Published in final edited form as:

Cancer Prev Res (Phila). 2010 November ; 3(11): 1409–1416. doi:10.1158/1940-6207.CAPR-10-0118.

An MLH1 Mutation Links BACH1/FANCI to Colon Cancer, Signaling, and Insight toward Directed Therapy

Jenny Xie¹, Shawna Guillemette¹, Min Peng¹, Candace Gilbert¹, Andrew Buermeyer², and Sharon B. Cantor¹

¹Department of Cancer Biology, University of Massachusetts Medical School, Women's Cancers Program, UMass Memorial Cancer Center, Worcester, Massachusetts

²Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon

Abstract

Defects in MLH1, as with other mismatch repair (MMR) proteins, are the primary cause of hereditary nonpolyposis colon cancer (HNPCC). Mutations in MMR genes often disrupt mismatch repair and MMR signaling functions. However, some HNPCC-associated mutations have unknown pathogenicity. Here, we uncover an MLH1 clinical mutation with a leucine (L)-to-histidine (H) amino acid change at position 607 that ablates MLH1 binding to FANCI. Given that a DNA helicase is not essential for mammalian MMR *in vitro*, we considered that loss of MLH1 binding to FANCI could alter MMR signaling. Consistent with this hypothesis, FANCI-deficient cells exhibit delayed MMR signaling and apoptotic responses that generate resistance to agents that induce O⁶-methylguanine lesions. Our data indicate that the delay in MMR signaling provides time for the methylguanine methyltransferase (MGMT) enzyme to reverse DNA methylation. In essence, FANCI deficiency alters the competition between two pathways: MGMT-prosurvival versus MMR-prodeath. This outcome could explain the HNPCC familial cancers that present as microsatellite stable and with intact MMR, such as MLH1^{L607H}. Importantly, the link between FANCI and HNPCC provides insight toward directed therapies because loss of the FANCI/MLH1 interaction also uniquely sensitizes cells to DNA cross-linking agents.

Introduction

In the absence of DNA repair proteins, cell cycle checkpoints or DNA damage repair pathways are not properly activated, propelling tumorigenesis. Moreover, in the absence of DNA repair proteins, cancer cells can develop resistance to DNA-damaging agents used as chemotherapy. For example, loss of the mismatch repair (MMR) pathway is associated with hereditary nonpolyposis colon cancer (HNPCC; ref. (1)) and with chemoresistance (2, 3). MMR-deficient cells not only resist DNA damage-induced arrest and evade apoptosis but

Corresponding Author: Sharon B. Cantor, Department of Cancer Biology, UMass Medical School, 364 Plantation Street, Worcester, MA 01605. Phone: 508-856-4421; Fax: 508-856-1310; Sharon. Cantor@umassmed.edu.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

also have a greatly enhanced mutation frequency. Thus, MMR-deficient cells often have a mutator phenotype associated with microsatellite instability (4).

MMR proteins are required to activate apoptosis in response to certain types of DNA lesions. For example, methyl nitrosourea (MNU) generates methylation at O⁶ in guanine of DNA to form O⁶-methylguanine (O⁶-meG). This lesion is sensed by MMR through the heterodimer MutSα (MSH2 and MSH6), which recruits the heterodimer MutLα (MLH1 and PMS2) to initiate MMR signaling and repair. However, the contribution of these responses to apoptosis is not fully understood (1). In one model, MMR proteins are hypothesized to facilitate misguided attempts to repair DNA methylation, ultimately leading to more severe secondary lesions, such as double-strand breaks (5). In an alternative model, MMR proteins are proposed to function directly in activating checkpoint and apoptosis independent of a repair function (6). Consistent with the latter model, in response to O⁶-meG lesions, the MutSα and MutLα complexes are required to recruit and activate the checkpoint kinase ATR (7). In either model, the initiation of a MMR response and apoptosis can be minimized if damage is reversed by the enzyme methylguanine methyltransferase (MGMT). MGMT can transfer a methyl group onto itself, thus repairing an O⁶-meG lesion in a single step.

