

# RNF4-mediated polyubiquitination regulates the Fanconi anemia/BRCA pathway

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**The Fanconi anemia/BRCA (FA/BRCA) pathway is a DNA repair pathway that is required for excision of DNA interstrand cross-links. The 17 known FA proteins, along with several FA-associated proteins (FAAPs), cooperate in this pathway to detect, unhook, and excise DNA cross-links and to subsequently repair the double-strand breaks generated in the process. In the current study, we identified a patient with FA with a point mutation in *FANCA*, which encodes a mutant *FANCA* protein (*FANCA*<sup>I9395</sup>). *FANCA*<sup>I9395</sup> failed to bind to the FAAP20 subunit of the FA core complex, leading to decreased stability. Loss of FAAP20 binding exposed a SUMOylation site on *FANCA* at amino acid residue K921, resulting in E2 SUMO-conjugating enzyme UBC9-mediated SUMOylation, RING finger protein 4-mediated (RNF4-mediated) polyubiquitination, and proteasome-mediated degradation of *FANCA*. Mutation of the SUMOylation site of *FANCA* rescued the expression of the mutant protein. Wild-type *FANCA* was also subject to SUMOylation, RNF4-mediated polyubiquitination, and degradation, suggesting that regulated release of FAAP20 from *FANCA* is a critical step in the normal FA pathway. Consistent with this model, cells lacking RNF4 exhibited interstrand cross-linker hypersensitivity, and the gene encoding RNF4 was epistatic with the other genes encoding members of the FA/BRCA pathway. Together, the results from our study underscore the importance of analyzing unique patient-derived mutations for dissecting complex DNA repair processes.**

## Introduction

Maintaining the integrity of the genome is critical for both normal cellular processes and for suppressing mutagenic events that lead to cancer. Damage to DNA can arise from multiple external sources, including ionizing radiation, UV light, and environmental toxins. Intrinsic damage, such as the interstrand cross-linking (ICL) of DNA by serum aldehydes (1, 2), is especially dangerous, and the repair of such interstrand cross-links (ICLs) requires several enzymatic steps.

Fanconi anemia (FA) is an autosomal recessive cancer susceptibility disorder characterized by congenital abnormalities, progressive bone marrow failure, and cellular hypersensitivity to DNA ICL agents (3, 4). There are at least 16 FA genes, and the encoded FA proteins cooperate in a DNA repair pathway, the so-called FA/BRCA pathway (4, 5). In order to accomplish the formidable task of excising a DNA cross-link, the FA/BRCA pathway must coordinate multiple DNA repair activities, including nucleotide excision (NER) repair, translesion DNA synthesis (TLS) repair, and homologous recombination (HR) repair.

At least 8 of the FA proteins (including the *FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCL*, and *FANCM* proteins) are assembled into a large constitutive protein complex, the so-called FA core complex, which is targeted to stalled replication forks containing a DNA cross-link. The FA core complex also contains additional FA-associated proteins (FAAPs), includ-

ing FAAP24, FAAP100, and FAAP20. The FA core complex and its subcomplexes are required for coordinating several DNA repair steps in the FA/BRCA pathway (6). First, the *FANCM* subunit and its direct binding partner, FAAP24, are required for sensing the DNA cross-link and for docking the FA core complex at the site of the arrested DNA replication fork (7–9). The *FANCL* subunit and its direct binding partner, FAAP100, are critical for the regulated monoubiquitination of the *FANCD2*/*FANCI* heterodimer (10). Monoubiquitinated *FANCD2* is subsequently required for binding to *SLX4*/*FANCP*, an endonuclease protein complex required for unhooking the DNA cross-link and for the downstream HR activity of the pathway (11). The *FANCA* subunit, in contrast, is a scaffold protein in the FA core complex, which is required for binding to the FAAP20 subunit. Recent studies have indicated that FAAP20 binds to *FANCA* and recruits *REV1* to the FA core complex, which is required for TLS activity (12).

The ubiquitin-like modifier, SUMO, has recently been shown to play critical roles in DNA repair processes (13–16). Indeed, DNA repair protein complexes, such as the HR complex, can undergo UBC9-mediated multiSUMOylation, leading to assembly of SUMOylated complexes at sites of DNA repair (17, 18). SUMOylation only affects a minor fraction of protein subunits in these assembled complexes, and SUMO-SUMO-interacting motif (SUMO-SIM) interactions among subunit members provide further stabilization.

Another critical step in DNA repair is the timed degradation and clearance of the DNA repair protein complex, resulting in recovery of the replication fork and resumption of normal DNA replication. In this case, the SUMO-conjugated subunits

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may be removed by SUMO-targeted ubiquitin ligase-mediated (STUbL-mediated) ubiquitin-dependent proteasomal degradation. One STUbL, RING finger protein 4 (RNF4), is a known regulator of multiple DNA repair pathways, through its timed polyubiquitination of DNA repair proteins, such as SUMO-MDC1 and SUMO-RPA, and their subsequent proteasomal degradation (19–22). Furthermore, genetic disruption of *RNF4* is known to play a more general role in the regulation of genomic stability and DNA cross-link repair.

In the current study, we identify a patient with a FA-derived missense mutation in *FANCA*, which elucidates a role of SUMO in the FA pathway. The point mutation, *FANCA*<sup>I939S</sup>, fails to bind the FAAP20 subunit of the FA core complex. Failure of FAAP20 binding exposes a SUMOylation site on *FANCA*. This site is SUMOylated rapidly, resulting in RNF4-mediated polyubiquitination and proteasome-mediated degradation. Our results also reveal a more general role of SUMOylation of FA proteins and their RNF4-mediated polyubiquitination in the regulation of the FA pathway.

## Results

*FANCA*<sup>I939S</sup> mutation disrupts FAAP20 binding. We identified a patient with FA with an atypical clinical phenotype (Figure 1). The patient was a 33-year-old woman who developed triple-negative breast cancer (estrogen receptor-negative, progesterone-negative, HER2-negative breast cancer). She was noted to have short stature and mild bone marrow dysfunction, suggesting a diagnosis of FA. Primary peripheral blood lymphocytes exhibited an increased level of DNA damage-inducible quadriradial chromosomes (Figure 1A), consistent with the diagnosis of FA (23, 24). Since subtype A is the most prevalent subtype of FA, accounting for 60% to 70% of patients with FA (25), we next performed genomic sequencing of *FANCA* from the patient's primary peripheral blood lymphocytes and her primary skin fibroblast cells (Figure 1B and data not shown). Two mutant *FANCA* alleles were identified. One mutant allele contained a previously known germline mutation in *FANCA*, the S947Stop mutation (26–28). The other mutant allele contained a thymine-to-guanine change at nucleotide 2816 (variant of unknown significance), predicted to encode a full-length mutant *FANCA* protein with one amino acid change (*FANCA*<sup>I939S</sup>). The mother of the patient with FA carries the *FANCA*<sup>I939S</sup> mutation (data not shown), and the son of the patient with FA is an obligate heterozygous carrier of one of these mutant *FANCA* alleles (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI79325DS1).

