL sensitivity in BRCA-FA cells (Adamo et al., 2010; Bunting & Nussenzweig, 2010; Aly & Ganesan, 2011), we asked whether loss of these NHEJ factors also reduced ICL sensitivity in FANCJ-deficient cells. For these studies, we exploited the human M059K (DNA-PKcs proficient) and M059J (DNA-PKcs deficient) glioblastoma cell lines (Anderson et al., 2001). Treatment of the M059K or M059J cells with two different FANCJ siRNAs resulted in sensitivity to MMC (Fig 1E and F). Likewise, treating the two stably 53BP1-depleted cell lines with FANCJ siRNA resulted in MMC sensitivity (Fig 1G and H). Thus, eliminating or depleting DNA-PKcs or 53BP1 does not suppress MMC sensitivity associated with FANCJ deficiency, consistent with findings in Caenorhabditis elegans (Adamo et al., 2010).

MSH2 depletion suppresses ICL sensitivity in cells lacking the FANCJ–MLH1 interaction

FANCJ binds directly to MLH1, and cells expressing a FANCJ mutant that cannot bind MLH1 are hypersensitive to MMC (Peng et al., 2007). Thus, we hypothesized that a function of the interaction was to inhibit the action of MSH2 at lesions induced by MMC. Immunoblot and co-immunoprecipitation experiments of FA-J patient cell lines confirmed that FANCJK141/142A was expressed similarly to FANCJWT, but was defective in MLH1 binding (Fig 2A). As expected, FA-J cells complemented with FANCJK141/142A remained sensitive to MMC, whereas FA-J cells complemented with wild-type FANCJ had enhanced resistance (Fig 2B; Peng et al,
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MSH2 depletion rescues aberrant checkpoint and DNA damage responses in cells lacking the FANCJ–MLH1 interaction

FA cells also have prolonged checkpoint responses and exacerbated DNA damage responses that are thought to contribute to the growth defects, ICL sensitivity, and bone marrow failure in FA patients (Cecchaldi et al., 2012; Kim & D’Andrea, 2012). The prolonged G2/M arrest in FA cells has been analyzed in response to ICL-inducing agents such as MMC or melphalan (Litman et al., 2005). We previously found that FA-J cells have a prolonged G2/M accumulation in response to melphalan that is corrected by introduction of FANCJ WT, but not the MLH1 binding mutant FANCJ K141/142A (Peng et al., 2007; Supplementary Figure S3). Moreover, as compared to vector, FANCJ K141/142A complemented FA-J cells have a more pronounced G2/M accumulation (Peng et al., 2007; Supplementary Figure S3). Thus, we asked whether these cell cycle recovery defects were also attributable to MSH2. While MSH2 depletion had only a modest effect on FA-J cells with vector, and little or no effect on FANCJ WT, the prolonged G2/M accumulation was reduced in FA-J cells with FANCJ K141/142A (Fig 3A and B), suggesting that MSH2 contributes to the ICL-induced checkpoint defect in cells lacking the FANCJ–MLH1 interaction.

The exacerbated DNA damage response in FA cells includes hyper-phosphorylation of the NHEJ protein, DNA-PKcs (Adamo et al., 2010). To address whether MSH2 contributes to this DNA damage response, we generated FA-J cells with FANCJ K141/142A and shRNA to MSH2 or control using vectors that also express green fluorescent protein (GFP). Cells positive for GFP and containing shRNA to MSH2 had markedly reduced MMC-induced DNA-PKcs phosphorylation relative to cells expressing shRNA control vector (Fig 3C). Immunoblot analysis revealed a similar reduction in phosphorylation of DNA-PKcs and that MMC treatment induced a damage response evidenced by phosphorylated H2AX, γ-H2AX, albeit slightly less in the MSH2-depleted cells (Fig 3D). These findings suggest that MSH2 mediates aberrant activation of NHEJ, perhaps by inducing DSBs in response to MMC.

Because NHEJ contributes to ICL-induced chromosomal aberrations in FA cells (D’Andrea & Grompe, 2003), we next tested whether MSH2 depletion limited the number or type of aberrations found in FA-J cells lacking the FANCJ–MLH1 interaction. Remarkably, we found fewer radial chromosomes and chromosomes with breaks induced by MMC treatment in the FANCJ K141/142A-complemented FA-J cells expressing shMSH2 than in cells expressing control shRNA (shCon; Fig 3E). Cells expressing control shRNA had ~9 chromosomal aberrations per mitotic spread as compared to ~4 in cells expressing MSH2 shRNA (Fig 3F). Collectively, these findings indicate that MSH2 contributes to the MMC sensitivity, prolonged G2/M accumulation, hyper-activation of DNA-PKcs, and radial chromosomes in FA-J cells lacking the FANCJ–MLH1 interaction.