Separation-of-function mutations in MMR genes show that both repair and checkpoint functions are critical for tumor suppression. For example, *Msh2*-null mice develop tumors with faster onset than mice that carry a missense mutation, *Msh2*^{G674A}, which disrupts repair but not checkpoint function (8). Moreover, *MLH1*-null mice develop a full range of tumors, whereas mice that carry a missense mutation that disrupts repair, *MLH1*^{G67R}, present fewer intestinal tumors (9). A separation-of-function mutant that disrupts checkpoint but not repair remains to be identified. Conceivably, such a mutant could exist among HNPCC sequence variants that are characterized by unknown pathogenicity and/or a microsatellite stable (MSS) phenotype.

Loss of DNA repair and checkpoint functions as well as cancer is also a characteristic associated with defects in the BRCA1-associated helicase FANCF (also known as BACH1/BRIP1). FANCF mutations were identified in breast cancer (10, 11) and also in the cancer-prone disease Fanconi anemia (12–14). Treatment of FANCF-null FA-J cells with DNA cross-linking agents, such as mitomycin C (MMC), generates cellular sensitivity and a prolonged checkpoint response. These outcomes are corrected by complementation with wild-type FANCF, but not with an MLH1-interaction defective mutant FANCF^{K141/142A} (15). Given this finding, we considered that loss of the FANCF/MLH1 interaction could be associated with cancer.

In this study, we uncover that loss of the FANCF/MLH1 interaction is associated with HNPCC. Specifically, we identified an MLH1 clinical mutation, *MLH1*^{L607H}, which ablates MLH1 binding to FANCF and alters the DNA damage response. We identify that expression of *MLH1*^{L607H} in *MLH1*-null cells generates sensitivity to MMC but resistance to MNU. The resistance to MNU is dependent on MGMT activity. Likewise, we find that FANCF-null and FANCF-depleted cells are resistant to MNU when MGMT is active. The data indicate that the MGMT dependence of our findings is due to reduced MMR function, which allows

greater time for MGMT lesion reversal. In particular, we find that FANCD1 deficiency delays MMR checkpoint and apoptotic responses. We suggest that this delay could explain some HNPCC familial cancers, such as MLH1^{L607H}, characterized as MSS and with intact repair. The link between FANCD1, MMR signaling, and colon cancer suppression also provides insight toward directed therapy because loss of the FANCD1/MLH1 interaction sensitizes cells to DNA cross-linking agents.

Materials and Methods

Cell lines

MCF7 and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/mL each). HCT116 cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/mL each). FA-D2, FA-D2+FANCD2, FA-C, and FA-G cell lines were obtained from Fanconi Anemia Research Fund (FARF) and grown in DMEM supplemented with 15% fetal bovine serum and penicillin/streptomycin (100 units/mL each). FA-J and fibroblast cell lines were cultured as previously described (12). FA-J cells were infected with the POZ retroviral vector (16) containing no insert, FANCD1^{WT}, FANCD1^{K52R}, and FANCD1^{K141/142A} and pCDNA3 vectors were described previously (15).

Immunoprecipitation, Western blot, and antibodies

Cells were untreated or treated with 1 mmol/L hydroxyurea (continuous treatment) or MNU (1 hour, serum-free), harvested, lysed, and processed for Western blot analysis as described previously (15). The antibodies used for immunoprecipitation and Western blot assays included FANCD1, monoclonal pool (1A3, 2G7, and IG5; ref. 10) and polyclonal, E67 (17), β -actin (Sigma), MLH1 (BD Biosciences), PMS2 (BD Biosciences), MSH6 (BD Biosciences), MSH2 (Calbiochem), Chk1 (Bethyl), pChk1317 (Bethyl), and MGMT (NeoMarkers). To prepare chromatin extracts, MCF7 cell lines were prepared as described (18). Briefly, cells were left untreated or treated with 1 mmol/L MNU for 1 hour, washed, and incubated for 24 hours. The cells were washed extensively with hypotonic buffer containing digitonin, collected, and sonicated.

Viability assays

FA-J complemented cells, stable MCF7 cells, or transiently transfected HCT116 cells were seeded onto six-well plates at 1,000 per well; incubated overnight; and treated with increasing doses of MNU (1 hour, serum-free), methyl methanesulfonate (MMS) (1 hour, serum-free), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (1 hour, serum-free), or *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (1 hour, serum-free). To suppress MGMT, 2 hours before exposure to DNA damage agents, cells were incubated with 20 μ mol/L *O*⁶-benzylguanine (*O*⁶-BZG). In addition, *O*⁶-BZG was also included during and after treatment. DNA-damaged cells were counted after 5 to 8 days using a hemocytometer or measured photometrically in a model 3550 microplate reader (Perkin-Elmer) as the relative growth (in luciferase units) using the CellTiter-Glo viability assay (Promega). Colonies were stained with Giemsa (LabChem) and counted. Percent growth or colony survival was calculated as (treated cells/untreated cells) $\times$ 100.