To test the possible functional significance of the *FANCA*<sup>I939S</sup> mutation, we compared the ability of wild-type *FANCA* (*FANCA*<sup>WT</sup>) and the *FANCA*<sup>I939S</sup> mutant to complement a protein-null human FA-A fibroblast line, GM6914 (Figure 1C). Unlike the wild-type protein, the mutant protein only partially rescued the mitomycin C (MMC) hypersensitivity of these cells, indicating that the I939S mutation is hypomorphic. *FANCA* associates directly with *FANCG* and FAAP20, and this interaction is critical for the stability of all 3 proteins (12, 29, 30). The I939S mutant protein rescued the expression of *FANCG* but failed to rescue the expression of FAAP20 (Figure 1D), suggesting that the I939S mutation falls in the FAAP20-binding site of *FANCA*. Indeed, the I939S missense mutation is located in exon 28 of the *FANCA* gene, a region impli-

cated previously in FAAP20 binding, and our experiments also demonstrated the binding domain to be between amino acids 913 and 1095 (refs. 12, 30–32, and Supplemental Figure 1B). Interestingly, other patients with FA have been identified with *FANCA* alleles harboring point mutations in this same region of *FANCA* (33).

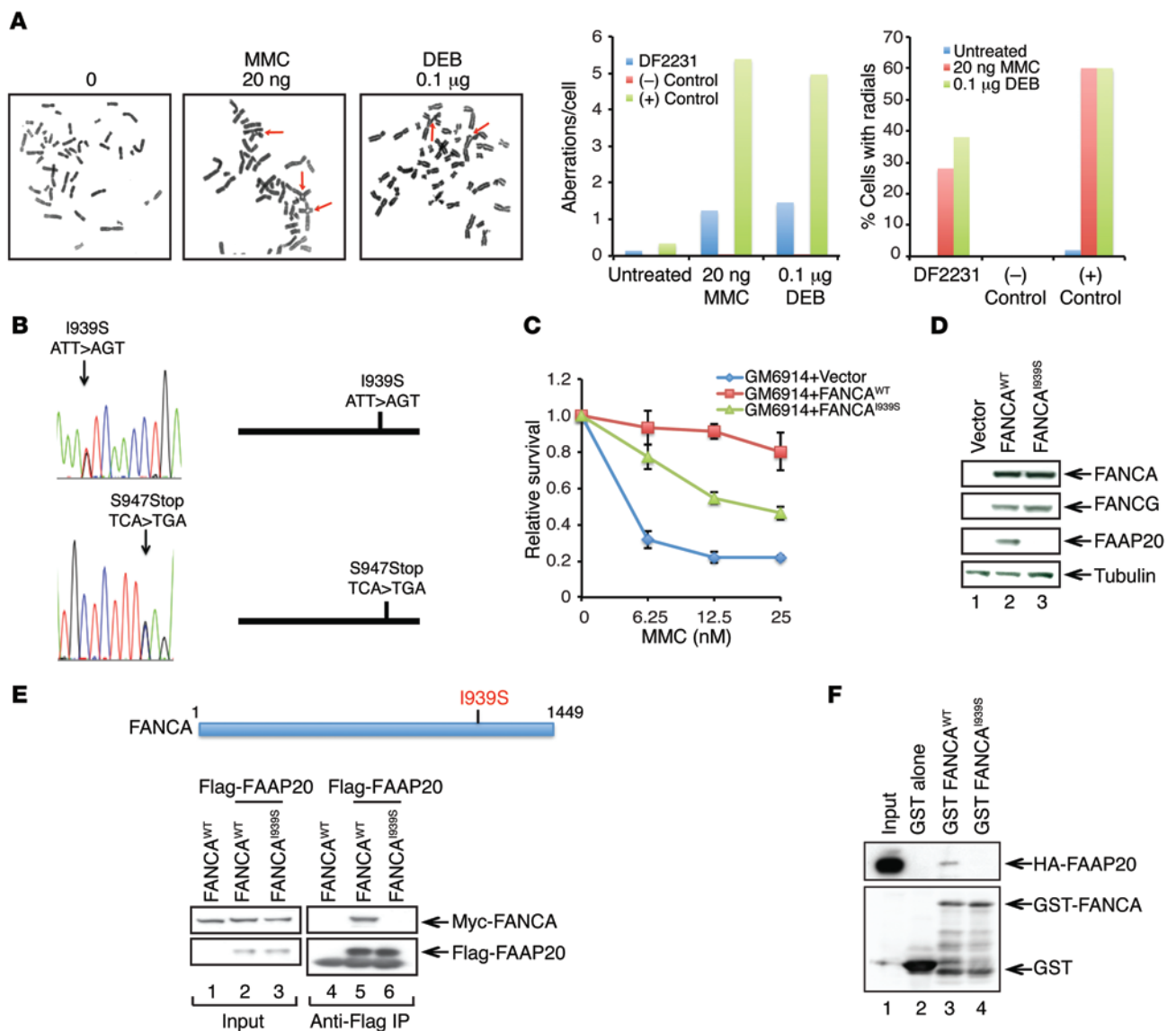
To test whether the *FANCA*<sup>I939S</sup> mutation blocks FAAP20 binding, we coexpressed Myc-tagged *FANCA* with Flag-tagged FAAP20 and tested for coimmunoprecipitation. As predicted, unlike *FANCA*<sup>WT</sup>, the *FANCA*<sup>I939S</sup> protein failed to bind to FAAP20 (Figure 1E). Similarly, a GST-*FANCA*<sup>I939S</sup> failed to pull down HA-tagged FAAP20 in vitro (Figure 1F). Taken together, the patient-derived point mutation in *FANCA* disrupts FAAP20 binding, accounting, at least in part, for its dysfunction (Supplemental Figure 1C) and the patient's clinical phenotype.

*Loss of FAAP20 binding disrupts the TLS function of the FA core complex.* The interaction of *FANCA* and FAAP20 is critical for stabilization of the FA core complex, thereby allowing *FANCD2* monoubiquitination and recruitment of the TLS for ICL repair (12). We next determined whether *FANCA*<sup>I939S</sup> could restore these functions in the FA pathway. Interestingly, *FANCA*<sup>I939S</sup> was able to complement *FANCD2* monoubiquitination, albeit at a lower level than *FANCA*<sup>WT</sup> (Figure 2A). Consistent with the rescue of *FANCD2* monoubiquitination, the *FANCA*-deficient GM6914 cells expressing *FANCA*<sup>I939S</sup> also exhibited DNA damage-inducible *FANCD2* foci (Figure 2B and Supplemental Figure 2). This result confirms that the *FANCA*<sup>I939S</sup> mutant can support FA core complex assembly and *FANCD2* monoubiquitination despite its failure to bind FAAP20.