MSH2 depletion could promote survival of MMC-treated cells by enhancing recombination-based repair. To address this possibility, FANCJ K141/142A-expressing FA-J cells positive for γ-H2AX foci were analyzed for co-staining Rad51 foci. At all time points examined, we found that cells positive for γ-H2AX foci have a similar percent of Rad51 foci whether they express shRNA control or MSH2 shRNA vectors (Fig 4A and B, and Supplementary Figure S4A). Moreover, at several time point post-MMC, the chromatin bound Rad51 appeared similar and γ-H2AX was induced in both cell lines at 16 h post-MMC (Fig 4C). While γ-H2AX was slightly reduced by 24 h post-MMC in MSH2-depleted FANCJ K141/142A-FA-J cells as compared to control FA-J cells, more striking was the reduction in phosphorylated RPA and DNA-PKcs (Fig 4D and Supplementary Figure S4B and C). Thus, MSH2 does not appear to alter the accumulation of Rad51, perhaps at resected DNA sites prior to ICL excision (Long et al., 2011), but may contribute to ICL-induced break formation.

MSH2 depletion reduces MMC sensitivity through a Rad18-dependent mechanism

The restart of stalled replication forks by TLS was shown to mediate the reduced cytotoxicity of cisplatin in MMR-deficient cells (Lin et al., 2007). Consistent with a rescue from MMC sensitivity, siRNA to MSH2 increased MMC resistance 3.6-fold in FA-J cells expressing the FANCJ K141/142A mutant (Fig 2C–E). In contrast, siRNA for MLH1 had no effect on MMC resistance in the FANCJ K141/142A FA-J cell lines, indicating again that with respect to rescue from MMC sensitivity, MLH1 is distinct from MSH2.

In contrast to FA-J cells complemented with FANCJ K141/142A, MSH2 depletion had only a small effect on FA-J cells complemented with vector (Fig 2D and E) and did not enhance MMC resistance in FA-J cells expressing a helicase inactivating mutant FANCJ K52R (Supplementary Figure S2B). These findings suggest that MSH2 depletion rescues the repair defect in cells in which FANCJ cannot bind to MLH1, but does not rescue loss of a FANCJ function(s) connected to its helicase activity. Perhaps MSH2 depletion is able to rescue FANCJ-depleted cells because there is sufficient residual helicase activity (Fig 1B and Supplementary Figure S1B).
TLS requires PCNA ubiquitination by the Rad18/Rad6 ubiquitin–ligase complex (Kannouche & Lehmann, 2004; Alt et al, 2007; Waters et al, 2009). Thus, we tested whether the gains in MMC resistance due to MSH2 depletion in the FANCJΔK141/142A-complemented FA-J cells are dependent on Rad18. When siRNAs and shRNAs to Rad18 or MSH2 were used in combination or alone, knockdown was achieved (Fig 4E). Compared with MSH2 depletion alone, MSH2 and Rad18 co-depletion reduced MMC resistance (Fig 4F). Furthermore, gain in MMC resistance in MSH2-deficient HeLa cells was also dependent on Rad18 (Supplementary Figure S5A–D).

Depleting Rad18 or the TLS polymerase REV1 also sensitized MCF7 or U2OS cells co-deficient in FANCJ and MSH2 (Supplementary Figure S5E and F). These findings suggest that in a non-cell-type-specific manner, MSH2 depletion promotes MMC resistance through a Rad18-dependent mechanism that requires TLS polymerases such as REV1.

**MSH2 depletion restores replication restart after aphidicolin-induced arrest**

Processing of ICLs is complex and involves lesion processing, repair, and the restoration of replication fork progression. By simply arresting cells with the DNA polymerase inhibitor aphidicolin, we sought to gain clarity as to the underlying defect in cells lacking the FANCJ–MLH1 interaction. Moreover, it was previously shown that defects in S phase progression characterize FANCJ-deficient cells following release from aphidicolin (Greenberg et al, 2006; Kumarswamy & Shiekhattar, 2007). Similar to treatment with MMC,
Figure 3. Aberrant DNA damage responses in cells lacking the FANCJ–MLH1 interaction are suppressed by MSH2 depletion.

A Immunoblot analysis of FANCJ and MSH2 expressions in the Fanconi anemia (FA)-J cell lines expressing indicated shRNAs.