Apoptosis assay

Stable MCF7 cells were plated onto 60-mm plates, incubated overnight, and treated with 1 mmol/L MNU (1 hour, serum-free). O^6 -BZG was used to suppress MGMT. Cells were washed 72 and 96 hours after treatment, and 1×10^6 cells were stained with Annexin V-FITC (Sigma) and propidium iodide (Sigma). Stained cells were analyzed by fluorescence-activated cell sorting.

Results

MLH1 clinical mutant L607H disrupts FANCDJ binding

Previously, we found that the FANCDJ/MLH1 interaction was required for FANCDJ function in DNA cross-link repair. Unlike wild-type FANCDJ, the MLH1-interaction-defective mutant FANCDJ^{K141/142A} failed to restore MMC resistance to FA-J cells (15). To perform the reciprocal experiment, we sought to identify a FANCDJ-interaction-defective mutant of MLH1. Deletion of MLH1 amino acids 478 to 744 disrupted not only FANCDJ but also PMS2 binding (ref. 15; Fig. 1A). To selectively disrupt FANCDJ binding, we considered whether an MLH1 missense mutation associated with HNPCC could be useful. Thus, MLH1 clinical mutants targeting the region of amino acids 478 to 744 and proficient for PMS2 binding were screened for FANCDJ binding. Wild-type or mutant MLH1 constructs were cotransfected with PMS2 into MutLa-deficient 293T or HCT116 cells. MLH1 antibody precipitation revealed that most of the MLH1 mutants precipitated PMS2 and FANCDJ similar to wild-type MLH1. Notably, mutation of MLH1 at amino acid 607, from leucine to histidine (MLH1^{L607H}), disrupted FANCDJ, but not PMS2, binding (Fig. 1B and C). In the reciprocal immunoprecipitation with FANCDJ antibody, FANCDJ precipitated wild-type MLH1, but not the MLH1^{L607H} mutant (Fig. 1C).

Expression of MLH1 L607H mutant sensitizes cells to MMC

Given that cells lacking the FANCDJ/MLH1 interaction were sensitive to MMC (15), we considered whether expression of MLH1^{L607H}, as compared with wild-type MLH1, differentially affected the MMC sensitivity of HCT116 cells. To address this possibility, vector, wild-type, or MLH1^{L607H} were cotransfected with PMS2; cells were plated at equal numbers; and cell proliferation was assessed in response to increasing dose of MMC. Expression of wild-type MLH1 as compared with vector control did not measurably alter the sensitivity of HCT116 cells to MMC. In contrast, expression of MLH1^{L607H} enhanced MMC sensitivity (Fig. 1D). Thus, the clinical MLH1^{L607H} mutant resembled the FANCDJ^{K141/142A} mutant in sensitivity to MMC, further indicating that the FANCDJ/MLH1 interaction is important for processing DNA cross-links.

FANCDJ deficiency alters the MNU response when MGMT is active

MLH1 functions in the MMR pathway, which is required for cellular sensitivity to DNA methylating agents such as MNU (19). To address whether this sensitivity requires an intact FANCDJ/MLH1 interaction, transfected HCT116 cells were treated with increasing doses of MNU. Introduction of wild-type MLH1 restored MNU sensitivity to HCT116 cells. As expected, the MNU-induced sensitivity was most dramatic when DNA methylation reversal

by MGMT was inhibited with $\mathcal{O}^6$ -BZG (Fig. 1E). Similar to wild-type MLH1, introduction of MLH1^{L607H} restored MNU sensitivity when MGMT was inhibited (Fig. 1E). Surprisingly, when MGMT was active, in contrast to wild-type MLH1, introduction of MLH1^{L607H} did not enhance MNU sensitivity. Instead, cells expressing MLH1^{L607H} were resistant to MNU, similar to vector-transfected cells (Fig. 1E). In summary, a defective FANCI/MLH1 interaction enhanced MMC sensitivity but reduced MNU sensitivity when MGMT was active.