We demonstrated previously that FAAP20 contains a ubiquitin-binding zinc finger 4 (UBZ4) required for recruiting the TLS scaffold protein, REV1, to sites of DNA cross-link repair (12). REV1 recruitment correlates with cellular TLS repair activity. We next compared the ability of the *FANCA*<sup>I939S</sup> protein to correct the DNA cross-linker-inducible assembly of REV1 nuclear foci with that of the *FANCA*<sup>WT</sup> protein. Complemented *FANCA*-deficient GM6914 cells were exposed to psoralen plus UVA, a mechanism known to induce DNA ICLs. Interestingly, while *FANCA*<sup>WT</sup> supported the assembly of psoralen-UV-inducible REV1 foci, *FANCA*<sup>I939S</sup> failed to rescue REV1 foci (Figure 2C). Previous studies have indicated that DNA damage-inducible REV1 foci assembly correlates with enhanced point mutagenesis, as measured by the *SupF* assay (34). Consistent with this defect, the *FANCA*<sup>I939S</sup> protein, unlike *FANCA*<sup>WT</sup>, failed to rescue UVC-inducible point mutagenesis, as measured by the *SupF* assay (Figure 2D).

Taken together, these results indicate that *FANCA*<sup>I939S</sup> is a separation-of-function mutant. The *FANCA*<sup>I939S</sup> protein functions efficiently in the assembly of the FA core complex and the DNA damage-inducible monoubiquitination of *FANCD2* protein, which is required for *FANCD2* foci assembly and recruitment of the downstream endonuclease complex (11). However, the *FANCA*<sup>I939S</sup> protein fails to bind to FAAP20, fails to recruit REV1, and fails to restore TLS activity.

*Loss of FAAP20 binding results in increased SUMOylation, polyubiquitination, and proteasome-mediated degradation of FANCA.* A primary skin fibroblast culture established from the patient with FA was immortalized with SV40 T antigen and tested for MMC-induced cytotoxicity. These transformed fibroblasts

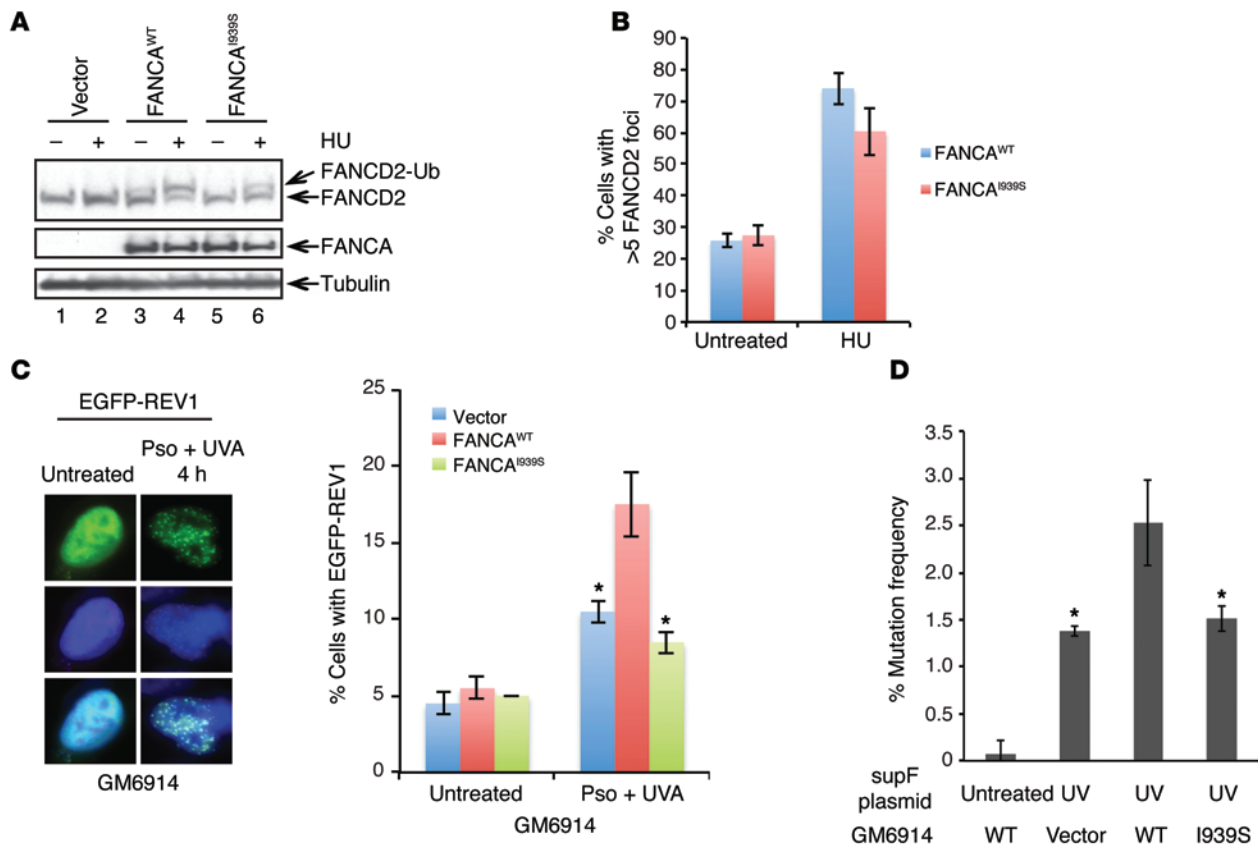


**Figure 1. Atypical clinical presentation of a patient with FA reveals a unique mutation in the *FANCA* gene that disrupts FAAP20 binding.** (A) Image and quantification of chromosomal aberrations and radial chromosomes (indicated by red arrows) of peripheral blood lymphocytes and primary skin fibroblast (DF2231) with a positive (+) and a negative (-) control cultures exposed to 20 ng/ml MMC or 0.1 μg/ml diepoxybutane (DEB). Original magnification, ×100. (B) The chromatograms indicate 2 mutant *FANCA* alleles, a T-to-G point mutation at nucleotide 2816 and a C-to-G point mutation at nucleotide 2840. (C) Relative survival of GM6914 (*FANCA*-deficient) cells complemented with vector, *FANCA*<sup>WT</sup>, or *FANCA*<sup>I939S</sup>; exposed to increasing doses of MMC; and plated for 5 days. Three independent experiments were performed. (D) Immunoblot of *FANCA*, *FANCG*, and *FAAP20* proteins derived from cell lysates of the complemented GM6914 cells. (E) Schematic representation of *FANCA*<sup>I939S</sup> and immunoblot of anti-Flag IP immunocomplexes isolated from 293T cell lysates transfected with the Myc-*FANCA* and Flag-*FAAP20* cDNAs. (F) Immunoblot of GST pull-downs, using bacterially expressed GST-*FANCA* mixed with 293T cell lysates expressing HA-*FAAP20*.