B Representative cell cycle profiles based on PI staining of DNA content for the indicated FA-J cell lines untreated (U) or at the indicated times following 0.25 μg/ml melphalan treatment.

C MSH2 depletion reduces DNA-PKcs Ser2056 phosphorylation after mitomycin C (MMC) treatment. Green fluorescent protein (GFP) expression indicates shRNA vector-infected FANCJ K141/142A FA-J cells. Representative immunofluorescence images are shown.

D Immunoblot analysis with indicated antibodies.

E Genomic instability is suppressed by MSH2 depletion after 250 nM MMC for 16 h. Representative metaphases show examples of (a) broken and (b) quad-radial chromosomes that were suppressed by MSH2 depletion.

F Graph shows number of breaks and radials quantified from 50 metaphases.

Data information: Where shown, error bars represent standard deviations from three independent experiments. The asterisk (*) represents a P-value < 0.01.
Figure 4. MSH2 depletion does not enhance RAD51 foci, but suppresses mitomycin C (MMC) sensitivity through a Rad18-dependent mechanism.

A Immunoblot analysis of MSH2 expression in the FANCJ-K141/142A Fanconi anemia (FA)-j cells treated with the indicated shRNAs.
B Quantification of the percentage of γH2AX foci-positive cells that have also RAD51 foci after 250 nM MMC treatment for 26 h.
C, D Immunoblot analysis with the indicated Abs of chromatin fractions from FANCJ-K141/142A FA-j cells stably expressing shRNA to Con or MSH2.
E Immunoblot analysis of Rad18 and MSH2 expressions in FANCJ-K141/142A FA-j cells stably expressing shRNA to Con or MSH2 treated with indicated siRNAs.
F Graph shows the percentage of viable cells 5 days after 125 nM MMC.

Data information: Where shown, error bars represent standard deviations from three independent experiments. The asterisk (*) represents a P-value < 0.01.

treatment with aphidicolin correlated with an aberrant induction of DNA-PKcs phosphorylation in FA-J cells lacking FANCJ WT (Fig 5A and Supplementary Figure S6A). Correspondingly, FA-J cells lacking FANCJ WT were severely defective in S phase progression following release from aphidicolin and did not incorporate Edu in a 1-h pulse (Fig 5B and C). Notably, as compared to vector, FANCJ-K141/142A complemented FA-J cells had an even more severe defect and did not recover by 48 h, but rather required from 72 to 96 h to gain a 4N DNA population (Fig 5D and data not shown), indicating that cells lacking the FANCJ–MLH1 interaction have a pronounced defect in resuming cell cycle progression. By analyzing the percent of G2/M cells following aphidicolin release, we found that MSH2 depletion effectively restored cell cycle progression to the FANCJ-WT FA-J cells, expressing FA-J cells (Fig 5E) consistent with MSH2 interfering with the restart of stalled replication forks when cells lack the FANCJ–MLH1 interaction (see model in Supplementary Figure S7).

MSH2 depletion reduces MMC sensitivity in human cells deficient in FANCJ, FANC2, or BRCA1, but not FANCA

To determine whether MSH2 underlies aberrant responses in other BRCA-FA cells, we tested whether MSH2 contributed to MMC sensitivity in cells lacking BRCA-FA proteins known to interact with MMR proteins, such as BRCA1 or the central FA pathway protein, FANC2 (Wang et al., 2000; Williams et al., 2011). We also tested whether MSH2 contributed to MMC sensitivity in cells lacking the FA upstream core component, FANCA, which has not been shown to interact with MMR proteins. As expected, U2OS cells treated with siRNA reagents to FANCJ, FANC2, FANCA, or BRCA1 were more sensitive to MMC than cells treated with control siRNA (Fig 6A and B, blue bars). Similar to our results in FANCJ-deficient cells, BRCA1- or FANC2-deficient U2OS cells treated with siRNAs to MSH2 have improved survival after MMC treatment as compared to cells treated with siRNA controls (Fig 6B, red bars, Supplementary Figure S8A). Furthermore, the MMC sensitivity of BRCA1-depleted MCF7 cells was fully suppressed by MSH2 depletion (Supplementary Figure S8B and C). In contrast, the MMC sensitivity of FANCA-deficient U2OS or FA-A patient cells depleted of MSH2 was not suppressed (Fig 6A and B, and Supplementary Figure S8D–G), suggesting FANCA functions in ICL processing in a manner distinct from FANCJ, BRCA1, or FANC2.