FANCI-null FA-J patient cells are uniquely resistant to MNU

If FANCI expression was required for a robust MNU-induced sensitivity, we reasoned that FANCI-null FA-J fibroblast cells should be more resistant to MNU than FA-J cells complemented with wild-type FANCI. To address this idea, we first confirmed that wild-type FANCI was functionally expressed in FA-J cells by immunoblot analysis and restored resistance to MMC (Fig. 2A; Supplementary Fig. S1). Next, we scored resistance to MNU with MGMT active or inhibited in proliferation assays. Remarkably, wild-type FANCI restored MNU sensitivity when MGMT was active but had no effect when MGMT was inhibited (Fig. 2B). In contrast, wild-type FANCD2 complementation in FANCD2-null FA-D2 patient cells had no effect on MNU resistance with active MGMT (Fig. 2C). Furthermore, in comparison with other Fanconi anemia patient cell lines, FA-C (PD331) and FA-G (PD352), or normal fibroblasts, FA-J cell lines (2833 and EUFA30F) were more resistant to MNU (Fig. 2D).

FANCI function is linked to MMR and MLH1 binding

The MGMT dependence of our findings could reflect a role for FANCI in modulating MGMT protein levels or activity. However, FANCI expression in FA-J cells did not dramatically change the expression of MGMT in response to MNU (Fig. 3A). Greater MGMT activity should reduce the toxicity of *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea, which generates DNA methylation processed by MGMT, but not by MMR (20). Instead, toxicity was greater in FANCI-deficient as compared with wild-type FANCI-expressing FA-J cells (Fig. 3B). Similar to MNU, however, FANCI deficiency enhanced resistance to *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Fig. 3B), a DNA methylating agent that, similar to MNU, generates $\mathcal{O}^6$ -meG lesions processed by MMR. Taken together, the data indicated that FANCI deficiency altered MMR function, but not MGMT function.

Given these findings, we sought to test the prediction that FANCI promoted MMR function through a direct FANCI/MLH1 interaction. Thus, FA-J cells were complemented with wild-type FANCI or the MLH1-interactive defective mutant FANCI^{K141/142A}, as well as the enzyme inactive mutant FANCI^{K52R} (15, 17). Whereas protein expression was similar (Fig. 3C), unlike wild-type FANCI, FANCI^{K52R} and FANCI^{K141/142A} failed to restore MNU sensitivity. Instead, MNU resistance in the mutant-expressing lines was similar to that in FA-J cells complemented with vector (Fig. 3D), indicating that both the MLH1 binding and enzyme activity of FANCI were required to restore MNU sensitivity.

FANCD1 functions in MMR-induced signaling response

Conceivably, the MNU resistance in FANCD1-deficient cells could reflect reduced processing by MMR, which in turn enables a greater number of lesions to be reversed by MGMT. To test this idea, we sought to directly compare how FANCD1 or MLH1 deficiency affected the MNU-induced checkpoint and apoptotic response. Western blot confirmed that shRNA reagents targeted FANCD1 and MLH1 proteins in MCF7 breast cancer cells (Fig. 4A). FANCD1 deficiency did not alter the expression of the MutL α complex (Fig. 4A). In contrast, shRNA targeting MLH1 suppressed both MLH1 and PMS2, confirming reports that MLH1 stabilizes PMS2 (ref. 21; Fig. 4A). As found in FA-J cells, FANCD1-deficient MCF7 cells were resistant to MNU when MGMT was active (Fig. 4C). In fact, colony survival data in MLH1- or FANCD1-depleted MCF7 cells were similar; both had ~2-fold enhanced colony survival as compared with nonsilencing controls (Fig. 4C). When MGMT was inhibited, FANCD1 depletion had no effect on MNU-induced colony survival as compared with MLH1 depletion, which dramatically enhanced the number of surviving colonies (Fig. 4D). Likewise, MLH1 depletion reduced apoptosis following MNU treatment irrespective of MGMT activity (Fig. 4B). Instead, FANCD1 depletion only had a measurable effect on apoptosis in MGMT-active cells (~2-fold) as compared with nonsilencing controls (Fig. 4B).