(DF2231) were hypersensitive to MMC, comparable to the hypersensitivity observed for a control *FANCI*-deficient fibroblast culture (Figure 3A). Immunoblotting and immunostaining revealed a detectable but decreased expression level of the full-length mutant *FANCA*<sup>I939S</sup> polypeptide (Figure 3B, lanes 5–8, and Supplemental Figure 3A) in DF2231 cells compared with the higher expression level of full-length *FANCA*<sup>WT</sup> in wild-type cells. Moreover, decreased monoubiquitination of the *FANCD2* protein was observed, consistent with a defect in the FA core complex function. The DF2231 cells also had reduced *FANCD2* nuclear foci, consistent with an upstream defect in the pathway (Figure 3B,

lanes 5–8, and Supplemental Figure 3B). The MMC hypersensitivity of the DF2231 cells was functionally complemented with the cDNA-encoding *FANCA*<sup>WT</sup> protein, further confirming the assignment of this patient with FA to subtype A (Supplemental Figure 3C). Complementation of the cells also resulted in restoration of the stable FA core complex, including *FANCA*, *FANCG*, and *FAAP20* (Supplemental Figure 3D and refs. 12, 30–32).

We hypothesized that the reduced expression of *FANCA*<sup>I939S</sup> in DF2231 cells results from decreased protein stability. Previous studies suggest that failure of *FAAP20* binding might account for the increased degradation of *FANCA* via the ubiquitin-protea-



**Figure 2. The FANCA<sup>I939S</sup> mutant polypeptide promotes monoubiquitination of FANCD2 but fails to activate REV1 recruitment and TLS-mediated mutagenesis.** (A) Immunoblot of FANCD2 and FANCA derived from cell lysates of the indicated complemented GM6914 FANCA-deficient cells treated with HU (1 mM). (B) Quantification of immunostaining of FANCD2 in GM6914 cells complemented with FANCA<sup>WT</sup> or FANCA<sup>I939S</sup> exposed to HU (1 mM). Three independent experiments were performed. (C) Representative image of GFP fluorescence microscopy of REV1 foci in complemented GM6914 cells 4 hours after psoralen and UVA treatment. Original magnification,  $\times 60$ . Quantification of cells displaying more than 5 REV1 foci. Data shown are mean  $\pm$  SEM from 3 independent experiments. \* $P < 0.05$ , compared with psoralen plus UVA-treated FANCA<sup>WT</sup> complemented GM6914 cells, 2-tailed  $t$  test (assuming unequal variance). (D) The mutation frequency in damaged (1,000 J/m<sup>2</sup> UVC) *SupF* plasmid recovered from GM6914 cells, complemented with vector, FANCA<sup>WT</sup>, or FANCA<sup>I939S</sup>. Data shown are mean  $\pm$  SEM from 3 independent experiments. \* $P < 0.05$ , compared with UVC-treated FANCA<sup>WT</sup> complemented GM6914 cells, 2-tailed  $t$  test (assuming unequal variance).

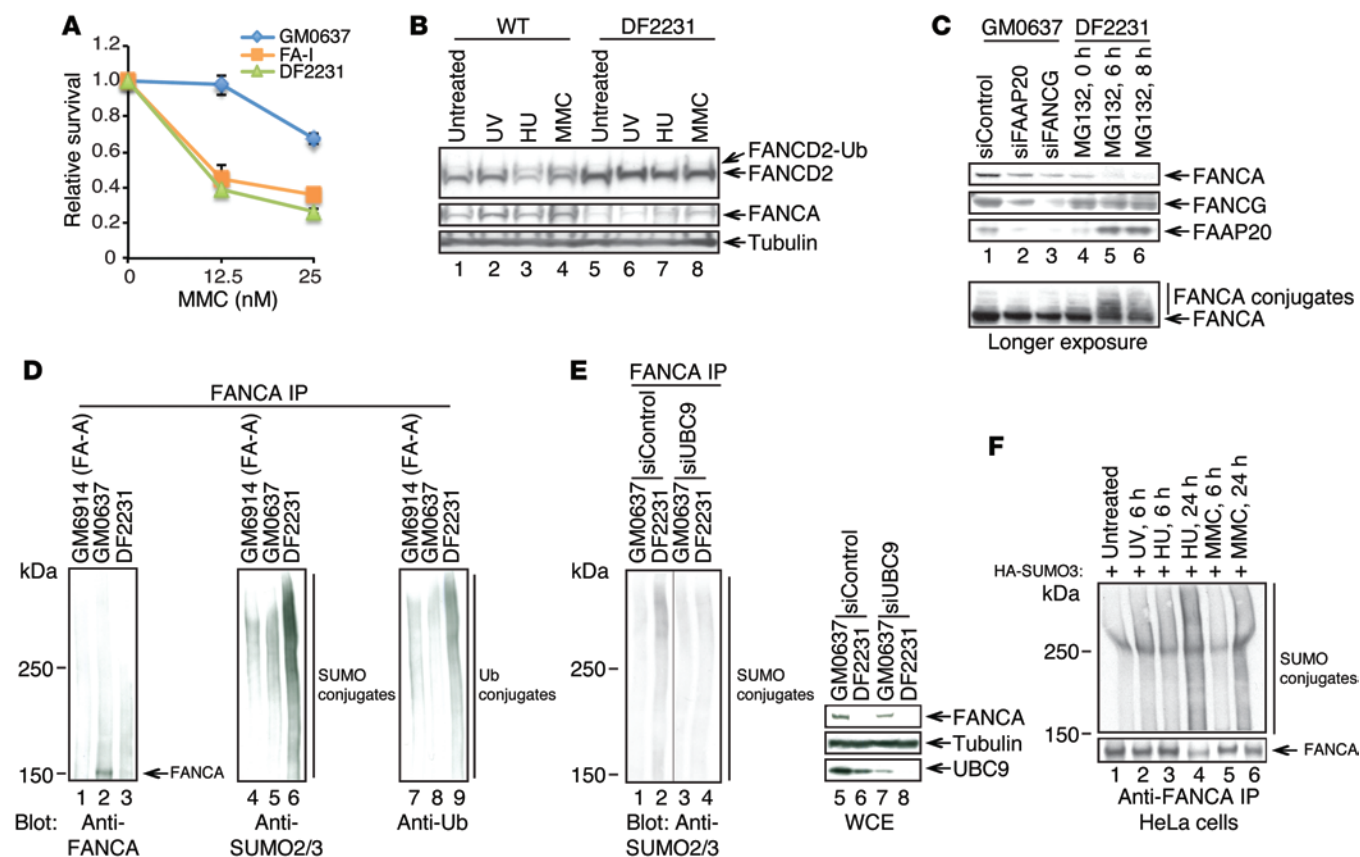
some system (12, 30, 32). Consistent with this hypothesis, the proteasome inhibitor MG132 stabilized the mutant FANCA protein and its interaction with FANCG and FAAP20 (Figure 3C, lanes 4–6). Cellular exposure to MG132 also resulted in accumulation of a high-molecular-weight FANCA protein ladder, suggesting that the mutant FANCA protein (FANCA<sup>I939S</sup>) may undergo polyubiquitination and proteasome-dependent degradation.