DNA-PKcs is aberrantly phosphorylated in FANC2-deficient cells in response to MMC (Adamo et al., 2010), similar to our findings in FANCJ mutant cells (Fig 3C and D). Thus, we asked whether MSH2 contributed to this aberrant response in FANC2-deficient cells, as it does in FANCJ mutant cells. Indeed, FA-D2 patient cells stably expressing shRNA GFP-fusion vectors to MSH2 show markedly less MMC-induced DNA-PKcs phosphorylation than cells expressing shRNA control vector after MMC treatment (Fig 6C and D). In addition, MSH2-depleted FA-D2 cells as compared to control
FA-D2 cells had a reduction in the phosphorylation of DNA-PKcs (Fig 6E), suggesting that MSH2 contributes to the abnormal DDR in FANCD2-deficient cells.

Msh2 deletion reduces MMC sensitivity and the DNA damage response in Fancd2-null mouse cells

To substantiate these findings, we analyzed the impact of deleting Msh2 in Fancd2-null mouse embryonic fibroblasts (MEFs). When mice heterozygous for Fancd2 and Msh2 were interbred and embryos were harvested between 13 and 14 days of gestation, we identified double-mutant embryos, which upon visual inspection resembled wild-type embryos. From 86 embryos, we obtained four double mutants (Fig 7A). We found that compared with wild-type, the fancd2+/+ MEFS were extremely sensitive to MMC, with only 22% of cells surviving after treatment with 25 nM MMC. We also found that resistance to MMC was enhanced in double-knockout MEFS to a level comparable with wild-type (Fig 7B), indicating that ICL sensitivity is reduced by Msh2 deletion as previously found for Mlh1 deletion (van de Vrugt et al., 2009).

To further ascertain whether FA-like phenotypes found in Fancd2-null MEFs were suppressed by msh2 deletion, we analyzed the DNA damage response. Compared with wild-type MEFs, MMC induced a greater damage response in fancd2+/+ MEFS, as detected...
by an antibody to ATR/ATM-phosphorylated substrates and γ-H2AX, consistent with a previous report (Ceccaldi et al., 2012). These phosphorylation events were reduced in the fancd2/C0/C0/msh2/C0/C0 MEFs (Fig 7C and D). Next, we analyzed 53BP1 foci, which mark unrepaired lesions remaining due to problems during replication (Lukas et al., 2011). As reported in FANCD2-deficient human cells (Ceccaldi et al., 2012) and FANCM-deficient cells (Blackford et al., 2012), we found more 53BP1 foci in the fancd2/C0/C0/msh2/C0/C0 MEFs than in wild-type MEFs. And here too, we detected fewer 53BP1 foci in the fancd2/C0/C0/msh2/C0/C0 MEFs (Fig 7E and F). Together, these findings suggest that Msh2 contributes to ICL sensitivity and the heightened DNA damage response in Fancd2-null cells.

**Discussion**

The BRCA-FA and MMR pathways intersect through several protein interactions. FANC2-FAN1, BRCA1, and FANCJ interact with MLH1, and SLX4/FANCP interacts with MSH2 (Wang et al., 2000; Svendsen et al., 2009; Kratz et al., 2010; Liu et al., 2010; O’Donnell &
Our data indicate that in the absence of BRCA-FA and MMR pathway coordination, MSH2 exacerbates the DNA damage response that characterizes FA cells. In particular, FA cells have a dysregulated NHEJ pathway in which DNA-PKcs is hyper-phosphorylated in response to DNA damage (Adamo et al., 2010). Aberrant DNA damage responses also include hyper-induction of ATR/ATM signaling pathways (Lukas et al., 2011; Ceccaldi et al., 2012), which were apparent in the Fancd2-null MEFs following MMC (Fig 7C and D). Even in unperturbed Fancd2-null MEFs, 53BP1 foci are enhanced, suggesting replication stress (Fig 7E and F; Lukas et al., 2011). Significantly, these aberrant DNA damage responses are suppressed by MSH2 loss (Fig 7C–F). Phosphorylation of DNA-PKcs is associated with its binding to DNA double-strand breaks (Meek et al., 2004). Therefore, DSB induction is likely a consequence of MSH2 activity in FA cells treated with MMC. In agreement, inhibiting MMR reduces the number of DSBs at stalled forks and promotes ICL resistance through enhanced recombination and TLS bypass pathways (Brown et al., 1997; Durant et al., 1999; Moreland et al., 1999; Wu et al., 2004; Lin et al., 2006). Likewise, we found that bypass pathways are essential for the mechanism of ICL resistance in MSH2-depleted cells.