MMR is required for Chk1 activation following MNU treatment (7). Consistent with this point, depletion of MLH1 reduced MNU-induced, but not hydroxyurea-induced, Chk1 phosphorylation (Fig. 4E and F). As expected, in comparison with MGMT-inhibited cells, MGMT-active cells required a higher dose of MNU (~1.5 mmol/L) to induce Chk1 phosphorylation (data not shown and Fig. 4E and F). Depletion of MLH1 reduced MNU-induced Chk1 phosphorylation by ~1.7- and ~5.5-fold at 1.5 and 2 mmol/L MNU, respectively (Fig. 4E and F). Interestingly, as compared with nonsilencing control, FANCD1 depletion also decreased Chk1 phosphorylation induced with 1.5 and 2 mmol/L MNU by ~1.7- and ~2.2-fold, respectively. Furthermore, depletion of FANCD1 with two distinct siRNAs also reduced MNU-induced Chk1 phosphorylation by ~1.7- and ~1.8-fold at 1.5 mmol/L MNU (Supplementary Fig. S2). Thus, FANCD1 is required for maximal MNU-induced checkpoint signaling, sensitivity, and apoptosis when MGMT is active.

Discussion

The complex role of MMR proteins in the DNA damage response has complicated efforts to understand which function(s) is required for tumor suppression. Furthermore, MMR-associated HNPCC tumors have heterogeneous defects and varying levels of MSI. Here, we uncover the potential pathogenic defect of an HNPCC-associated MLH1 mutant, MLH1^{L607H}, to be lack of FANCD1 binding. Loss of FANCD1 binding is unlikely to affect repair given that the MLH1^{L607H} mutant was shown previously to be intact for MMR, have normal expression, and generate tumors that are MSS (22–24). Instead, our data indicate that loss of FANCD1 binding reduces, but does not inactivate, MMR signaling. Most notably, in the absence of FANCD1, cells can initiate processing, checkpoint activation, and undergo cell death. However, the timing of these events seems to be delayed. With this delay, the MMR-independent methylation reversal by MGMT likely has time to enhance DNA methylation

resistance (see model in Fig. 5). An intriguing question is whether this delay in MMR signaling could be a mechanism linked to cancer and/or chemoresistance.

Our findings further show that a functional relationship exists between FANCI and MLH1 in the DNA damage response. Similar to the response to DNA cross-links, the response to DNA methylation requires FANCI helicase and MLH1 binding activities. Why does loss of the FANCI/MLH1 interaction enhance sensitivity to agents that induce DNA cross-links but reduce sensitivity to agents that induce *O*⁶-meG? In response to DNA cross-links, the delay in MMR signaling likely interferes with repair and/or recovery. In contrast, in response to *O*⁶-meG lesions, the delay in MMR signaling is prosurvival because MGMT reversal is available. If MGMT is inactivated, however, *O*⁶-meG lesions will inevitably be processed by MMR and induce apoptosis. Because MGMT is typically inhibited in most studies analyzing MMR contribution to DNA methylation signaling, it is unclear whether other MLH1 or HNPCC-associated mutations will generate resistance to MNU when MGMT is active. If so, tumors derived from such patients may also be selectively sensitive to MMC.

Our data show that FANCI contributes to MMR signaling. Whether FANCI facilitates loading, translocation, or stabilization of MMR complexes on chromatin is not clear. Loss of FANCI or the FANCI/MLH1 interaction may not overtly limit MMR activity, given that patients with the MLH1^{L607H} mutation are characterized as MSS (25) and MMR activity for this mutant is similar to wild-type MLH1 (24). It is possible, however, that delayed MMR signaling in MLH1^{L607H} patient cells might provide a selective advantage in the initial stages of tumorigenesis, or under high mutation stress, enhance the mutation frequency. Conceivably, more DNA damage could be tolerated without activating the MMR-induced checkpoint. This lowered checkpoint barrier could enhance genomic instability and cancer. Mouse model studies will be useful to estimate the risk associated with the MLH1^{L607H} mutant allele.

Acknowledgments

Grant Support

Grant RO1 CA129514-01A1 (S.B. Cantor). We thank Mr. and Mrs. Edward T. Vitone, Jr. for charitable contributions.