Many cellular proteins are known to undergo UBC9-mediated SUMOylation prior to their polyubiquitination and degradation by STUbL enzymes (35, 36). STUbL enzymes are ubiquitin E3 ligases, which contain SIMs, and are directed to their substrates by SUMO-SIM interactions. Furthermore, recent studies indicate that some FA proteins are SUMOylated following cellular heat shock (37). To test the hypothesis that FANCA is degraded through this mechanism, we immunoprecipitated FANCA under denaturing conditions from various SV40-transformed fibroblast lines and immunoblotted for SUMO or ubiquitin (Figure 3D). Immunoprecipitation of FANCA from patient-derived DF2231 cells, followed by immunoblotting with anti-SUMO or anti-ubiquitin, indicated that FANCA is modified with SUMO and ubiquitin (lanes 6 and 9) and that it may coprecipitate with other conjugated proteins. In

contrast, the full-length FANCA<sup>WT</sup> immunoprecipitated from wild-type GM0637 cells (lanes 5 and 8), with reduced coprecipitation of SUMO or ubiquitin conjugates. Moreover, an siRNA that silences the expression of UBC9, the SUMO-conjugating enzyme, blocked the SUMOylation of FANCA (Figure 3E). As further evidence of FANCA SUMOylation, we immunoprecipitated endogenous FANCA under denaturing conditions from HeLa cells transfected with HA-tagged SUMO3. In response to DNA damage, FANCA showed enhanced SUMO conjugation, especially 24 hours after hydroxyurea (HU) and MMC exposure (lanes 4 and 6) (Figure 3F). Taken together, these results support the hypothesis that the FANCA<sup>I939S</sup> protein is degraded by UBC9-dependent SUMOylation followed by polyubiquitination by a STUbL enzyme.

*Loss of FAAP20 binding leads to increased UBC9/PIAS1-dependent SUMOylation of FANCA at K921.* Protein SUMOylation occurs on a lysine residue within the context of a local consensus sequence,  $\Psi$ -K-X-D/E ( $\Psi$  is a hydrophobic acid). Using the SUMO prediction program, we identified a potential SUMOylation site on FANCA at K921. Interestingly, K921 is located near the I939S mutation and falls within the putative FAAP20-binding domain of FANCA (Figure 4A). We next demonstrated that the FANCA<sup>WT</sup>





**Figure 3. The full-length mutant FANCA protein in DF2231 cells is unstable and has enhanced SUMO and ubiquitin conjugation.** (A) Relative survival of wild-type human GM0637 cells, FA-I cells, or DF2231 cells derived from a patient with FA-A treated with increasing doses of MMC and plated for 5 days. Three independent experiments were performed. (B) Immunoblot analysis of wild-type (GM0637) or DF2231 fibroblasts treated with various DNA damage-inducing agents. (C) Immunoblot of FANCA, FANCG, and FAAP20 in lysates of DF2231 cells untreated or pretreated with 20  $\mu$ M MG132 and GM0637 cells treated with siRNAs to control, FAAP20, or FANCG. (D) Endogenous FANCA was immunoprecipitated under denaturing conditions from cell lysates of FA-A (GM6914), wild-type (GM0637), or DF2231 cells, followed by anti-FANCA, -SUMO, or -ubiquitin immunoblot. (E) Endogenous FANCA was immunoprecipitated under denaturing conditions from cell lysates of wild-type (GM0637) or DF2231 cells transfected with siRNA oligos against control or UBC9, followed by anti-SUMO immunoblot. The thin black line indicates that noncontiguous lanes from the same blot are shown. (F) HeLa cells were transfected with HA-tagged SUMO3, and the cells were treated with UV, HU, or MMC for the indicated times. Endogenous FANCA SUMOylation was determined under denaturing conditions by anti-FANCA immunoprecipitation, followed by anti-FANCA or anti-HA immunoblot.

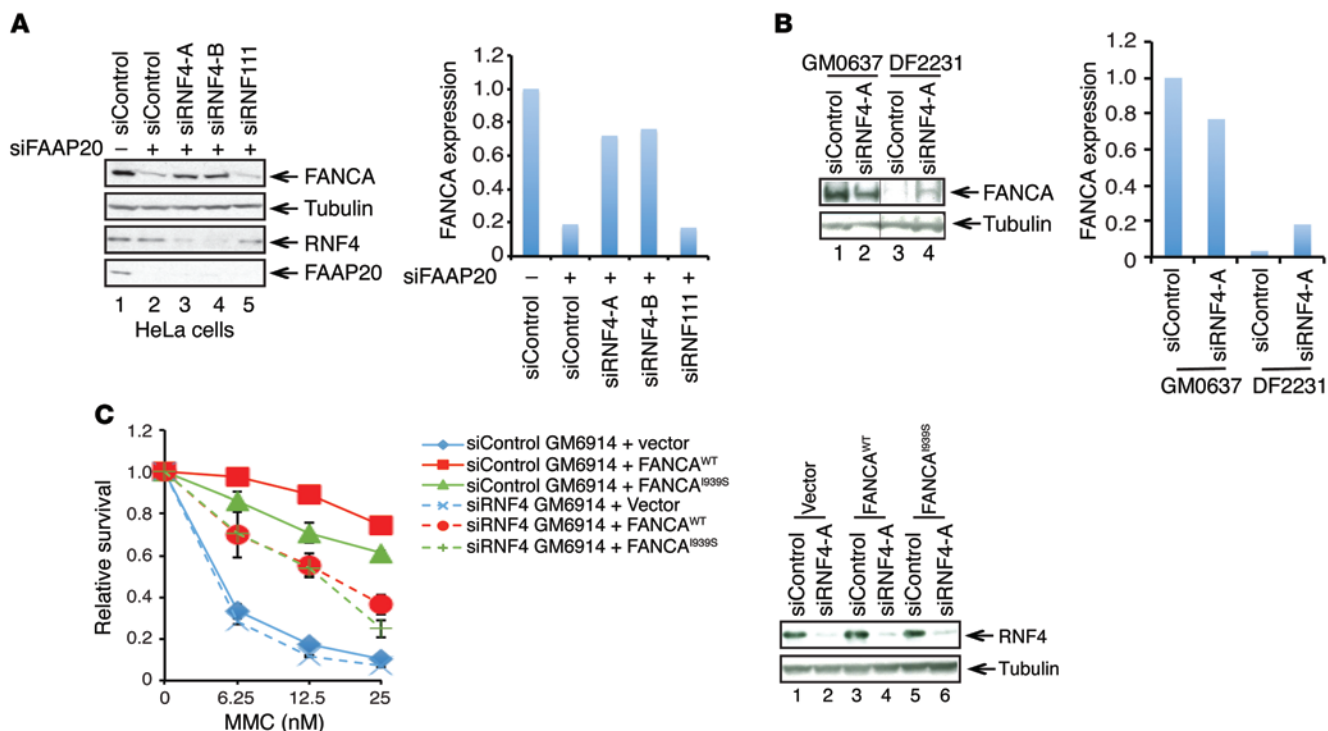
protein is SUMOylated and that the mutant protein, FANCA<sup>K921R</sup>, has reduced SUMOylation (Figure 4, B and C, and Supplemental Figure 4, A–C). Moreover, FANCA<sup>K921R</sup> exhibited reduced polyubiquitination (Figure 4D), indicating that SUMOylation of FANCA is required for optimal polyubiquitination. The FANCA mutant protein (K921R) still interacted with FAAP20, suggesting that the lysine residue or its SUMOylation is not required for this interaction (Supplemental Figure 4D).