**Figure 7. Sensitivity to mitomycin C (MMC) and the aberrant DNA damage response in Fancd2-null mouse cells are suppressed by Msh2 deletion.**

A. Chart shows embryos obtained from indicated cross.

B. Graph shows the percentage of viable primary mouse embryonic fibroblasts (MEFs) with the designated genotypes 5 days after MMC treatment. Three independent MEFs per genotype were analyzed.

C, D Representative immunofluorescence images (C) and quantification (D) of cells with foci positive for the ATM/ATR substrate phosphorylation and γ-H2AX following 250 nM MMC treatment.

E, F Representative immunofluorescence images (E) and quantification (F) of cells with 53BP1 foci and γ-H2AX in untreated MEFs.

Data information: Where shown, error bars represent standard deviations from three independent experiments. The asterisk (*) represents a P-value < 0.01.

Durocher, T.; Shereda, et al., 2010; Smogorzewska et al., 2010; Yoshikyo et al., 2010). Moreover, the MMR pathway activates the BRCA-FA pathway, inducing FANC2 monoubiquitination and localization of FANCJ to sites of DNA crosslinks (Huang et al., 2011; Williams et al., 2011; Suhasini et al., 2013; Guillemette et al., 2014). In this study, we provide further evidence that crosstalk between BRCA-FA and MMR pathways is critical for coordinating the DNA damage response. In particular, we find that lack of coordination between these pathways, as in BRCA-FA cells, makes MSH2 toxic. Consistent with this point, ICL sensitivity is suppressed by MSH2 depletion in human cells deficient in the FANCJ-MLH1 interaction, FANCJ, or other BRCA-FA proteins, such as BRCA1 or FANC2 that interact with MMR proteins (Figs 1B, 2D and E). Also, eliminating the MMR pathway in Fancd2-deficient mouse cells suppresses ICL sensitivity (Fig 7B; van de Vrugt et al., 2009), whereas loss of NHEJ does not (Houghtaling et al., 2005; Bunting et al., 2012). Interestingly, MSH2 depletion fails to rescue FANCA-deficient cells (Fig 6B and Supplementary Figure S8D–G). Thus, in a distinct set of BRCA-FA pathway deficient cells, loss of MMR function could synergize and contribute to tumors in FA patients.
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Our results also indicate that MSH2 depletion does not dramatically improve Rad51-based repair. We did not find evidence that recombination was heightened, as MMC-induced Rad51 foci and chromatin loading did not change upon MSH2 deletion (Fig 4B and C). In support of MSH2 blocking a restart step in ICL processing, when FANCJ mutant cells are arrested by aphidicolin, we also find induction of DNA-PKcs phosphorylation and defects in the restart of DNA replication (Fig 5A–C). Upon MSH2 depletion, however, mutant FA-J cells progress to G2/M (Fig 5E). Interestingly, MSH2 deficiency abolishes the anticancer and pro-aging activity of short telomeres by reversing proliferative defects, but not by enhancing recombination (Martinez et al., 2009). Thus, we suggest that in cells deficient for FANCJ, BRCA1, or FANC2D, MSH2 loss improves the ability of stalled forks to restart, but does not increase DNA repair.

The finding that MSH2-, but not MLH1-depletion rescues cells deficient for FANCJ or the FANCJ–MLH1 interaction (Figs 1B and 2D) suggests that FANCJ functions with MLH1 to prevent corruption/blockage of ICL repair by MSH2. Conceivably, FANCJ helicase and/or translocase activities could displace MSH2 heterodimers or unwind DNA structures that are substrates for MSH2. Of note, MSH2 binds branched DNA structures associated with replication forks (Alani et al., 1997; Kolodner & Marsischky, 1999), including G4 DNA structures that are substrates for FANCJ unwinding (Larson et al., 2005; Wu et al., 2009; Sarkies et al., 2012). Thus, to temper an MSH2 DNA damage response, FANCJ could restrict MSH2 heterodimers in several ways. First, to promote ICL repair, FANCJ might remove MSH2 bound to ICLs. Second, to promote error-prone recombination or TLS extension reactions that are required for ICL processing (Sharma & Canman, 2012), FANCJ might remove MSH2 that is bound to DNA mismatches. Finally, to restart replication following ICL processing, FANCJ might similarly prevent MSH2 binding to secondary structures that form at arrested forks that are barriers to replication. Unwinding DNA substrates and/or displacing MSH2 could explain the role of FANCJ in coupling replication past fork barriers (Schwab et al., 2013). The ability of FANCJ to dismantle MSH2 could underlie the importance of the FANCJ–MLH1 interaction for ICL repair as MLH1 links FANCJ to the MSH2 heterodimer (Peng et al., 2007). We suspect that the relevant heterodimer displaced is MSH2–MSH6. This conclusion is not based on MSH6 depletion studies because MSH6 siRNAs also reduced MSH2 expression (Supplementary Figure S9), and thus, the experiment would be uninformative. However, MSH3 depletion did not alter MSH2 or MSH6 levels, and we found that MMC resistance was not enhanced (Supplementary Figure S9).