References

1. O'Brien V, Brown R. Signalling cell cycle arrest and cell death through the MMR System. *Carcinogenesis*. 2006; 27:682–92. [PubMed: 16332722]
2. Kat A, Thilly WG, Fang WH, Longley MJ, Li GM, Modrich P. An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. *Proc Natl Acad Sci U S A*. 1993; 90:6424–8. [PubMed: 8341649]
3. Branch P, Aquilina G, Bignami M, Karran P. Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature*. 1993; 362:652–4. [PubMed: 8464518]
4. Schofield MJ, Hsieh P. DNA mismatch repair: molecular mechanisms and biological function. *Annu Rev Microbiol*. 2003; 57:579–608. [PubMed: 14527292]
5. Karran P, Marinus MG. Mismatch correction at *O*⁶-methylguanine residues in *E. coli* DNA. *Nature*. 1982; 296:868–9. [PubMed: 7040986]
6. Karran P. Mechanisms of tolerance to DNA damaging therapeutic drugs. *Carcinogenesis*. 2001; 22:1931–7. [PubMed: 11751422]

7. Yoshioka K, Yoshioka Y, Hsieh P. ATR kinase activation mediated by MutS α and MutL α in response to cytotoxic O⁶-methylguanine adducts. *Mol Cell*. 2006; 22:501–10. [PubMed: 16713580]
8. Lin DP, Wang Y, Scherer SJ, et al. An Msh2 point mutation uncouples DNA mismatch repair and apoptosis. *Cancer Res*. 2004; 64:517–22. [PubMed: 14744764]
9. Avdievich E, Reiss C, Scherer SJ, et al. Distinct effects of the recurrent Mlh1G67R mutation on MMR functions, cancer, and meiosis. *Proc Natl Acad Sci U S A*. 2008; 105:4247–52. [PubMed: 18337503]
10. Cantor SB, Bell DW, Ganesan S, et al. BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell*. 2001; 105:149–60. [PubMed: 11301010]
11. Seal S, Thompson D, Renwick A, et al. Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat Genet*. 2006; 38:1239–41. [PubMed: 17033622]
12. Litman R, Peng M, Jin Z, et al. BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product. *FANCI Cancer Cell*. 2005; 8:255–65. [PubMed: 16153896]
13. Levitus M, Waisfisz Q, Godthelp BC, et al. The DNA helicase BRIP1 is defective in Fanconi anemia complementation group. *J Nat Genet*. 2005; 37:934–5. [PubMed: 16116423]
14. Levrán O, Attwooll C, Henry RT, et al. The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. *Nat Genet*. 2005; 37:931–3. [PubMed: 16116424]
15. Peng M, Litman R, Xie J, Sharma S, Brosh RM Jr, Cantor SB. The FANCI/MutL α interaction is required for correction of the cross-link response in FA-J cells. *EMBO J*. 2007; 26:3238–49. [PubMed: 17581638]
16. Nakatani Y, Ogryzko V. Immunoaffinity purification of mammalian protein complexes. *Methods Enzymol*. 2003; 370:430–44. [PubMed: 14712665]
17. Cantor S, Drapkin R, Zhang F, et al. The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations. *Proc Natl Acad Sci U S A*. 2004; 101:2357–62. [PubMed: 14983014]
18. Hidaka M, Takagi Y, Takano TY, Sekiguchi M. PCNA-MutS α -mediated binding of MutL α to replicative DNA with mismatched bases to induce apoptosis in human cells. *Nucleic Acids Res*. 2005; 33:5703–12. [PubMed: 16204460]
19. Jiricny J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol*. 2006; 7:335–46. [PubMed: 16612326]
20. Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair (Amst)*. 2007; 6:1079–99. [PubMed: 17485253]
21. Trojan J, Zeuzem S, Randolph A, et al. Functional analysis of hMLH1 variants and HNPCC-related mutations using a human expression system. *Gastroenterology*. 2002; 122:211–9. [PubMed: 11781295]
22. Cravo M, Afonso AJ, Lage P, et al. Pathogenicity of missense and splice site mutations in hMSH2 and hMLH1 mismatch repair genes: implications for genetic testing. *Gut*. 2002; 50:405–12. [PubMed: 11839723]
23. Fidalgo P, Almeida MR, West S, et al. Detection of mutations in mismatch repair genes in Portuguese families with hereditary non-polyposis colorectal cancer (HNPCC) by a multi-method approach. *Eur J Hum Genet*. 2000; 8:49–53. [PubMed: 10713887]
24. Takahashi M, Shimodaira H, Andreutti-Zaugg C, Iggo R, Kolodner RD, Ishioka C. Functional analysis of human MLH1 variants using yeast and *in vitro* mismatch repair assays. *Cancer Res*. 2007; 67:4595–604. [PubMed: 17510385]
25. Barnetson RA, Cartwright N, van Vliet A, et al. Classification of ambiguous mutations in DNA mismatch repair genes identified in a population-based study of colorectal cancer. *Hum Mutat*. 2008; 29:367–74. [PubMed: 18033691]