The mammalian SUMO conjugation pathway has 1 known E2 SUMO-conjugating enzyme (UBC9) and 6 known E3 SUMO ligases (PIAS1–PIAS4, ZMIZ1, and NSE2) (17). PIAS1 and PIAS4 are critical for DNA double-strand break response, and suppression of either enzyme results in cisplatin sensitivity (38, 39). Furthermore, PIAS1 interacts with SNM1A, and this association is critical for ICL repair (40). In contrast to that of PIAS4, suppression of PIAS1 with siRNA decreased FANCA SUMOylation to a similar extent as UBC9 suppression (Figure 4E). Taken together, we hypothesized that the absence of FAAP20 binding, resulting from the FANCA<sup>I939S</sup> point mutation derived from a patient with

FA, results in increased exposure of the K921 residue. Exposure of K921 results in increased SUMOylation by UBC9, increased polyubiquitination of the mutant protein, and decreased protein stability. Consistent with this hypothesis, the FANCA<sup>I939S</sup> mutant protein has a decreased half-life, and its enhanced degradation is rescued by the concomitant mutation of K921R in the same polypeptide (Figure 4F and Supplemental Figure 4E). FANCA<sup>I939S</sup>, FANCA<sup>K921R</sup>, and FANCA<sup>K921R/I939S</sup> complemented cells all exhibited similar MMC sensitivity (Figure 4G).

*SUMOylation results in RNF4-mediated polyubiquitination and proteasome-dependent degradation of FANCA.* STUbL E3 ligases, such as RNF4, promote the polyubiquitination and degradation of polySUMOylated proteins (35, 36). RNF4 is a strong regulator of DNA damage response (19–22, 41–43), suggesting that it polyubiquitinates DNA repair proteins, which are themselves polySUMOylated. We therefore hypothesized that RNF4, which contains a tandem repeat of SIMs (44), might be the STUbL that promotes the degradation of polySUMOylated FANCA (Figure 5). To test this hypothesis, HeLa cells were transfected with siRNA oligos





**Figure 5. RNF4 polyubiquitinates SUMOylated FANCA and regulates its cellular expression level.** (A) Immunoblot of FANCA derived from lysates of HeLa cells transfected with the indicated siRNA oligos and relative FANCA level quantified by ImageJ. (B) Immunoblot of FANCA from wild-type (GM0637) or DF2231 cells exposed to siRNAs against control or RNF4 and relative FANCA level quantified by ImageJ. The thin black line indicates that noncontiguous lanes from the same blot are shown. (C) GM6914 (FA-A) fibroblasts were stably transfected with the cDNA encoding either FANCA<sup>WT</sup> or the FANCA<sup>I939S</sup> mutant. Cells were treated with the indicated siRNA and subjected to MMC cytotoxicity testing. Three independent experiments were performed. The efficient knockdown of RNF4 expression by the siRNA-RNF4 is indicated by immunoblot.

The interaction was increased after HU treatment (Supplemental Figure 5, A and B), and it was dependent in part on SUMO-SIM interactions (Supplemental Figure 5C). RNF4 suppression also partially rescued the expression of mutant FANCA<sup>I939S</sup> in DF2231 cells (Figure 5B). Furthermore, RNF4 suppression increased the MMC sensitivity of GM6914 cells expressing FANCA<sup>WT</sup> but had little effect on the MMC sensitivity of GM6914 cells expressing the mutant FANCA<sup>I939S</sup> protein (Figure 5C). Interestingly, RNF4 suppression did not further increase the MMC sensitivity of GM6914 cells expressing FANCA<sup>K921R</sup> (Supplemental Figure 5D). Taken together, these results support the hypothesis that RNF4 functions as the STUB1 for FANCA.

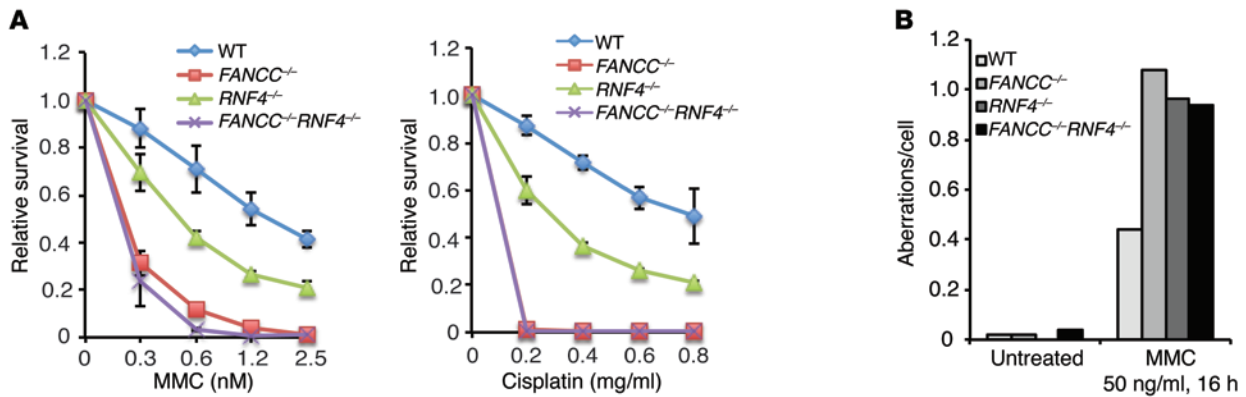
**A normal function of RNF4 in the FA/BRCA pathway.** We next hypothesized that RNF4 may promote the degradation of FANCA<sup>WT</sup> in normal (non-FA) human cells, albeit at a slower rate than the degradation of the mutant FANCA<sup>I939S</sup> protein in the cells from patients with FA. Indeed, RNF4-mediated degradation of FANCA<sup>WT</sup> may be required to clear the FA core complex from the site of damaged chromatin as part of the DNA repair process (17). Failure to clear FANCA and the FA core complex may result in disruption of the pathway and persistence of collapsed replication forks. To test this hypothesis, HeLa cells were transfected with a siRNA to RNF4 and treated with HU or MMC. Interestingly, the reduction of RNF4 resulted in hypersensitivity to DNA-damaging agents and increased chromosome aberrations and radials, consistent with a FA phenotype (Supplemental Figure 6, A-C). Furthermore, RNF4 suppression in HeLa cells

resulted in increased levels of prolonged retention of FANCD2-Ub in the chromatin fraction, suggesting persistence of the active FA core complex (Supplemental Figure 6D).