Aside from functioning with MLH1 to limit MSH2, our data suggest that FANCJ has a separate helicase function that is also important for ICL repair. Most notably, MSH2 depletion has little or no effect on FANCJ-null cells or cells that lack FANCJ helicase activity (Supplementary Figure S2B). This further supports the hypothesis that FANCJ functions with MLH1 to remove MSH2 from a DNA lesion, perhaps with its translocase activity, and also unwinds DNA barriers with its helicase activity. As compared to FANCJ-null cells, cells lacking the FANCJ–MLH1 interaction have a more severe ICL processing defect (Peng et al., 2007; Fig 3B) and a more severe replication restart defect following arrest by aphidicolin treatment (Fig 5D), suggesting that stalled replication forks are differentially processed (see model in Supplementary Figure S7). We speculate that stalled replication forks remain intact in FANCJ-null cells, a point supported by studies in DT40 cells (Schwab et al., 2013). Restart at these forks likely involves loss of genomic integrity, consistent with loss of G4 structures in FANCJ-null cells (Cheung et al., 2002; London et al., 2008; Wu et al., 2008; Sarkies et al., 2012). Replication barriers could be cleaved by opportunistic nucleases that gain access to stalled forks, as found in BRCA-FA cells (Schlacher et al., 2011, 2012). Instead, in cells lacking the FANCJ–MLH1 interaction, replication barriers could be insurmountable. FANCJ could physically block nucleases and MSH2 could in turn block FANCJ. Thus, restart via nucleases and break induction fails and forks collapse. This model suggests that in FANCJ(k141/142A) mutant cells, MSH2 depletion, but not MLH1, will ‘unlock’ the secondary structure and enable FANCJ to unwind the replication barrier and restart replication. In FANCJ-null cells, MSH2 depletion has little effect because without FANCJ helicase activity, the replication barrier remains and restart will be largely dependent on nucleases. In sum, MSH2 depletion may rescue cells that have FANCJ helicase activity (FANCJ(K141/142A) FA-J), but not cells without FANCJ helicase activity (vector or FANCJ(K52R) FA-J cells). Rescue may be achieved in FANCJ siRNA-depleted cells because sufficient residual FANCJ supports its helicase function.

FANC2D-deficient cells are effectively rescued by loss of MSH2 (Figs 6 and 7) or by loss of MLH1 when p53 is also inactivated (van de Vrugt et al., 2009). While MLH1 loss alone did not rescue cells deficient for FANCJ or the FANCJ–MLH1 interaction, we did not address if co-depletion of p53 would. Any differences could reflect the fact that some BRCA-FA proteins function with MMR complexes to initiate the DNA damage response aside from a role in regulating MMR. Indeed, FANC2D forms complexes with MMR proteins at several nodes. FANC2D binds MLH1 (Huang et al., 2011; Williams et al., 2011), and following DNA damage, the monoubiquitinated FANC2D is found in a complex containing the MLH1-associated endonuclease FAN1 (Kratz et al., 2010; Smogorzewska et al., 2010). To process ICLs, FAN1 is predicted to function with other endonucleases, such as SLX4, that associate with MSH2 (Kim & D’Andrea, 2012). Thus, FANC2D and associated partners could serve to link upstream and downstream MMR complexes. In FANCJ-deficient cells, in which FANC2D undergoes a normal DNA damage-induced monoubiquitination (Litman et al., 2005), MLH1 loss could be insufficient to disarm an MSH2–SLX4–FANC2D–FAN1-dependent DNA damage response.