Fig. 1. MLH1 L607H mutant is defective for FANCD binding and alters DNA damage response. A, MLH1 cartoon showing the FANCD and PMS2 interacting domains. 293T cells (B) or HCT116 cells (C) cotransfected with PMS2 with different versions of MLH1 were collected and analyzed by Western blot with the indicated antibodies or immunoprecipitated with MLH1 or FANCD antibody and blotted with the indicated antibodies. Transfected HCT116 cells were plated at low density, treated with the indicated doses of (D) MMC or (E) MNU with MGMT active or inhibited, and allowed to grow for 5 d. Percent growth was analyzed by collecting and counting the cells or by measuring ATP content.

Fig. 2.

When MGMT is active, FA-J cells are uniquely resistant to MNU. A, FANCD2-null FA-J (EUFA30F) cells were complemented with vector or FANCD2^{WT}; cells were either collected and analyzed by immunoblot with the indicated antibodies or (B) plated at low density, treated with the indicated doses of MNU with MGMT active or inhibited, and allowed to grow for 8 d. The cells were collected and counted to analyze percent growth. C, FA-D2 (PD20) and FA-D2+FANCD2 cells were either collected and analyzed by immunoblot with the indicated antibodies or treated with the indicated dose of MNU. The cells were collected and counted to analyze percent growth 8d later. D, Fibroblast (2822), FA-J (2833), FA-J (EUFA30F), FA-C (PD331), or FA-G (PD352) cells were plated at low density, treated with the indicated dose of MNU; and allowed to grow for 8 d. Percent growth was assessed as described in B.

Fig. 3.

The helicase activity and interaction with MLH1 of FANCI are required for its role in MNU response. A, FANCI-null FA-J (EUFA30F) cells complemented with vector or FANCI^{WT} were treated with 1 mmol/L MNU with MGMT active. Cells were collected at various time points and analyzed by immunoblot with the indicated antibodies. B, vector or FANCI^{WT} complemented FA-J cells were plated at low density and treated with the indicated dose of *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU) or *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG). Cells were collected and counted to analyze percent growth 4 to 8 d later. C, FA-J cells were complemented with vector, FANCI^{WT}, FANCI^{K52R}, or FANCI^{K141/142A} cells, which were either collected and analyzed by immunoblot with the indicated antibodies or (D) plated at low density and treated with the indicated dose of MNU. Percent growth was assessed as described in B.

Fig. 4.

FANCD1 functions with MMR proteins in MNU response. A, MCF7 cells stably expressing shRNA reagents targeting control, MLH1, or FANCD1 were either analyzed by immunoblot with the indicated antibodies or B, apoptosis was analyzed after cells were treated with the indicated dose of MNU with MGMT active or inhibited and allowed to grow for the indicated time periods. The cells were collected, fixed, and stained with Annexin V and propidium iodide (percent apoptotic cells were analyzed by fluorescence-activated cell sorting). C and D, stable MCF7 cells were treated with the indicated dose of MNU with MGMT active or inhibited. Cells were fixed and stained with Giemsa 8 d later, and then the colonies were counted. The graph represents three independent experiments. E, stable MCF7 cells were treated with the indicated dose of hydroxyurea (HU) or MNU, collected after 24 h, and analyzed by immunoblot with the indicated antibodies. F, the graph represents the densitometry of pChk1 versus total Chk1.

Fig. 5.

Model showing the enhancement of MMR signaling and apoptosis by FANCI. In FANCI-proficient cells, MMR proteins efficiently process O⁶-meG lesions leading to checkpoint activation and apoptosis. In FANCI-deficient cells, MMR proteins are less efficient at processing O⁶-meG lesions, allowing more time for MGMT processing and thus enhancing cell survival. This study identified MLH1 L607 as a residue required for MLH1 binding to FANCI.