We next examined the phenotype of avian DT40 cells, which have a stable knockout of FANCC or RNF4 (Figure 6 and refs. 45, 46). Similar to the observation in HeLa cells, the decreased expression of either protein resulted in MMC and cisplatin hypersensitivity and a FA phenotype. In order to examine the possible epistatic relationship between RNF4 and other FA genes, we generated double knockouts of RNF4 and FANCC in avian DT40 cells (Figure 6). We compared the MMC or cisplatin sensitivity of DT40 cells containing a knockout of FANCC, RNF4, or both FANCC and RNF4. Interestingly, the double-knockout cells had MMC (or cisplatin) sensitivity comparable to that of the single FANCC knockout cells. Taken together, these data demonstrate that RNF4 is a component of the FA/BRCA pathway.

## Discussion

In the current study, we identified a patient with FA with a relatively mild clinical presentation. Systematic evaluation of the patient's primary cells revealed subtype FA-A, resulting from germline mutations in both FANCA alleles. One of the mutant FANCA alleles encoded a mutant FANCA protein (FANCA<sup>I939S</sup>). The FANCA<sup>I939S</sup> mutant protein is a unique separation-of-function mutant. On the one hand, the protein promotes the assembly of the FA core complex and allows the monoubiquitination of the FANCD2 protein, leading to endonuclease recruitment (3,



**Figure 6. RNF4 functions in the FA/BRCA pathway. (A)** Relative survival of wild-type, *FANCC*<sup>-/-</sup>, *RNF4*<sup>-/-</sup>, or *FANCC*<sup>-/-</sup>/*RNF4*<sup>-/-</sup> DT40 cells treated with increasing doses of MMC or cisplatin and plated for 3 days. Three independent experiments were performed. **(B)** Quantification of chromosomal aberrations of wild-type, *FANCC*<sup>-/-</sup>, *RNF4*<sup>-/-</sup>, or *FANCC*<sup>-/-</sup>/*RNF4*<sup>-/-</sup> DT40 cells exposed to 50 ng/ml MMC for 16 hours.

11). On the other hand, the *FANCA*<sup>1939S</sup> fails to bind to FAAP20 and thereby fails to efficiently recruit the REV1 translesion polymerase complex (12). We speculate that this reduced efficiency of TLS activity, in the setting of otherwise normal FA core complex assembly and FANCD2 monoubiquitination, may account for this patient's relatively mild clinical and cellular phenotype. An alternative mechanism of TLS polymerase recruitment, or an alternative TLS polymerase, may partially rescue the FA pathway in this patient's cells and thus account for this unique phenotype.

Failure of *FANCA*<sup>1939S</sup> to bind to FAAP20 resulted in its enhanced degradation in the patient-derived cells. Interestingly, the instability of the *FANCA* mutant polypeptide resulted from its hyperSUMOylation by UBC9 and its increased polyubiquitination by the SUMO-targeted E3 ubiquitin ligase, RNF4, leading to proteasomal degradation. Previous studies had indicated that RNF4 is a regulator of the DNA damage response. For instance, RNF4 regulates the degradation of the DNA repair protein MDC1, and inactivation of RNF4 results in ionizing radiation hypersensitivity (21, 22). Our study provides new evidence that RNF4 is also a regulator of ICL repair through its SUMOylation of *FANCA* in the FA/BRCA pathway.

RNF4 is the human homolog of the yeast protein complex SLX5/SLX8 (47). SLX5, SLX8, and SLX4 were identified originally through a synthetic lethality screen in yeast. Specifically, inactivation of any of these 3 genes in a yeast *Sgs1* mutant background, in the presence of HU, resulted in synthetic lethality (47). Interestingly, human *SLX4* was recently shown to be identical to the FA gene, *FANCP* (48, 49), further suggesting that SLX5/SLX8 might also be involved in the FA/BRCA pathway. Furthermore, recent studies in human cells suggest that the FA pathway is synthetically lethal with BLM (the human ortholog of yeast *Sgs1*) (46), consistent with the synthetically lethal relationship observed between *SLX* genes and *Sgs1* in yeast. The yeast SLX5/SLX8 complex also accumulates in DNA damage-inducible foci (50), analogous to the assembly of RNF4 foci in mammalian cells following DNA damage. These results further suggest that SLX5/SLX8 is a component of the DNA damage response. Taken together, these studies suggest that SLX4, SLX5, and SLX8 are components of an ICL repair pathway in yeast, which strongly resembles the FA/BRCA pathway in humans. While *FANCA* itself (a human RNF4 substrate) is

not found in yeast, other unrelated DNA repair protein(s) involved in DNA cross-link repair may be SUMOylated and polyubiquitinated by SLX5/SLX8 in the yeast pathway.

Other FANCD proteins also have consensus SUMO conjugation sites (Supplemental Figure 6E), suggesting that SUMOylation of multiple subunits of the FA core complex may regulate the pathway. Consistent with this hypothesis, we have recently shown that FANCD2 is SUMOylated and subsequently polyubiquitinated by RNF4 (J. Xie and A.D. D'Andrea, unpublished observations). Our work is therefore highly consistent with recent work from the Jentsch group (17, 18). Specifically, SUMOylation often collectively targets an entire group of physically interacting proteins, such as the HR proteins or, in our case, the FA core complex. We speculate that such a SUMO "spray" may stimulate the protein complex assembly. SUMO-SIM stabilized complexes may be subsequently polyubiquitinated by a SUMO-targeted E3 ligase, such as RNF4, and subsequently degraded. Protein-group SUMOylation seems particularly relevant to DNA repair complexes, whose regulated degradation allows for resumption of DNA replication.

Finally, our study underscores the importance of identifying specific *FANCD* gene mutations in adults with FA who have a relatively mild clinical phenotype (i.e., late onset of mild bone marrow dysfunction). The mutant *FANCA* allele found in the patient with FA (proband) in this study was highly informative. The mutant protein, *FANCA*<sup>1939S</sup>, was a separation-of-function mutant, which was functional for core complex assembly and FANCD2 monoubiquitination but dysfunctional for REV1 recruitment and TLS. Moreover, the mutant protein elucidated a normal mechanism of FA/BRCA pathway regulation — namely, the normal binding of the FAAP20 protein to *FANCA* and the regulated release of FAAP20, leading to *FANCA* SUMOylation and ubiquitin-dependent proteasomal degradation of *FANCA*. The identification of other hypomorphic mutations in the *FANCA* gene from patients with FA-A may therefore elucidate other mechanisms of FA/BRCA pathway regulation (Supplemental Figure 6F).