Our study indicates that MSH2 depletion rescues a subset of BRCA-FA cells, FANCJ-, BRCA1-, and FANC2D-deficient, but not FANCA-deficient cells. Notably, MSH2 loss also does not restore ICL resistance to FANCN-null DT40 cells (Huang et al., 2011). This distinction could reflect that FANCJ, BRCA1, and FANC2D function at least in part through their MMR protein interactions, whereas to our knowledge, FANCA and FANCM do not interact with MMR proteins. BRCA1 and FANC2D, similar to FANCJ, could restart replication through MMR protein interactions that serve to dismantle MSH2. By contrast, FANCA and FANCM could contribute to replication restart by engaging bypass pathways. Indeed, FANCM is required for template switch mechanisms (Whitby, 2010; Blackford et al., 2012). Likewise, FANCA through complex formation with REV1 activates bypass pathways (Kim et al., 2012; Fig 6F). A
fundamental role in bypass pathways could explain why MSH2 depletion fails to rescue.

Taken together, these findings are relevant for understanding FA disease and progression to cancer. FA patient hematopoietic stem and progenitor cells have a hyperactivated DNA damage response that is dampened in transformed FA cells (Ceccaldi et al., 2012). Determining whether this abnormal DNA damage response and/or the progression to bone marrow failure in FA patients are generated by MMR factors that promote a barrier to tumorigenesis will be an important future research direction. If so, loss of MMR could predict the onset of tumorigenesis in patients. Moreover, it will be important to identify whether MMR inactivation or suppression will be useful for therapy to retard the progression to bone marrow failure.

Materials and Methods

Cell culture

MCF7, HeLa, A549, and U2OS cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml each). M059J and M059K cells were cultured in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum and antibiotics. Human endometrial HEC59 and HEC59+chr2 cell lines, FA-J (EUFA30-F), FA-D2 (PD20), and FA-A (PD6914) cell lines were cultured in DMEM supplemented with 15% fetal bovine serum. FA-J cells were infected with the POZ retroviral vectors as described previously (Peng et al., 2007). Stable shRNA cells were selected with puromycin.

siRNA and shRNA

siRNA transfections were carried out with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Analyses were performed 48–72 h after siRNA transfection. siRNA reagents for MSH2 (siRNA MSH2 pool, target sequence of (a) GAAGAGACCUUAACUUGC, or (b) GGAGGUAAAUCAACAUUAUA), MLH1 (siRNA MLH1 smartpool), Rad18 (siRNA Rad18 smartpool), FANCD2 (siRNA FANCD2 smartpool), BRCA1 (siRNA BRCA1 smartpool), FANCA (siRNA FANCA smartpool or two distinct siRNA, target sequence of (a) AGAGGAAGAUGGCUAUAAUU, or (b) GGACUAAGCUCACUCUUCU)), and luciferase (Luc) were obtained from Dharmacon (Lafayette, CO, USA). The FANCJ siRNA reagents were described previously (Litman et al., 2005). U2OS, FA-D2, or FA-J stable cells were infected with pGIPZ vectors expressing GFP and containing shRNAs against non-silencing control, MSH2 (a) (mature antisense sequence, CATGTAATAGTGTGCTA) or MSH2 (b) (ATTACTTCAGGTTTAGC), 53BP1 (a) (mature antisense sequence, AGGACGACCCACAGACTATA), or 53BP1 (b) (AGAGTAGAAAGGGAAGTA), MSH6(a) (mature antisense sequence, TTTAACTGTATTTCTTGC), MSH6(b) (mature antisense sequence, TTCTACTTAGTATTTCTTC), MSH3 (a) (mature antisense sequence, ATGACCTTATTTCTTCTTGC) or MSH3(b) (mature antisense sequence, TTTCCCTAATTTAATGAGTGG). shRNAs were obtained from the UMMs shRNA core facility.

Cell growth and cell cycle analysis

Cells were seeded into six-well plates, incubated overnight, and left untreated or treated with MMC (Sigma, St Louis, MO, USA) for 1 h and CPT for 5 h (Invitrogen). Cells treated with MNU (1 h, serum free) were first pre-treated with 20 μM O6-benzylguanine (O6-BZG) to block methylguanine methyltransferase (MGMT). In addition, O6-BZG was also included during and after treatment. Cells were counted after 5 days using a hemocytometer and compared with untreated controls, and cell survival was analyzed as before (Xie et al., 2010). FA-J stable cell lines were either mock-treated or treated with 0.25 μg/ml of melphalan (Sigma) or 18 h with aphidicolin (3 μg/ml; Sigma) at which time fresh media was introduced. Cells were collected at various times and fixed with 90% methanol in PBS overnight and then incubated 10 min with PBS containing 30 μg/ml DNase-free RNase A and 50 μg/ml propidium iodide. 1 × 10⁶ cells were analyzed using a FACSCalibur instrument (Becton-Dickinson, San Jose, CA, USA). Aggregates were gated out, and the percentage of cells in G2/M was calculated using ModFit software. Errors represent standard deviation of the mean. Statistical analysis was performed using Student’s two-tailed, unpaired t-test.