## Methods

**Cell lines and plasmids.** HeLa, HEK293T, GM0637, PD326, and GM6914 cells were purchased from ATCC or from the Fanconi Anemia Research Fund. FA-I cells were a gift from Tony Huang (New York Uni-



versity Medical School, New York, New York, USA). DF2231 were skin fibroblast cells generated the D'Andrea lab. All cell lines were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum. Indicated cDNAs were cloned into pcDNA3.1+Flag, pcDNA3+Myc<sup>-</sup> vectors (Invitrogen). Stable cell lines were generated by retroviral transduction of pMMP-FANCA variants followed by 2 mg/ml puromycin selection. Mutations were generated by the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed by DNA sequencing.

**Antibodies and reagents.** Antibodies used for Western blotting include FANCA (Bethyl), FANCG (Fanconi Anemia Research Fund), FAAP20 (Sigma-Aldrich), FANCD2 (FI-17, Santa Cruz), Flag (M2, Sigma-Aldrich), Myc (9E10, Sigma-Aldrich), HA (6E2, Cell Signaling),  $\beta$ -actin (Cell Signaling),  $\beta$ -tubulin (Sigma-Aldrich), SUMO 2/3 (Millipore), ubiquitin (Cell Signaling), PIAS1 (Cell Signaling), PIAS4 (Cell Signaling), UBC9 (Cell Signaling), and RNF4 (gift from Ronald Hay, University of Dundee, Dundee, United Kingdom). Plasmid transfection and siRNA duplex transfection were done using Lipofectamine LTX (Invitrogen) or Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols. siRNA duplexes were synthesized by Qiagen with the following target sequences: control, 5'-CAGGGTATC-GACGATTACAAA-3'; RNF4-A, 5'-AAGAATGGACGTCTCATC-GTT-3'; RNF4-B, 5'-AAGCGTCGTGGTGGAGCAATA-3'; RNF4-C, 5'-CCCTGTTTCTAAGAACGAAA-3'; FAAP20, 5'-CACGGTGAGC-CCGAGCTGAT-3'; FANCA, 5'-CAGCGTTGAGATATCAAAGAT-3'; PIAS1 (pool of 4 predesigned Qiagen PIAS1 siRNA oligos 1, 6, 7, and 8); PIAS4 (pool of 4 predesigned Qiagen PIAS4 siRNA oligos 2, 3, 5, and 6); RNF111, 5'-TAGGAGATGACTCAAGGAGAA-3'; and FANCG, 5'-CCCAGGTAATCGAGACACTTA-3'.

**Drug sensitivity assays.** Stable human cells (500–1,000 cells) were seeded in 96-well plates and treated with MMC the following day. Three days later, cellular viability was assayed using CellTiterGlo (Promega). Chicken DT40 cellular sensitivity was analyzed 4 days after drug treatment with CellTiterGlo.

**Immunoblot analysis and immunoprecipitation.** Cells were lysed with lysis buffer (1% NP40, 50 mM Tris, pH 7.5, 300 mM NaCl, 0.1 mM EDTA) supplemented with protease inhibitor cocktail (Roche). The lysates were resolved by NuPAGE (Invitrogen) gradient gels and transferred onto nitrocellulose membrane, followed by antibody detection using enhanced chemiluminescence (Western Lightning, Perkin Elmer). For immunoprecipitation, cells were lysed with 300 mM NaCl lysis buffer but diluted subsequently to 150 mM NaCl. Lysates were incubated with anti-Flag M2 antibody-conjugated agarose beads (Sigma-Aldrich) overnight at 4°C and washed 4 times with PBS before immunoblot analysis.

**In vivo SUMOylation assay under denaturing conditions.** Denatured SUMOylation assays were performed as previously described with modifications (51, 52). Transfected 293T or HeLa cells and DF2231, GM0637, or GM6914 cells were incubated with 20  $\mu$ M MG132 (Sigma-Aldrich) for 4 hours before being lysed by boiling in 1% SDS in PBS for 15 minutes. Lysates were cleared by high-speed centrifugation and diluted 10-fold in PBS containing 10 mM iodoacetamide and 20 mM N-ethylmaleimide. Lysates were incubated with anti-HA antibody-conjugated agarose beads (Sigma-Aldrich) or anti-FANCA antibody with protein G sepharose beads (GE Healthcare) for 6 hours at 4°C and washed 6 times with 0.1% SDS in PBS before immunoblot analysis.

**Glutathione S-transferase pull-down assay.** Glutathione S-transferase-tagged (GST-tagged) FANCA variants were expressed in BL21

(DE3) strains and purified using glutathione-sepharose beads (GE Healthcare) in PBS/0.1% Triton X-100 with sonication. Beads were then incubated with 293T cell lysates in 150 mM NaCl buffer for 3 hours at 4°C before immunoblot analysis.

**Fluorescence microscopy.** For GFP fluorescence, complemented GM6914 cells were transfected with EGFP-REV1. 48 hours after transfection, cells were treated with psoralen plus UVA and fixed 4 hours later with 3% paraformaldehyde for 10 minutes at room temperature, washed 3 times with PBS, and mounted with DAPI-containing mounting medium (Vector Laboratories). For FANCA and FANCD2 immunofluorescence, cells were treated with HU or MMC and preextracted with 0.3% Triton X-100 in PBS for 3 minutes on ice, followed by fixation with 4% paraformaldehyde for 10 minutes on ice. Cells were stained with FANCA (Bethyl, 1:1,000) or FANCD2 antibody (Novus, 1:500). All fluorescence and immunofluorescence images were captured using a Zeiss AX10 fluorescence microscope and analyzed with AxioVision software.

**Chromosome breakage analysis.** DF2231 cells were treated with 20 ng/ml MMC or 0.1  $\mu$ g/ml diepoxybutane. DT40 cells were treated with 50 ng/ml MMC. HeLa cells were transfected with siRNAs and treated with 20 ng/ml MMC. Numbers of chromosome breakages were analyzed in untreated and treated cells as previously described (12).

**SupF mutagenesis assay.** Complemented GM6914 cells were transfected with undamaged or damaged (UVC 1,000 J/m<sup>2</sup>) pSP189 plasmids using GeneJuice (Novagen). After 48 hours, plasmid DNA was isolated by a miniprep kit (PureYield Plasmid Miniprep System, Promega) and DpnI digested. After ethanol precipitation, retrieved plasmids were transformed into  $\beta$ -gal<sup>-</sup> MBM7070 indicator strain plasmids via electroporation (GenePulsor X Cell, Bio-Rad) and plated onto LB plates containing 1 mM isopropyl- $\beta$ -D-thiogalactoside, 100  $\mu$ g/ml X-gal, and 100  $\mu$ g/ml ampicillin. White and blue colonies were scored, and the mutation frequency was calculated as the ratio of white (mutant) to total (white + blue) colonies.

**Statistics.** All results are presented as mean  $\pm$  SEM and were analyzed by 2-tailed Student's *t* test. Statistical significance was accepted at *P* < 0.05.

**Study approval.** The Dana-Farber Cancer Institute Ethical Review Committee and IRB approved this study. Written informed consent was obtained from the patient.

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