Western blot and antibodies

Cells were harvested, lysed, and processed for immunoblot analysis as described previously using an NETN lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing 10 mM NaF and 1 mM NaVO₃ (Litman et al., 2005). Antibodies for Western blot analysis included FANCJ (E67), BRCA1 (ms110) (Cantor et al., 2004), MLH1 (Santa Cruz), MSH2 (Calbiochem), 53BP1 (Novus Biologicals), FANCD2 (FARF), FANCA (FARF), DNA-PKcs Ser2056 (Abcam), phospho DNA-PKcs (Abcam; 1:200), c-H2AX (Upstate 1:100), c-ATR/ATM Substrate (Cell Signaling; 1:100), cells were washed and then incubated with secondary antibody. After washing, cover slips were mounted onto glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories). Cells with >10 foci per cell were scored as positive. For Edu labeling, cells were left untreated or treated with aphidicolin for 18 h and released at indicated times. Edu imaging kit (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence

Immunofluorescence was performed as described previously (Cantor et al., 2001). Cells grown on cover slips were either untreated or treated with MMC (250 nM) for 16 h. Then, cells were fixed and permeabilized. After incubation with primary antibodies against phospho DNA-PKcs (Abcam; 1:200), γ-H2AX (Upstate 1:100), Rad51 (Abcam; 1:1,000), 53BP1 (Novus Biologicals; 1:100), or P-ATR/ATM Substrate (Cell Signaling; 1:100), cells were washed and then incubated with secondary antibody. After washing, cover slips were mounted onto glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories). Cells with >10 foci per cell were scored as positive. For Edu labeling, cells were left untreated or treated with aphidicolin for 18 h and released at indicated times. Edu labeling was carried out with Click-iT Edu imaging kit (Invitrogen) according to the manufacturer’s instructions.
Metaphase spread preparation

Cells were left untreated or treated with MMC. Then, cells were incubated in media containing 100 ng/ml Colcemid for 1.5 h. After incubation, cells were harvested by trypsinization, lysed in 75 mM KCl, and fixed with fixative solution (75% methanol, 25% acetic acid). Fixed cells were dropped onto slides at 55°C, allowed to dry, and stained with Giemsa. Chromosome abnormalities were scored based on standard guidelines (Levitt et al, 2007).

Generation of mice and PCR genotyping

Msh2-deficient mice (Reitmair et al, 1996) were obtained from T. Mak, University of Toronto, Toronto, Canada. Fancd2-deficient mice (Houghtaling et al, 2003) were obtained from M. Grompe, Oregon Health and Sciences University, Portland, OR, USA. The mouse strains were backcrossed to C57BL/6 for at least eight generations. Mice were maintained as heterozygotes, and double heterozygotes (Msh2+/Fancd2−/−) were bred to obtain embryos of all six genotypes studied here. Mice were housed in the same room of the IACUC-approved SPF facility at University of Massachusetts Medical School and were bred and used under guidelines formulated by the University of Massachusetts Animal Care and Use Committee. As described in Reitmair et al, 1996 and Houghtaling et al, 2003, 50 ng of gDNA was prepared from embryo’s head or mom’s tail and used as a template in PCR to genotype mice. For Fancd2, forward primer MG968 (5′-TCAGCCTCACATGGAGTTTAACG-3′) and two reverse primers MG1280 (5′-GCTACACAGCATTGCCATAAG-3′) and MG1008 (5′-CAGGATGAAAGGTCTTACG-3′) were used to amplify a wild-type band of 303 bp or a mutant band of 459 bp. The reaction conditions were 95°C for 2 min; 36 cycles of 94°C for 25 s, 58°C for 25 s, and 72°C for 35 s; and a final extension at 72°C for 2 min. For Msh2, forward primer Msh2 COM (5′-AAATGGCAGT- CATTGGG-3′) and two reverse primers Msh2 WT (5′-GCTCACT TA- GACCATTGT-3′) and Msh2 MT (5′-GCCCTCTGAGGATCTTCTC- 3′) were used to amplify a wild-type band of 174 bp or a mutant band of 460 bp. The reaction conditions were 95°C for 2 min; 36 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

MP and JX helped design and conduct the experiments. AU maintained the mouse colony and generated crosses. JS advised on experimental design and edited the manuscript. SC designed and led the study and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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